THEORY AND RESEARCH IN HEALTH SCIENCES

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Theory and Research in Health Sciences

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Chapter 1

BLOOD AND TISSUE PROTOZOA

Gülseren AKTAŞ[1]

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THE GENERA *LEISHMANIA, TRYPANOSOMA, TOXOPLASMA, PLASMODIUM*, AND *BABESIA*

The genus *Leishmania*

Leishmania spp. and *Trypanosoma* spp. are protozoa belonging to the family *Trypanosomatidae*.

For *Leishmania* in the Old World (exept America continent and Avustralia), there is only one subgenus, *Leishmania*; however, in the New World, the genus has been split into two subgenera as *Leishmania* and *Viannia* according to the development of the organism in the digestive tract of the sand fly. Leishmaniasis is a vectorborne disease that is transmitted by female phlebotomine sand flies and caused by obligate intracellular protozoa of the genus *Leishmania*. The taxonomy of leishmanias is controversial and in a state of dynamic flux. There are over 20 *Leishmania* species causes Leishmaniasis. Depending on the geographic area, many different species can infect humans, producing a variety of diseases (cutaneous, diffuse cutaneous, mucocutaneous, and visceral diseases). Species differentiation is currently based on molecular techniques rather than geographical distribution and clinical presentation. The different species are morphologically indistinguishable, but they can be differentiated by isoenzyme analysis, molecular methods, or monoclonal antibodies. In Table 1, it is seen the features of human leishmanial infections caused by more than 20 Leishmania species.

Table 1. Features of human leishmanial infections.

Species	Disease type(s)[a]	Recommended specimen(s)	Geographic distribution
L. (L.) donovani	VL	Bone marrow or spleen biopsy specimen	Africa, Asia
	CL, MCL, DL	Skin or mucosal biopsy specimen	
L. (L.) infantum[b]	VL	Bone marrow or spleen biopsy specimen	Africa, Europe, Mediterranean area, Southeast Asia, Central and South America
L. (L.) killicki	CL	Skin biopsy specimen	Algeria, Tunisia
L. (L.) tropica	CL	Skin biopsy specimen	Afghanistan, India, Turkey, former USSR
L. (L.) major	CL	Skin biopsy specimen	Afghanistan, Africa, Middle East, former USSR
L. (L.) aethiopica	CL, DCL, MCL	Skin or mucosal biopsy specimen	Ethiopia, Kenya, former USSR, Yemen
L. (L.) mexicana	CL, DCL, MCL	Skin or mucosal biopsy specimen	Belize, Guatemala, Mexico, Texas
L. (V.) braziliense	CL, MCL	Skin or mucosal biopsy specimen	Central and South America
L. (V.) peruviana	CL	Skin biopsy specimen	Colombia, Costa Rica, Panama
L. (V.) garnhami	CL	Skin biopsy specimen	Venezuela
L. (V.) colombiense	CL	Skin biopsy specimen	Colombia, Panama
L. (V.) panamensis	CL	Skin biopsy specimen	Colombia, Costa Rica, Panama
L. (V.) guyanensis	CL, MCL	Skin or mucosal biopsy specimen	Brazil, Colombia, French Guiana, Peru
L. (V.) venezuelensis	CL	Skin biopsy specimen	Venezuela
L. (V.) lainsoni	CL	Skin biopsy specimen	Brazil
L. (V.) shawii	CL	Skin biopsy specimen	Brazil
L. (L.) amazonensis	CL, DCL	Skin or mucosal biopsy specimen	Brazil, Venezuela
L. (V.) naffi	CL	Skin biopsy specimen	Brazil, Caribbean Islands
L. (L.) pifanoi	CL, DCL	Skin or mucosal biopsy specimen	Brazil, Venezuela

[a]CL, cutaneous leishmaniasis; DCL, diffuse cutaneous leishmaniasis; DL, diffuse leishmaniasis; MCL, mucocutaneous leishmaniasis; VL, visceral leishmaniasis.
[b]*L. (L.) infantum* in the Old World is *L. (L.) chagasi* in the New World.

Morphology

The parasite has two distinct phases in its life cycle, amastigote (Leishmania) and promastigote (Leptomonas) (Figure1).

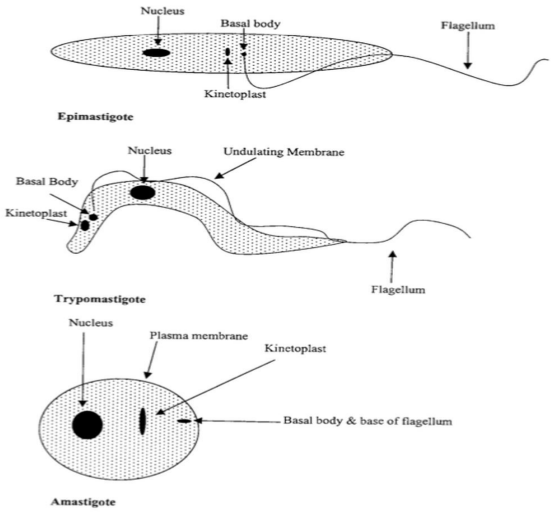
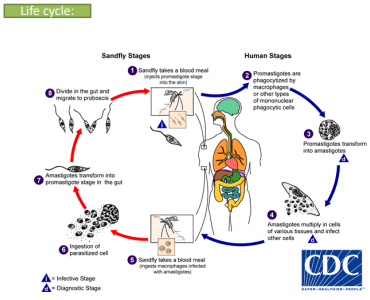


Figure 1. Life cycle stages of Leishmania spp.

The amastigote stage (Leishman-Donovan body) is found in reticuloendothelial cells of the host. The amastigote form is small, round or oval, measures 3 to 5 µm. Upon ingestion during a blood meal by the insect vector, the amastigote transforms into the flagellated promastigote stage in the gut of the insect and multply, then, transform to metacyclic promastigotes (infectious form), and migrate to the hyposome of the sand fly, where they are released when the next bloodmeal is taken.

Life cycle



Leishmaniasis is transmitted by the bite of infected female phlebotomine sand flies to human.

1. The sand flies inject the infective stage [i.e., promastigotes-(leptomonas)] from their proboscis during blood meals.

2. Promastigotes are phagocytized by macrophages and other phagocytic cells.

3. Promastigotes transform in these cells into the tissue stage of the parasite [i.e., amastigotes-(leishmania)],

4. which multiply by simple division and proceed to infect other mononuclear phagocytic cells.

5-6. Sand flies become infected by ingesting macrophages infected with amastigotes during blood meals.

7-8. In sand flies, amastigotes transform into promastigotes, develop in the gut and migrate to the proboscis.

The complete life cycle in the sand fly is 4 to 18 days. Upon inoculation into the bite site, the promastigote changes to the amastigote form.

Transmission and epidemiology

The disease is considered primarily a zoonosis with natural rezervoirs, including rodents, dogs, anteaters, sloths (Figure 2). In certain areas of the world where the disease is endemic, the infection can be transmitted by a human-vector-human cycle also rarely human to human (this type of transmission is called anthroponotic). The infection may also be transmitted by direct contact with an infected lesion or mechanically through bites by stable or dog flies. Some 70 animal species, including humans, have been found as natural reservoir hosts of *Leishmania* parasites.

Figure 2. Natural rezervuars: anteaters, sloths, racum as rodent.

Greater than 90% of cutaneous leishmaniasis cases occur in Afghanistan, Algeria, Brazil, Iran, Iraq, Peru, Saudi Arabia, and Syria (Figure 3). There has been an increase in the number of cases among military personnel deployed in Afghanistan, Iraq, and Kuwait. Autochthonous human infections have been described in USA. Most of the diagnosed cases of mucocutaneous leishmaniasis are from Bolivia, Brazil, and Peru.

Figure 3. Leishmaniasis: High risk areas.

Leishmania parasites are transmitted through the bites of infected female phlebotomine sandflies, which feed on blood to produce eggs. All infected female sand flies transmitting leishmaniasis belong to the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World (America,Australia continent). There are more than 30 species of sand flies that can transmit leishmaniasis.

Disease

There are 3 main forms of leishmaniasis in people. **Visceral form** , invide throughout the reticuloendothelial system, **Cutaneous**, which result from infection of macrophages in the dermis,

Mucosal, infection in the naso-oropharyngeal mucosa. For all three forms, the infection can range from asymptomatic to severe. Cutaneous and mucosal leishmaniasis can cause substantial morbidity, whereas

visceral leishmaniasis can be life threatening. Some people have a silent infection, without any symptoms or signs.

Visceral leishmaniasis (kala-azar)

Visceral leishmaniasis is also known as kala-azar and the most serious form of the disease.

The term *kala-azar*— means black (*kala*) fever (*azar*) in Hindi.

It is usually caused by the species *L. donovani* and *L. infantum* [*L. chagasi* (in the new world-American) generally is considered synonymous with *L. infantum* (in the old world)].

Visceral leishmaniasis affects reticuloendothelial system organs (usually, spleen, liver, and bone marrow) and can be life threatening. The illness typically develops within months (sometimes as long as years) of the sand fly bite. Affected people usually have; fever, weight loss, enlargement (swelling) of the spleen and liver, and low blood counts (anemia, leucopenia, thrombocytopenia). HIV-coinfected patients may have atypical manifestations, such as involvement of the gastrointestinal tract and other organ systems (Figure 4).

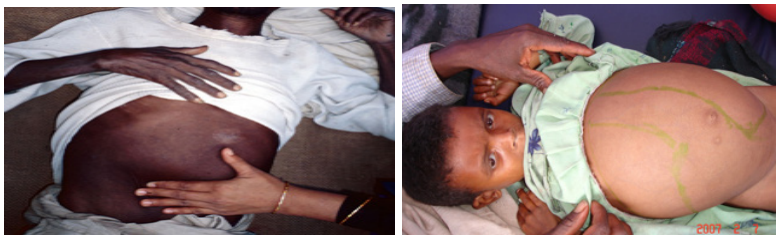


Figure 4. Patients who have visceral leishmaniasis are seen in the pictures.

Cutaneous leishmaniasis

It is usually caused by the species *L. tropica* in the New World (American) and *L. major* in the Old World (the Eastern Hemisphere). It is the most common form of leishmaniasis and causes skin lesions, leaving life-long scars and serious disability or stigma (Figure 5). The lesion may start out as papules or nodules and may end up as ulcers (like a volcano, with a raised edge and central crater); skin ulcers might be covered by scab or crust. Lesions are usually painless but can be painful. If untreated, severe cases of visceral leishmaniasis typically are fatal, either directly from the disease or indirectly from complications, such as secondary bacterial infection or hemorrhage.

Figure 5. Patients who have cutaneous leishmaniasis are seen.

Mucosal (mucocutaneous) leishmaniasis

It is produced most often by the *Leishmania braziliensis*. The primary lesions are similar to those found in other infections of cutaneous leishmaniasis. Untreated primary lesions may develop into the mucosal form in up to 80% of cases. Metastatic spread to the nasal or oral mucosa may occur in the presence of the active primary lesion or many years later after the primary lesion has healed (latent). In mucosal leishmaniasis, the lesions can lead to partial or total destruction of the mucous membranes of the nose, mouth and throat cavities and surrounding tissues (Figure 6). This disabling form of leishmaniasis can lead to the sufferer being rejected by the community. A small number of mucosal leishmaniasis cases have been reported with L. donovani. Over 90% of mucosal leishmaniasis cases occur in Bolivia, Brazil, Ethiopia and Peru.

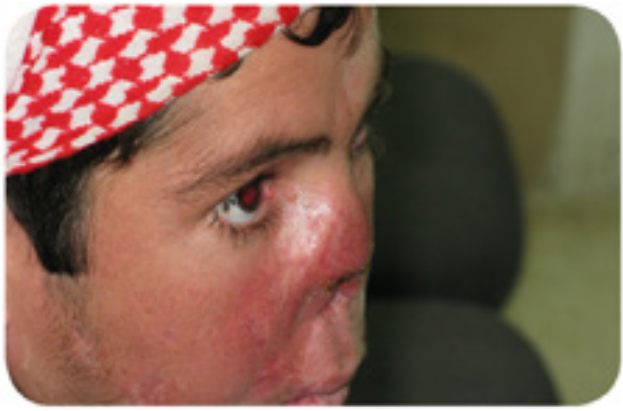


Figure 6. Mucosal leishmaniasis.

Post-kala-azar dermal leishmaniasis (PKDL)

Post-kala-azar dermal leishmaniasis (PKDL) is usually a sequel of visceral leishmaniasis that appears as hypopigmented macular, papular or nodular rash usually on face, upper arms, trunks and other parts of the body (Figure 7). It occurs mainly in East Africa and on the Indian subcontinent, where 5-10% of patients with kala-azar are reported to develop the condition. It usually appears 6 months to 1 or more years after kala-azar has apparently been cured. People with PKDL are considered to be a potential source of *Leishmania* infection. Some patients develop post kala-azar dermal leishmaniasis (PKDL), a syndrome characterized by skin lesions that develop at variable intervals after (or during) therapy for visceral leishmaniasis. Persons with chronic PKDL can serve as important reservoir hosts of infection Figure 7.

Figure 7. Patient with post-kala azar-dermal leishmaniasis.

Diagnosis

Leishmaniasis is diagnosed by detecting *Leishmania* parasites (or DNA) in tissue specimens—such as from skin lesions for cutaneous leishmaniasis, or from bone marrow for visceral leishmaniasis—via light-microscopic examination of stained slides, molecular methods, and

specialized culture techniques. Definitive diagnosis depends on detecting either the amastigotes in clinical specimen or the promastigotes in culture.

Direct Examination

Amastigote stages (leishmania) are found within macrophages (monocytes, leukocytes with polymorphic nucleus) or close to distrupted cells. The cytoplasm will stain light blue, and the nucleus and kinetoplast will stain red or purple with Giemsa stain. In Figure 8. it is seen a Light-microscopic examination of a stained bone marrow specimen from a patient with visceral leishmaniasis—showing a macrophage containing multiple *Leishmania* amastigotes (the tissue stage of the parasite). Note that each amastigote has a nucleus (red arrow) and a rod-shaped kinetoplast (black arrow). Visualization of the kinetoplast is important for diagnostic purposes, to be confident the patient has leishmaniasis.

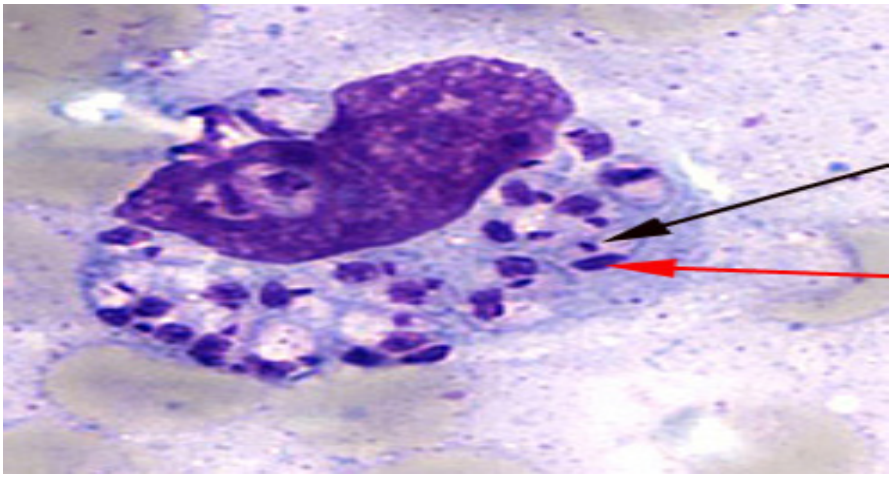


Figure 8. Amastigote. Giemsa stained

Culture (promastigote-leptomonas): Culture media successfully employed to recover organisms include Novy, MacNeal, and Nicolle's (NNN) medium and Schneider's Drosophila medium suppemented with bovine serum. Cultures, incubated at 25 °C, should be examined for up to 4 weeks before the culture is declared negative. Promastigote stages can be detected microscopically in wet mounts, and then, stained with Giemsa stain to observe their morphology (Figure 9).

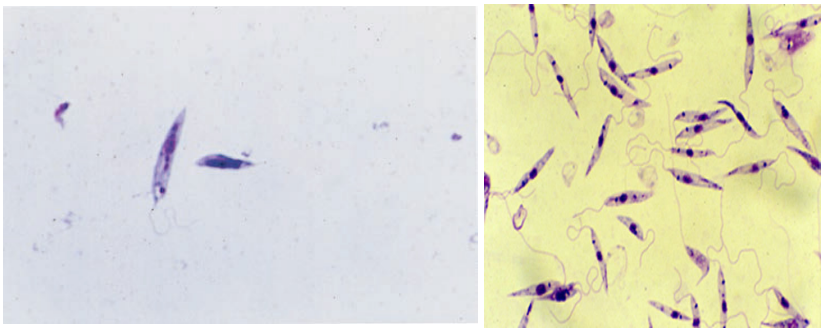


Figure 9 -Promastigot-(leptomonas) with filagella (In culture and in sand flies)

Indirect Diagnostic Tests

Test-I: Serologic tests are available for research, or epidemiologic purposes; however, they are not very useful for the diagnosis of mucocutaneous and visceral leishmaniasis. For visceral leishmaniasis, serologic testing can provide supportive evidence for the diagnosis. In kala-azar, there is a large increase in gamma globulins (both immunoglobulin G (IgG) and IgM. This is the basis for the formol-gel test, which has been used as a screening test in areas of endemicity. The **formol-gel test** is a test to detect the greatly increased serum proteins in visceral leishmaniasis; one drop of full-strength formalin is added to 1 mL of serum, with rapid and complete coagulation indicating the positive reaction. A number of serologies, including indirect fluorescent-antibody assay (IFA), enzyme-linked immunoassay (ELISA), and immunoblot tests have been developed for diagnostic purposes; however, they are not widely available exept in areas of endemicity.

Tests-II: PCR testing, if available, can be used as a supplemental diagnostic procedure.

Skin testing: the leishmanin test **(Montenegro)**, a delayed-type hypersensitivity reaction, is useful for epidemiological surveys of a population to identify groups at risk of infection. This test is of no value for the diagnosis of visceral leishmaniasis. **Montenegro skin test** is intradermal application of a solution containing antigenic preparation of promastigote forms of *Leishmania*. The result should be evaluated within 48 hours with a ballpoint pen, being positive if the papule formed is equal or greater than 5mm. No skin tests for leishmaniasis are approved for use in the United States, primarily due to the lack of standardization of these tests. Delayed-type hypersensitivity is a common immune response that occurs through direct action of sensitized T cells when stimulated by contact with antigen. It is referred to as a delayed response in that it will usually require 12–24 hours at a minimum for signs of inflammation to occur locally.

Viceral leishmaniasis patients coinfected with HIV may have no detectable antileishmania antibodies. Serologic testing is available at some referral laboratories and Center for Disease Control and Prevention (CDC).

The detection of urinary antigens has been used for the diagnosis of visceral leishmaniasis.

Treatment: In treatment, these agents have been using pentavalent antimonial (SbV) compounds,liposomal amphotericin B, azoles (ketoconazole, itraconazole, and fluconazole).

Prevention & Control: No vaccines or drugs to prevent infection are available. Inoculatin the serous exudate from naturally acquired lesions of cutaneous leishmaniasis into an inconspicuous area of the body of a nonimmune person has been effective; however vaccines against other forms of leishmaniasis have not worked. The best way for travelers to prevent infection is to protect themselves from sand fly bites applying insect repellents to the skin, and using fine-mesh bed metting. Vector control helps to reduce or interrupt transmission of disease by decreasing the number of sand flies. Control methods include insecticide spray, use of insecticide-treated nets, environmental management and personel protection.

Major risk factors: Socioeconomic condition (poverty); Malnutrition: Diets lacking protein-energy, iron, vitamin A and zinc increase the risk; Population mobility: Epidemics of both cutaneous and visceral leishmaniasis are often associated with migration and the movement of non-immune people into areas with existing transmission cycles; Climate change: Leishmaniasis is climate-sensitive as it affects the epidemiology.

The genus *Trypanosoma*

Trypanosomes are unicellular parasitic protozoa belonging to the *Trypanosoma* genus of the Trypanosomatidae Class. *Trypanosoma* spp. are hemoflagellate protozoa that live in the blood and tissue of the human host. The trypomastigote form (in blood) which measures 15 to 30 µm x 1.5 to 3.5 µm, has an elongated body with a flagellum and a undulating membrane that runs the length of the body (Figure 10).

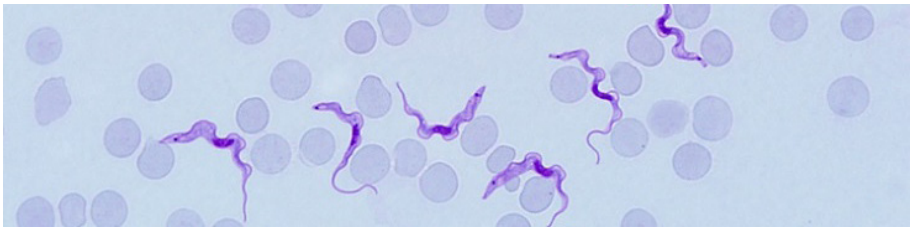


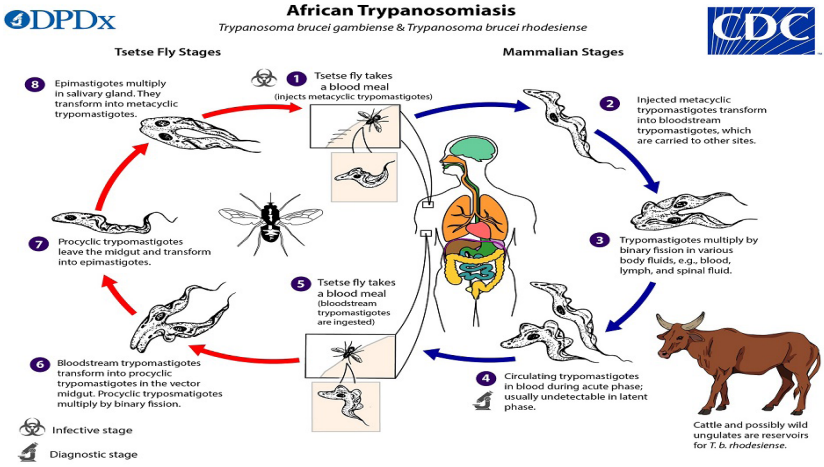
Figure 10. Giemsa stained blood smear preparation of Trypanosoma spp.

A central nucleus and a posterior kinetoplast are usually easily seen. These diagnostic forms can be seen in stained smears of peripheral blood, and in the spinal fluid in certain stages of the infection.

In addition to taking thin and thick blood films, determining **the buffy coat concentration** (the buffy coat is the fraction of an anticoagulated blood sample that contains most of the white blood cells following centrifugation) is recommended to detect the parasites.

The life cycle involves animals and humans as the hosts, in which the mature trypanosomes circulate and divide in the peripheral blood and ultimately invade the CNS (central-nervous-system); an intermediate host and vector, the tsetse fly, is also involved. The immature forms develop in the salivary gland of the tsetse fly, ultimately forming infective metacyclic trypanomastigotes.

Life cycle



- During a blood meal on the mammalian host, an infected tsetse fly injects metacyclic trypomastigotes into skin tissue. The parasites enter the lymphatic system and pass into the bloodstream.
- Inside the host, they transform into bloodstream trypomastigotes.
- They are carried to other sites throughout the body, reach other body fluids (e.g., lymph, spinal fluid), and continue the replication by binary fission.
- 4,5- The entire life cycle of African trypanosomes is represented by extracellular stages. The tsetse fly becomes infected with bloodstream trypomastigotes when taking a blood meal on an infected mammalian host.

- In the fly's midgut, the parasites transform into procyclic trypomastigotes, multiply by binary fission,
- They leave the midgut, and transform into epimastigotes.
- The epimastigotes reach the fly's salivary glands and continue multiplication by binary fission.

Diseases

Trypanosomes are parasites of humans, and wild and domestic mammals, in which they cause several important diseases, including sleeping sickness by *Trypanosoma brucei* subsp. *gambiense* and *T. brucei* subsp. *rhodesience* in Africa; and American trypanosomiasis (Chagas' disease) is caused by *Trypanosoma cruzi* in the Americas. *T. brucei rhodesiense* and *T. brucei gambiense* are closely related and morphologically indistinguishable.

African trypanosomiasis (sleeping sickness)

African trypanosomiasis (African sleeping sickness) is caused by *Trypanosoma brucei* subsp. *gambiense* and *T. brucei* subsp. *rhodesience*. Tsetsi flies transmit *T. brucei* to humans.

Tsetse fly

Geographic distribution and Clinical forms

Georgraphic distribution

Trypanosoma brucei gambiense is endemic in the (tropical rainforests of) central and Western Africa, whereas *T. brucei rhodesiense* is endemic in

the (savannah region of) central and east Africa (Figure 11). *Trypanosoma cruzi* is common in parts of Mexico, Central America, and South America (Figure 12).

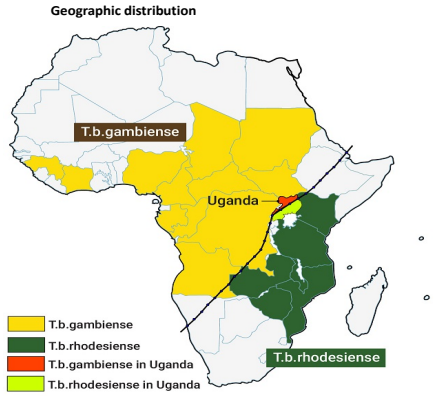


Figure 11. It is seen the Georgraphic distribution of African trypanosomiasis.

Figure 12. Geografical distribution of T. cruzi (chagas disease)

Clinical forms

African trypanosomiasis (sleeping sickness) has two clinic forms.

The West African (Gambian) form of sleeping sickness is responsible for 99% of the sleeping sickness cases, which is caused by *T. brucei gambiense*. It is more chronic. Its infections can last for months to years with slow CNS involvement, characterized by neurologic deterioration, whereas,

The East African (Rhodesian) form of sickness causes acute morbidity and mortality within months of infection, which is caused by *T. brucei rhodesiense*. It tends to be more rapid onset with a greater tendency to become rapidly progressive, even leading to death (rapidly).

Transmission and Epidemiology: The development cycle in the tsetse fly varies from 12 to 30 days. Fewer than 10% of tsetse flies become infective after obtaining blood from infected patients. Both female and male tsetse flies can transmit the infection. Although there is no evidence of animal-to-human transmission of *T. brucei gambience*, trypanosomal strains isolated from kobs, chickens, dogs, cows, and domestic pigs in West Africa are identical to those isolated from humans in the same area. The tsetse fly vectors of Rhodesian trypanosomiasis may transmit the disease from human to human or from animal to human. Infections can also occur through placental transfer from mother to fetus and by needle sticks. Chagas' disease also known as American trypanosomiasis, is a tropical parasitic disease caused by *Trypanosoma cruzi*. It is transmitted to humans by triatomine bugs living in close associated with reservoirs as dogs, cats, armadillos, opossums raccoons, and rodent.

Diagnosis: Definitive diagnosis depends upon demonstration of trypomastigotes in blood, lymph node aspirate, sternum bone marrow, chancre fluid and cerebrospinal fluid (CSF).

Microscopic detection: In addition to taking thin and thick blood films, determining the buffy coat concentration is recommended to detect the parasites. In suspected and confirmed cases of trpanosomiasis, a lumber puncture is mandatory to rule out CNS involvement. Molecular methods have been used to detect infections and differentiate species. Small laboratory animals have been used to detect infections. Cultivation is not practical. Serologic techniques that have been used for epidemiologic screening include IFA, and ELISA.

Treatment and control: The drugs used and the course of treatment are dependent on the trypanosomal species and the clinical stages of the disease. The avoidance of infection through control of the vectors is particularly important.The most effective control measures include an

integrated approach to reduce the human reservoir of infection and the use of insecticides to eliminate the reduviid vector. Vaccines are not available.

The genus *Toxoplasma*

Toxoplasma gondii is included in the family *Sarcocystidae,* subfamily *Toxoplasmatinae*, and genus *Toxoplasma. Toxoplasma gondii* is a protozoan parasite that infects most species of warm-blooded animals, including humans and causes the disease toxoplasmosis. It is one of the most common parasitic infections in humans and is most typically asymptomatic. However, in select clinical situations it can cause severe and disabling disease, making accurate and timely diagnosis vital.

Members of the cat family *Felidae* are the only known definitive hosts for *T. gondii* and thus are the main reservoirs of infection. *(Felidae* is a *family* of mammals in the order, colloquially referred to as cats)

The three stages of this obligate intracellular parasite are

(i) Tachyzoites (trophozoites), which rapidly proliferate and destroy infected cells during acute infection;

(ii) Bradyzoites, which slowly multiply in tissue cysts; and

(iii) Sporozoites in oocysts (Fig. 13).

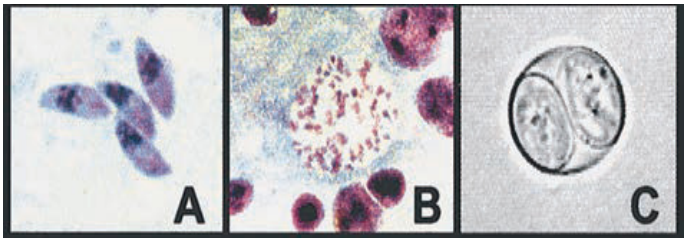


Figure 13- Three life stages of T. gondii. (A) Tachyzoites (Giemsa stain); (B) cyst with bradyzoites in brain tissue (Giemsa stain); (C) sporulated oocysts, unstained.

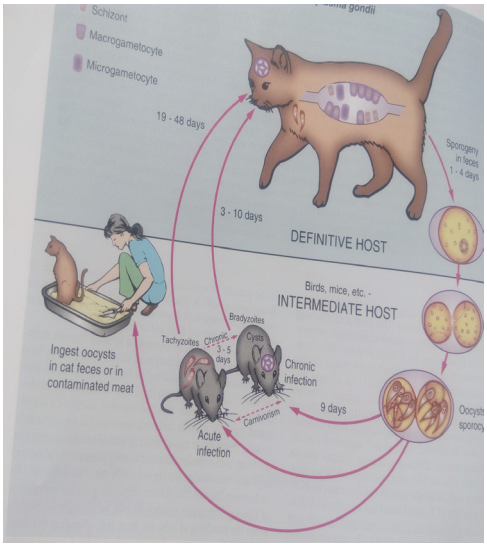
Tachyzoites and bradyzoites occur in body tissues; oocysts are excreted in cat feces. Cats become infected with *T. gondii* by carnivorism or by ingestion of oocysts.

Life cycle of *Toxoplasma gondii*

The life cycle of *T.gondii* has both sexual and asexual stages. The sexual stage occurs in the intestine of cats, where infective oocysts, replicate within the intestinal epithelial cells and are excreted in the feces. The asexual stage commonly occurs in a variety of herbivorous and

carnivorous animals that ingest the infective oocysts. Humans may also become infected by ingesting food or water contaminated with oocysts. Cockroaches, earthworms, snails and slugs may also serve as transport hosts for oocysts.

Life cycle



After tissue cysts or oocysts are ingested by the cat, viable organisms are released and invade epithelial cells of the small intestine, where they undergo an asexual cycle followed by a sexual cycle and then form oocysts, which are then excreted. The unsporulated (i.e., noninfective) oocyst takes 1 to 5 days after excretion to become sporulated (infective). Although cats shed oocysts for only 1 to 2 weeks, large numbers may be shed, often exceeding 100,000 per g of feces. Oocysts can survive in the environment for several months to more than a year and are remarkably resistant to disinfectants, freezing, and drying but are killed by heating to 70°C for 10 min.

Epidemiology and risk factors

Serologic prevalence data indicate that toxoplasmosis is one of the most common infections of humans throughout the world. In various places throughout the world, it has been shown that more than 60% of some populations have been infected with *Toxoplasma.* The prevalence of positive serologic titers increases with age. In many areas of the world, infection is more common in warm climates and lower altitudes, because the oocysts survive better in these types of environments. Toxoplasmosis is not passed from person-to-person, except in instances of mother-to-child (congenital) transmission and blood transfusion or organ transplantation.

Risk factors for *T. gondii* infection include eating raw or under cooked pork, mutton, lamb, beef, oysters, clams, mussels, cleaning a cat litter box, contact with soil (gardening and yard work), and eating raw or unwashed vegetables or fruits.

Transmission

Human infection may be acquired in several ways:

a) ingestion of undercooked contaminated meat (pork, lamb, venison) containing *T.gondii* cysts*;* (zoonotic transmission)

b) ingestion of oocysts from hands, food, soil, or water contaminated with cat feces; (foodborne transmission)

c) transplacental transmission; (congenital transmission)

d) organ transplantation or blood transfusion; (rare instances of transmission)

Transmission

The two major routes of transmission of *Toxoplasma* to humans are oral and congenital. In humans, ingesting either the tissue cyst or the oocyst results in the rupture of the cyst wall, which releases organisms that invade the intestinal epithelium, disseminate throughout the body, and multiply intracellularly. The host cell dies and releases tachyzoites, which invade adjacent cells and continue the process. The tachyzoites are pressured by the host's immune response to transform into bradyzoites and form tissue cysts, most commonly in skeletal muscle, myocardium, and brain; these cysts may remain throughout the life of the host. Recrudescence of clinical disease may occur if the host becomes immunosuppressed and the cysts rupture, releasing the parasites.

Toxoplasmosis

Healthy people (nonpregnant) who become infected with *Toxoplasma gondii* often do not have symptoms because their immune system usually keeps the parasite from causing illness. When illness occurs, it is usually mild with"flu-like" symtoms (i.e., tender lymph nodes, muscle aches, etc.) that last for weeks to months and then go away. However, the parasite remains in the person's body in an inactive state. It can become reactivated if the person becomes immunosuppressed. Persons with compromised immune systems may experience sever symptoms if they are infected with *Toxoplasma* while immune suppressed. Persons who acquire HIV infection and were not infected previously with *Toxoplasma* are more likely to develop a severe primary infection.

Mother-to-child (congenital transmission)

Generally, if a woman has been infected before becoming pregnant, the unborn child will be protected because the mother has developed immunity. If a woman becomes newly infected with *Toxoplasma* during or just before pregnancy, she can pass the infection to her unborn baby (congenital transmission).

Potenatial results can be

A miscarriage,(abortus)

A stillborn child,

A child born with signs of congenital toxoplasmosis (i.e., abnormal enlargement-(hydrocephalus) or smallness (microcephaly) of the head).

Eye disease from *Toxoplasma* infection can result from congenital infection or infection after birth. Eye lesion from congenital infection are often not identified at birth but occur in 20-80% of congenitally-infected persons by adulthood.

Laboratory diagnosis

Serum, plasma, CSF, ocular fluid, and amniotic fluid may be tested for

antibodies and/or parasite DNA.

Microscopy: Only very rarely can the diagnosis of toxoplasmosis be documented by the direct observation of parasites in patient specimens. Tachyzoites may be observed as free organisms or within host cells, such as leukocytes on the slide stained with Giemsa stain. Well-preserved tachyzoites are crescent shaped and stain well, but degenerating organisms may be oval and stain poorly. Tissue imprints stained with Giemsa stain may reveal *T. gondii cysts.*

Serologic tests: Serologic testing for *T. gondii*-specific antibodies is the most commonly used method for diagnosis of toxoplasmosis. Although elevated *Toxoplasma*-specific IgG levels have been suggested as an indicator of recent infection, high levels may last for many years after primary infection. The diagnosis of infection in the neonate can be established if a positive IgM test at any titer is obtained after birth (in the absence of a placental leak), or the organisms demonstrated in tissue biopsies or smears of aspirates or by PCR.

Antigen detection: Immunologic techniques such as fluorescein isothiocyanate-labeled (or peroxidase) antisera may be useful in detecting tachyzoites in tissue sections (FA) (Figure 14). Enzyme immunoassay

(EIA) antigen detection techniques lack sensitivity for human samples and are not recommended.

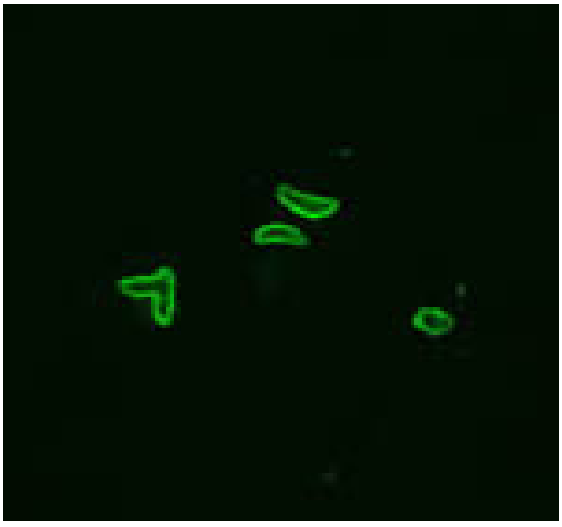


Figure 14: Toxoplasma-positive reaction, stained by immunofluroescence.

Nucleic acid detection techniques: PCR technology for *Toxoplasma* has been used to detect disease.

Isolation procedures: Parasites can be isolated with limited success by inoculating patient tissue or body fluids into either mice or tissue culture cells. *T. gondii* grows in a variety of tissue culture cells.

Prevention : Recommendations for prevention of toxoplasmosis for all persons, including pregnent women include following:

(i) meat should be cooked at safe temperatures;

(ii) fruits and vegetables should be peeled or washed thoroughly before being eaten;

(iii) cutting boards, dishes, counters, utensils, and hands should always be washed with hot soapy water after they have come into contact with raw meat, poultry, seafood, or unwashed fruits or vegetables;

(iv) individuals should wear gloves when gardening and during any contact with soil or sand, because cat waste might be in soil or sand, and wash hands afterwards;

(v) pregnant women should avoid changing cat litter. Pregnant women who must change cat litter should use gloves and then wash their hands thoroughly.

Several outbreaks have been reported in association with drinking untreated water contaminated by oocysts. Freezing meats for several days at subzero temperatures greatly reduces the risk of infection.

THE GENERA *PLASMODIUM* AND *BABESIA*

Plasmodium and *Babesia* are intraerythrocytic protozoan parasites that cause malaria and babesiosis, respectively. Although they differ in their epidemiology and life cycle, there is overlap between both clinical and diagnostic features of these two parasites, and for these reasons, they are discussed together.

The genus *Plasmodium*

Members of the *Plasmodium* genus infect a wide range of birds, mammals, reptiles, and amphibians worldwide using blood-feeding vectors. Despite there being at least 200 named *Plasmodium* species, only five regularly infect humans: ***P. falciparum, P. vivax, P. ovale*, *P. malaria*** **and** ***P. knowlesi.*** The Plasmodium parasite is transmitted primarily through the bite of an infected *Anopheles* mosquito (15).

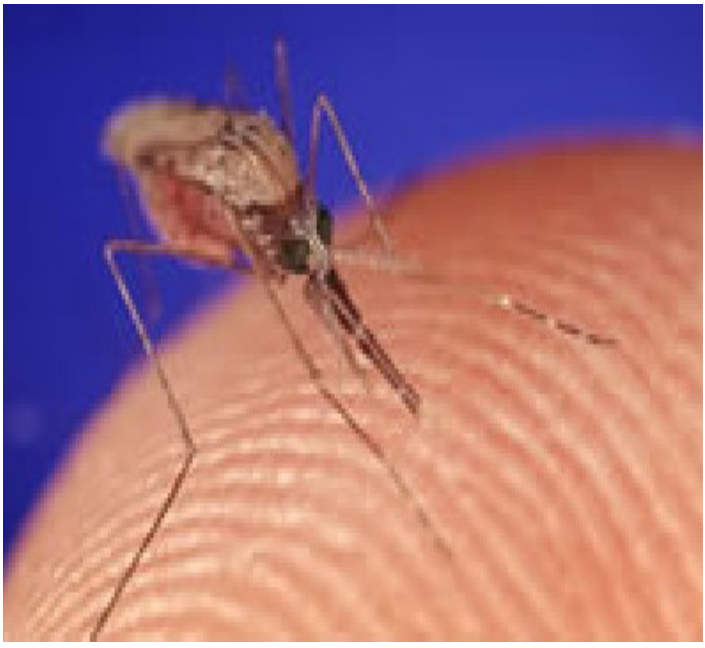


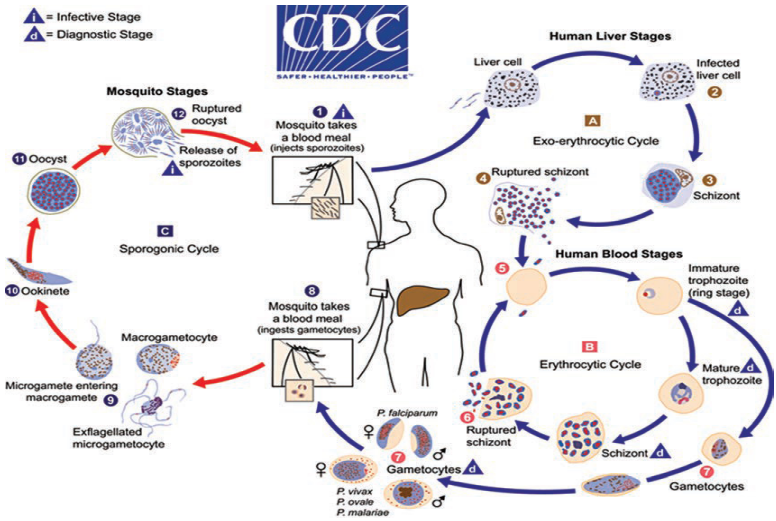
Figure 15. The mosquito taking its blood meal.

Epidemiology and Transmission: For practical purposes, malaria is endemic only in tropical areas of the developing world. However, the anopheline (any mosquito of the genus anopheles) mosquito vector necessary for malaria transmission is also present in the developed

countries such as the United States after the introduction of large numbers of infected persons (e.g., following the Korean and Vietnam wars and the more recent influx of refugees from Southeast Asia).

Malaria may also be transmitted by the transfusion of infected blood or blood products, by the sharing of syringes among drug addicts, or across the placenta to the developing fetus (congenital malaria). In most countries where malaria is nonendemic, the blood supply is partially protected through use of screening questionnaires if they have recently traveled to malaria-endemic countries or have had malaria recently.

Life cycle



The malaria parasite life cycle involves two hosts. During a blood meal, a malaria-infected female *Anopheles* mosquito inoculates sporozoites into the human host 1. Sporozoites infect liver cells 2 and mature into schizonts 3, which rupture and release merozoites 4. (Of note, in *P. vivax* and *P. ovale* a dormant stage [hypnozoites] can persist in the liver (if untreated) and cause relapses by invading the bloodstream weeks, or even years later.) After this initial replication in the liver (exo-erythrocytic schizogony A), the parasites undergo asexual multiplication in the erythrocytes (erythrocytic schizogony B). Merozoites infect red blood cells 5. The ring stage trophozoites mature into schizonts, which rupture releasing merozoites 5. Some parasites differentiate into sexual erythrocytic stages (gametocytes) 7. Blood stage parasites are responsible for the clinical manifestations of the disease. The gametocytes, male (microgametocytes)

and female (macrogametocytes), are ingested by an *Anopheles* mosquito during a blood meal 8. The parasites' multiplication in the mosquito is known as the sporogonic cycle C. While in the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes 9. The zygotes in turn become motile and elongated (ookinetes) 10 which invade the midgut wall of the mosquito where they develop into oocysts 11. The oocysts grow, rupture, and release sporozoites12, which make their way to the mosquito's salivary glands. Inoculation of the sporozoites 1 into a new human host perpetuates the malaria life cycle.

Clinical significance: Patients are asymptomatic for the initial 7 to 30 days incubation period during parasites replicate in the liver. It is only with subsequent infection and destruction of host erythrocytes that parasite antigens such as glucose phospate isomerase are released into the blood and stimulate cytokine production and resultant fever. The classic malarial fever paroxysm begins with rigors and chills, followed by an abrupt onset of fever which lasts for 1 to 2 hours. The paroxysm resolves with profuse sweating and a return to normal temperature. Fever is commonly preceded or accompanied by severe headache, malaise and myalgia. Infection can range from mild or asymptomatic disease, usually in individuals with pre-existing immunity, to severe, life-threatening disease. *P. falciparum* infection has the highest morbidity and mortality of the human plasmodium species.

Laboratory Identification: Preparation of thick and thin blood films is considered the gold standard for diagnosis of malaria. The Standards Institute (CLSI) recommends preparation of two thin and two thick blood films on initial evaluation of the patient, with preparation of repeat films every 6 to 8 hours for up to 3 days before excluding malaria from the clinical differential diagnosis, because parasitemia may be intermittent, and the number of circulating parasites may vary depending on the phase of the parasitic cycle.

Direct examination: Since *P. falciparum* infection can cause rapidly progressive fatal disease, blood films for malaria should always be performed. It should be accompanied by important patient information such as clinical signs and symptoms, travel history, and receipt of malaria chemoprophylaxis or therapeutic antimalarial agents, which might suppress parasitemia or alter parasite morphology**.**

Microscopy: Microscopic examination of Giemsa;stained **thick** and **thin blood films** is the traditional method for malaria diagnosis. The tick blood film is approximately 10 to 20 times more sensitive than the thin film. It is ideal for screening and parasite detection.

Staining of thick and thin films is best performed with Giemsa at a pH of 7.0 to 7.2. Wright-Giemsa stain may also be used.

Thick blood film preparation: The detection of the presence of a blood parasite is paramount, which is achieved most effectively through the analysis of the thick blood film. Once a parasite is detected through the screening of the thick blood film, then the thin blood film used to achieve species-level identification.

Thin blood film preparation: The most important feature in the differentiation of *Plasmodium* species is the morphological characteristics of plasmodiums in the erythrocytic cycle (schizogony). The one important characteristic is the size of the infected erythrocytes compared with uninfected erythrocytes. If the infected erythrocytes are the same size as uninfected erythrocytes, then the infecting species is either *P. falciparum* or *P. malariae.* If the infected erythrocytes are enlarged compared with the uninfected erythrocytes, then the infecting species is either *P. vivax* or *P. ovale*.(Table II)

Table II. Comparative morphology of Plasmodium spp. in Giemsa-stained thin films[a].

Diagnostic criterion	*P. falciparum*	*P. malariae*[b]	*P. vivax*	*P. ovale*	*P. knowlesi*
Size and shape of infected erythrocytes	Normal size and shape	Normal or slightly smaller size, normal shape	Normal or enlarged size, may appear molded against neighboring erythrocytes	Normal or enlarged size, frequently oval, may be fimbriated	Normal size and shape
Cytoplasmic inclusions	Occasional Maurer's clefts; larger (comma-shaped) and less numerous than Schüffner's dots	Ziemann's dots rarely seen; requires deliberate over-staining	Schüffner's dots/ stippling; may not be present in early trophozoites	Dark Schüffner's/ James' dots/ stippling; may not be present in early trophozoites	Irregular stippling in late trophozoites and schizonts
Parasite stages in peripheral blood	Early trophozoites and gametocytes	All stages	All stages	All stages	All stages
Multiply infected erythrocytes	Common	Rare	Occasional	Occasional	Common
Early trophozoite characteristics	Delicate rings, <1/3 diameter of the erythrocyte, frequently with double chromatin dots ("headphone" form); often at edge of erythrocyte ("appliqué/accolé form")	Rings ≤1/3 diameter of the erythrocyte; chromatin dot may appear unattached in center of ring ("bird's eye" form)	Rings ≥1/3 diameter of the erythrocyte; larger chromatin dot than *P. falciparum*	Rings ≥1/3 diameter of the erythrocyte; similar to *P. vivax*	Rings ≤1/3 diameter of the erythrocyte; double chromatin dots, rare appliqué forms; resembles *P. falciparum* early trophozoites
Mature trophozoites	Not typically seen in peripheral blood, compact thick rings	Compact cytoplasm, round, oval, basket or band-shaped, dark brown pigment	Amoeboid trophozoites, fine golden-brown pigment	More compact and less amoeboid than *P. vivax*, dark brown pigment	Slightly amoeboid; band forms common; scattered clumps of golden-brown pigment; resembles *P. malariae* mature trophozoites
Schizont characteristics	Not typically seen in peripheral blood, 8–24 merozoites	6–12 merozoites, often radially arranged around central pigment ("rosette" or "daisy head" schizont)	12–24 merozoites	6–14 merozoites	10–16 merozoites
Gametocyte characteristics	Crescent- or banana-shaped; distorting the shape of the erythrocyte	Round to oval; filling most of the erythrocyte	Round to oval; filling most of the erythrocyte	Round to oval; filling most of the erythrocyte	Round to oval; filling most of the erythrocyte

a)Adapted from MCM; b) Identification of *P. malariae* in patients with recent travel to Southeast Asia should raise the possibility of *P. knowlesi* infection, given the morphologic similarities of these two parasites. In this situation, severe clinical disease and a high parasite burden are consistent with *P. knowlesi* infection.

It is quite important that infections with *P. falciparum* be recognized as early as possible because the disease can be particularly severe, rapidly progressing to a fatal outcome.

The peripheral blood film interpretation may also be confusing if a given individual is infected with more than one *Plasmodium* species.

***P. falciparum*,** which is found worldwide in tropical and subtropical areas, and especially in Africa where this species predominates. *P. falciparum* can cause severe malaria because it multiples rapidly in the blood, and can thus cause severe blood loss (anemia). In addition, the infected parasites can clog small blood vessels. When this occurs in the brain, cerebral malaria results, a complication that can be fatal.

P. vivax, which is found mostly in Asia, Latin America, and in some parts of Africa. Because of the population densities especially in Asia it is probably the most prevalent human malaria parasite. Developental stages of *P. vivax* have been showed in Figure 16. The early stage trophozoite / ring (1), maturing ring (2), mature amoeboid trophozoites (3, 4), mature schizonts (5, 6), Macrogametocyte (7), microgametocyte (8). The infected erythrocytes are slightly larger than noninfected cells. *P. vivax* (as well as *P. ovale*) has dormant liver stages (hypnozoites) that can activate and invade the blood (relapse) several months or years after the infecting mosquito bite.

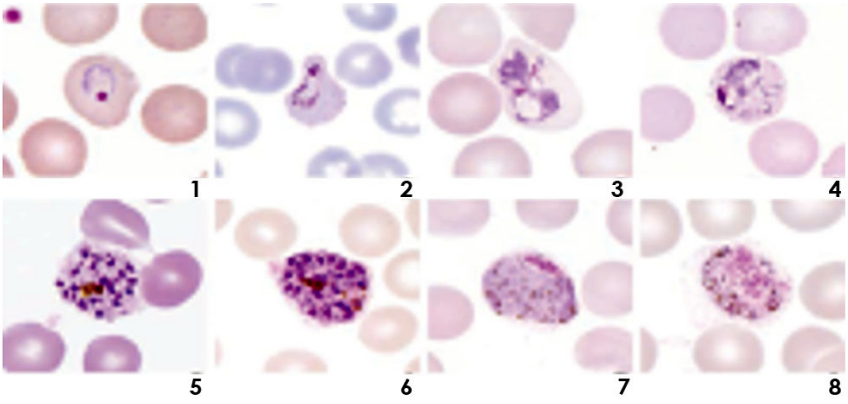


Figure 16. Developental stages of P. vivax in Giemsa-stained thin blood films.

P. ovale is found mostly in Africa (especially West Africa) and the islands of the western Pacific.

It is biologically and morphologically very similar to *P. vivax.*

P. malariae, found worldwide, is the only human malaria parasite species that has a quartan cycle. The three other species have a tertian cycle. If untreated, *P. malariae* causes a long-lasting, chronic infection that in some cases can last a lifetime.

P. knowlesi: There are approximately 156 named species of *Plasmodium* which infect various species of vertebrates. Four species are considered true parasites of humans, as they utilize humans almost exclusively as a natural intermediate host: *P. falciparum, P. vivax, P. ovale* and *P. malariae*.

However, there are periodic reports of simian (maymun) malaria parasites being found in humans, most reports implicating *P. knowlesi*. *P. knowlesi* has only recently been recognized as a significant cause of zoonotic malaria in parts of Southeast Asia particularly in Malaysia.

This species predominantly infects macaque monkeys (genus *Macaca*) but has been reported as the cause of malaria in 27.7% of cases in one study in Malaysia, and it has been identified in patients from Singapore, Thailand, Vietnam, and the Philippines. Indıvidual cases have also been reported from western travelers to Southeast Asia, including from Finland, Sweden, Austria, Spain, Great Britain, and the United States. *P. knowlesi* has a 24-hour replication cycle and so can rapidly progress from an uncomplicated to a severe infection; fatal cases have been reported.

Serologic tests: Serologic testing plays little role in diagnosis of acute malaria given that antibodies are frequently absent at the time of patient presentation. Also, a positive test does not differentiate between acute and past infection. For these reasons, serology has its greatest utility in epidemiologic studies, blood donor screening, and occasionally for evaluating relapsing, recrudescent, or untreated malaria in nonimmune patients. Indirect fluorescent antibody (IFA) tests using antigens prepared from the four human *Plasmodium* species are available.

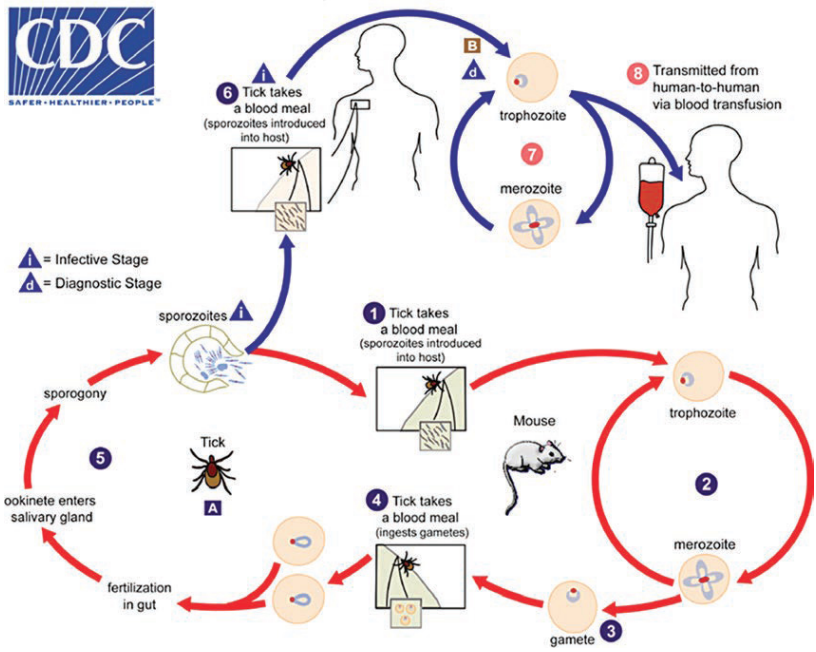
Treatment and Antimicrobial Susceptibilities: Parasite resistance to antimalarial medications is a serious problem worldwide. Chloroquine was a mainstay of treatment and prophylaxis for many years, but chloroquine-resistant strains of *P. falciparum* have been found in all regions endemic for malaria. Only rare instances of quinine resistance have been reported, allowing this drug to remain a therapy for severe malaria. There are a variety of other prophylactic and therapeutic options for malaria. Today, cases of malaria in the United States and Europe are almost exclusively

imported from individuals traveling from countries with ongoing malaria transmission. The majority of these imported malaria is detected in individuals who travel to endemic countries to visit friends and relatives.

The genus *Babesia*

Members of the genus *Babesia* infect a wide range of wild and domestic animals worldwide using primarly ixodid tick vectors. Despite there being more than 100 named *Babesia* species, only a few have been identified as causing human infections, including *B. microti*, *B. divergens*, *B. duncani*, and a currently un-named strain designated MO-1. Babesiosis in the U.S. is caused by *Babesia microti*. *B. divergens* causes a more severe disease in patients in Europe. The primary hosts are cattle and deer.

Life cycle



The normal zoonotic life cycle involves two hosts, which includes a rodent and an *Ixodes* tick.

(1)The rodent becomes infected when an infected tick introduces *B. microt*i sporozoites when taking a blood meal. (2)Sporozoites enter the rodent erythrocytes and most undergo asexual reproduction. (3) while some differentiate into female and male gametes. (4) Ticks become infected when ingesting *Babesia* gametes in the blood of an infected rodent. (5) Within the tick (A), the gametes undergo sexual reproduction to produce sporozoites. (6) As with rodents, humans become infected through the bite

of an infected *Ixodes* tick. (7) Sporozoites enter human erythrocytes (B) and undergo asexual replication. (8) Less commonly, human to human transmission is well recognized to occur through blood transfusions.

Geographic distribution: Worldwide, but little is known about the prevalence of *Babesia* in malaria-endemic countries, where misidentification as *Plasmodium* probably occurs. In Europe, most reported cases are due to *B. divergens* and occur in splenectomized patients. In the United States, *B. microti* is the agent most frequently identified (Northeast and Midwest), and can occur in nonsplenectomized individuals. *Babesia duncani* has been isolated in patients in Washington and California. MO-1 has been isolated from patients in Missouri.

Transmission: Babesia is transmitted by the bite of an infected ixodid tick vector; which is the same vector for *Borrelia burgdorferi*, the spirochete that causes Lyme disease. It has been shown that up to 40% of infected ticks are coinfected with both parasites. Both agents have the same main reservoir host " white-footed mouse" , *Peromyscus leucopus,* and other rodends. The seasonality of babesiosis corresponds with the activity of the tick vectors, with most cases occurring in the spring, summer, and fall. Less commonly, human babesiosis is acquired through receipt of infected blood products or by vertical transmission across the placenta. It has been established that *B. microti* can remain infective at 4°C for 30 days, the normal storage time for donor blood.

Clinical significance: Symptoms of babesiosis can range from asymptomatic or mild to life-threatening. Although many infections with *Babesia* are subclinical, severe-to-life-threatening disease occurs in patients without a spleen. When present, symptoms may include fever and a nonspecific influenza-like illness. Severe disease may behave like malaria, with high fever, chills, night sweats, myalgia, hemolytic anemia, hepatosplenomegaly, and jaundice (icterus).

Laboratory diagnosis

Microscopy: As with malaria, the conventional method for diagnosis of babesiosis is microscopic examination of Giemsa-stained thick and thin blood films. *Babesi*a spp. parasites may present a diagnostic challenge on blood films due to the many morphologic similarities they share with *Plasmodium* species (specifically *P. falciparum*) on thick and thin films. It is important to note that only ring-type forms are seen in babesiosis (Figure 17). The ring forms are resembling the early trophozoites of *P. falciparum.* The presence of hemozoin pigment, schizonts, or plasmodial-type gametocytes exludes the diagnosis of babesiosis. When the morphology is insufficient for differentiation of *Babesia* and Plasmodium parasites, it is

helpful to obtain a complete travel history examine multiple blood samples for parasites, or perform an alternative detection method (e.g., PCR).

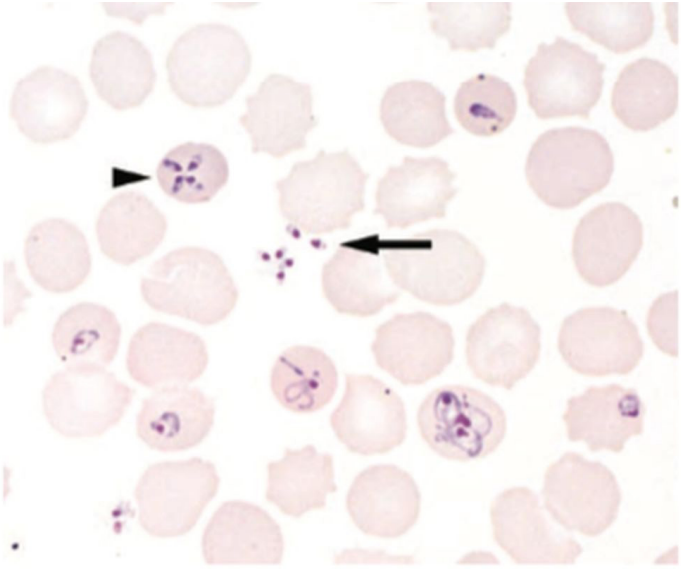


Figure 17: Babesia sp. in a thin blood smear stained with Giemsa.

Serologic tests: Serologic testing plays little role in the diagnosis of acute babesiosis, but may be useful for epidemiologic studies and in cases of chronic disease. Antibodies to *B. microti* typically appear 2 weeks after the onset of illness and may be detectable for several years after infection. The indirect fluorescence immunoassay (IFA) is the recommended method for detection of serum antibadies against *B. microti* and has a relatively high sensitivity and specificity. *B. microti* IFA serology does not demonstrate significant cross-reactivity with antibodies to *B. divergens*, or other species. If expertise for differentiation of *Babesia* and *Plasmodium* parasites is not available in the primary laboratory, it is important to inform the clinician that *Plasmodium* or *Babesia* parasites are identified and blood and/or films are being sent to a reference or public health laboratory for further analysis.

Treatment: Most asymptomatic persons do not require treatment. For ill patients, babesiosis usually is treated for at least 7-10 days with a combination of two prescription medications. Clindamycin plus quinine. This combination is the standard of care for severely ill patients.

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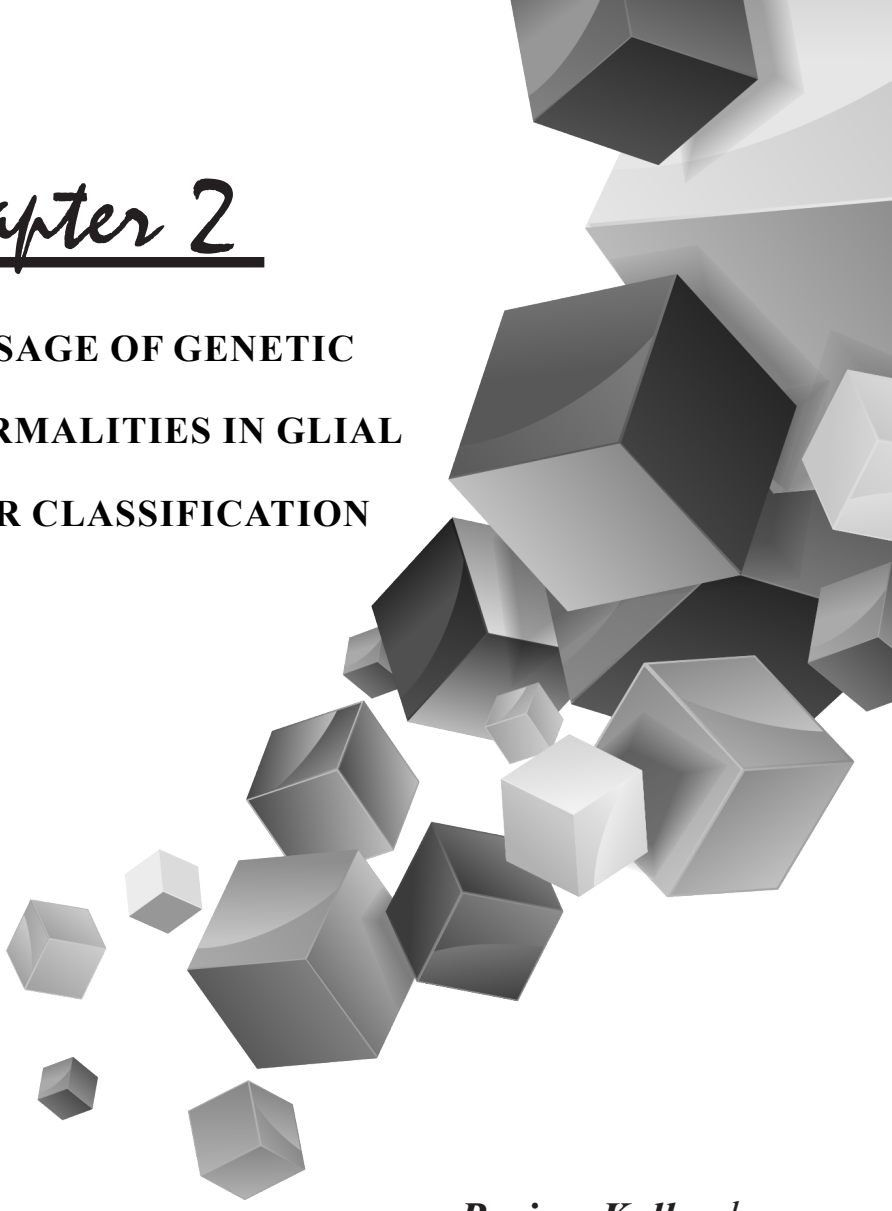
Chapter 2

THE USAGE OF GENETIC ABNORMALITIES IN GLIAL TUMOR CLASSIFICATION



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Introduction

The brain and spinal cord tumors which are derived from glial cells are called glioma. These tumors are highly invasive and leads to irregular tumor margins which are not precisely identifiable by medical imaging, thus it causes difficulties for the resection of the tumor. The understanding of tumor pattern is essential for both radiological therapy as well as surgical treatment and targeted treatment strategies (Engwer et al 2016).

Gliomas display several characteristics, which includes histological, immuno-histochemical, and genetic alterations (Maher et al 2001). The classification of the brain tumors is based on the World Health Organization (WHO) and identification of the tumor includes nuclear atypia, mitosis, necrosis, and microvascular proliferation (WHO 2007). Glioma classification updated in 2016 by WHO and genetic variants have been added in tumor classification (Molnár, 2011, Louis et al 2016, Wesseling and Capper 2018).

Gliomas are classified according to their origin and depending on their histological features and degree of malignancy they are classified into IV different categories (Maher et al 2001, WHO, 2016). The biological origin of oligodendroglial tumors and gliomas is different and there is no defined molecular marker to distinguish oligodendrogliomas from astrocytoma's. Due to this limitation, the histopathological diagnosis of oligodendroglioma is difficult (Lu et al 2001). Glial fibrillary acidic protein (GFAP) and S-100 β are astrocytic glial cell markers and expressed in astrocytomas. These markers are useful for the diagnostic evaluation of astrocytomas (Lu et al 2001). Lu et al. identified oligodendrocyte specific expression of OLIG1 and after screening 23 glial tumors they observed OLIG gene expression is occurs in only oligodendroglial tumors (Lu et al 2001). This shows us that the genetic abnormalities are tumor specific and they can be useful for classification of the tumor subtypes.

In gliomagenesis there are several pathways indentified which they have important role for glioma progression which includes; Platelet-derived growth factor (PDGF) and epidermal growth factor (EGF), receptor tyrosine kinase (RTK) signaling pathways; p53 and RB pathway (Maher et al 2001) and WIP/YAP/TAZ pathway (Rivas et al 2018). Studies demonstrated that the miRNAs are also plays a key role during the development and therapy resistance of GBM (Ondracek et al 2017) (Table 1).

The other important driving force of the gliomas is the activation of hypoxia-inducible factor 1 (HIF-1) pathway which was related with the glioma angiogenesis. HIF pathway is related with promoting proangiogenic properties and invasive properties of gliomas. Malignant gliomas are rich for hypoxic regions and these regions shows increased

activity of HIF. Increased activity of HIF results with increased expression of HIF target genes which they contribute tumor growth and vascularization of tumor (Kaur et al 2005). HIF pathway plays an important role during the maintenance of glioblastoma stem cells and could be a possible targeted treatment choice in gliomas.

Recent studies highlighted that the glioma stem cells (GSCs) which are related with the recurrence of disease and resistant to radiation and chemotherapy (Cho et al 2013) because of self-renewal properties as well as multi-lineage differentiation (Lathia et al 2015). Glioma stem cells has been shown a several molecular markers which they expressed CD133, CD15, A2B5, Nestin, ALDH1, proteasome activity, ABC transporters, label-retention (Lathia et al 2015) and recent data's combines these characteristics with genetic abnormalities like IDH mutations for evaluation of the disease progression (Bralten et al 2011). Increased molecular technologies gives huge amount of information for glioma development, glioma stem cells, molecular markers and highlighted combined targeted therapy strategies against to the glial stem cells.

Growth factor pathways:

Platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) plays an important role during gliomagenesis. During the neural stem cell proliferation and survival EGF plays a key role (Maher et al 2001). Increased level PDGF expression in low-grade gliomas and increased level EGFR expression in GBM makes possible targets for these pathways during the glioma development (Maher et al 2001).

Ras/MAPK (MAP kinase), PI3-K (phosphoinositide 3-kinase), PLC (Phospholipase C), and JAK-STAT (signal transducers and activators of transcription) are related with RTKs signaling pathway and they have a critical role during the proliferation, migration and cytokine stimulation of tumor (Pandey et al 2016). PDGF important for tumor migration and activation of downstream pathways which includes Ras, PI3- K, JAK-STAT and PLC pathways (Willis et al. 2000, Westermark 2014). EGFR is one of the members of tyrosine kinase (TK) family (Brennan et al 2013) and plays an important role during the tumor growth and reduces apoptosis in glioma (Nagane et al. 1996, Maire and Ligon 2014, Cimino et al 2015). Overexpression, deletion or amplification of EGFR detected in high-grade astrocytomas (Brennan et al 2013, Libermann et al. 1985; Wong et al. 1987, Bigner et al. 1990, Westphal et al 2017, Wong et al. 1987, Francis et al 2014, Huang et al 2016). Epidermal Growth Factor like domain 7 (EGFL7) is another important prognostic marker in cancer and it was associated with tumor grade (Wang et al 2017). Recent studies showed that the upregulation of the EGFR pathway and related

downstream pathways are important for self-renewal of glioma cancer stem-like cell (GSC). Soeda and colleagues showed that the inhibition of p38 MAPK pathway causes increasing of EGFR expression and reduced proliferation. They conclude that the p38 MAPK controls the EGFR signaling activity and changes GSC cell cycle (Soeda et al 2017).

p53 Pathway:

P53 is the guardian of the genome and has an important function during the G1-S phase of the cell cycle. P53 checks the DNA damage before replication if any damage was detected P53 blocks the cell cycle and gives chance to repair abnormality and if the abnormality does not repair the cells should be died via apoptosis. P53 and the downstream target genes, like MDM2, p21, Bax, were important to prevent the malign transformation or uncontrolled cell division.

P53 is one of the widely studied biomarker in human glioma (Jin et al 2016). Loss of chromosome 17p and p53 mutations are early event in gliomagenesis because both of the abnormalities were detected in low grade gliomas and anaplastic astrocytomas (Malkin et al. 1990, Louis 1994, Jin et al 2016). The low level P53 amplification were detected in astrocytomas and GBMs. According to the previous studies the p53 expression was associated with pathological grade of gliomas (Jin et al 2016, Fu et al 2015).

The mutational hotspots of TP53 are located in exons 5-8 in gliomas. Gillet and colleagues demonstrated mutational status of TP53 were correlated with expression of p53, tumor type, 1p/19q co-deletion and clinical features of patients. They conclude that TP53 and p53 status were not used as a prognostic marker in low grade gliomas (Gillet et al 2014). Zhu and colleagues demonstrated that the gain-of-function TP53 mutations are another driver for tumor growth and missense substitutions gives a selective advantage during tumor progression (Zhu et al 2015).

Rivas and colleagues detected activation of actin cytoskeleton-associated protein WIP (WASP-interacting protein) was related with mutation on p53 and correlated with tumor growth. Studies showed that the mutant p53 and WIP knockdown cells showed decreased level proliferation and limited growth of cancer stem cell (CSC)-like cells. Rivas and colleagues demonstrated new CSC signaling pathway which is located downstream of mutant p53 and regulates WIP and controls YAP/TAZ stability (Rivas et al 2018).

RB pathway (RB, CDK4/6, and INK4a):

Retinoblastoma gene (Rb1) is one of the important regulators of the cell-cycle. Allelic losses of chromosome 9p, 13q and amplification of 12q

was detected during the transition of gliomas (anaplastic astrocytomas) and these abnormalities are critical components of the RB pathway (Schmidt et al. 1994; Ueki et al. 1996, Liaguno and Parada 2016).

Retinoblastoma gene (RB1) is an important regulator of cell-cycle progression and localized on chromosome 13q14 (Sherr 1996). The phosphorylation of pRB is the key event of S phase which triggers entry into S phase. CDK inhibitors (CKIs) are important for the negative regulation of the CDKs and inhibits the G1 entrence. The RB mutations, CKIs and the epigenetic modifications on CKI and CDKs are triggers uncontrolled cell cycle entry (Sherr 1996).

In 25% of high-grade astrocytomas carries RB mutations (James et al. 1988; Henson et al. 1994, Sippl et al 2018). Ferreira and colleagues proposed that the evaluation of the RB/E2F pathway related genes could be used for the disease progression in astrocytomas (Ferraira et al 2015).

Chromosome 10q (PTEN, Mxi1, DMBT1)

PTEN (phosphatase and tensin homology) is located on chromosome 10q and LOH of PTEN identified in 75%–90% of gliomas (Fults and Pedone 1993; von Deimling et al. 1993, Lang et al. 1994, Han et al 2018). PTEN is a critical therapeutic target in gliomas and plays a key role during the tumorigenesis (Nan et al 2017). Loss of 10q is most common genetic abnormalities in GBM (Arslantas et al 2004, Arslantas et al 2007). Cluster of differentiation 164 (CD164), is associated with proliferation, apoptosis, adhesion and differentiation procedures in cancer. Tu et al showed that increased expression CD164 was associated with proliferation and apoptosis via PTEN/phosphoinositide 3-kinase/AKT pathway, and it should be a potential therapeutic and diagnostic target of glioma (Tu et al 2017).

Chromosome 1p/19q

Deletion of chromosome 1p/19q is a common finding in oligodendrogliomas (Louis and Cavenee 2000; Reifenberger et al. 2000). This co-deletion has been added WHO 2016 classification (WHO 2016). This deletion occurs in low-grade tumors which means they are early alterations in oligodendroglioma development (Louis and Cavenee 2000; Reifenberger et al. 2000, Arslantas et al. 2007). Leeper and colleagues were suggested that 1p/19q co-deletion, IDH mutation, and ATRX loss together with neuropathological evaluation should be added in WHO grade II diffuse glioma classification (Leeper et al 2015).

The deletion of 1p19q is a favorable prognostic marker in oligodendrogliomas (Ino et al 2001, Smith et al 2000) and associated with

PCV (procarbazine, lomustine (CCNU), vincristine) and temozolomide response (Cairncross et al 2013, Intergroup Radiation Therapy Oncology Group Trial 9402).

IDH1 and IDH2 mutations

Isocitrate dehydrogenase 1 (IDH1) mutation was discovered by Parsons and colleagues at 2008 in GBM samples and they showed the association between mutation and an increased overall survival in secondary GBM samples (Parsons et al 2008). IDH1 R132H mutation found in 90 % of gliomas and 65–80% of grades II and III gliomas (Hartmann et al 2009, Yan et al 2009, Watanabe et al 2009). IDH1 mutation was detected in 5% of primary GBM (Hartmann et al 2009, Yan et al 2009, Kim et al 2012, Zhang et al 2013, Kalkan et al 2015) and in 64–93% of oligodendrogliomas (WHO II and III) (Balss et al 2008, Bleeker et al 2009, Hartman et al 2009). IDH mutation is associated with different genetic abnormalities in gliomas as shown in table 3.

In general mutations of IDH is specific to low grade gliomas and secondary GBM. Mutations of IDH genes are independent predictor of response to temozolomide or radiation therapy and associated with the better prognosis (Network et al 2015, Zou et al 2013, Houillier et al 2010, Leu et al 2013, Sun et al 2013).

IDH1 and IDH2 mutations lead to the enzyme affinity for alpha ketoglutarate and causes accumulation of D-2-OH-glutarate (D-2-HG) (Dang et al 2009) and inhibits TET enzymes which remove methyl groups from DNA (Xu et al 2011) and triggers hypermethylation phenotypes in IDH mutant cells. The induction of histone methylation by 2-HG at H3K9 and H3K27 (Turcan et al 2012) prevents differentiation of cell and expression of a stem cell-like phenotype (Lu et al 2012).

Today testing of IDH1 mutation is important tool during the classification of glioma which gives important prognostic information and clinical decision making for gliomas. IDH mutation is an early event and driving force during the glioma progression.

Gliomas can be developed in different pathways from a same precursor cell. The initial event is IDH mutation, depending on the presence or absence of the IDH mutation, gliomas develop into two different pathways. Oligodendroglial tumors carries 1p/19q co-deletion and TERT mutations, astrocytomas carries ATRX and p53 mutations and oligoastrocytomas contains p53 and TERT mutations. Low-grade gliomas can dedifferentiate into anaplastic oligodendroglioma or astrocytoma and secondary GBM. The GBMs can be classified into two subgroups, as a primary and a secondary GBMs. These two GBM subtypes are carried different genetic

abnormalities. The primary GBM develop directly from precursor cell and 5% of these tumors contains IDH mutation and secondary GBM develop from low grade astrocytic tumors (Appin et al 2015, Appin et al 2015, Karsy et al 2017) (Table 2).

Recent studies highlighted that, in poor prognosis GBM cases together with IDH1 mutations, CDKN2A/2B homozygous deletions were also detected and CDK4, PDGFRA, MYCN and CCND2 amplification were detected in IDH1 mutated glioblastomas (Korshunov et al 2018).

ATRX

The protein α thalassemia/mental retardation syndrome X linked (ATRX) is the member of the SWI2/SNF2 family of DNA helicase and plays an important role during the chromatin remolding. ATRX gene mutations are associated with the ALT (lengthening of telomeres) phenotype (Heaphy et al 2011) and it was identified in pediatric and adult glioblastomas in 2011 (Heaphy et al 2011, Eckel-Passow et al 2015). Tumors which carries IDH mutations and loss of ATRX is associated with improved progression-free and overall survival (Cai et al 2016, Wiestler et al 2013). This situation is associated with mutations in IDH and TP53 genes. These abnormalities can be used to identification of astrocytic origin of tumors (Eckel-Passow et al 2015). ATRX mutation was detected in 71% of grade II-III astrocytomas, 57% of secondary GBM cases (Ceccarelli and 2016, Kannan et al 2012, Liu et al 2012). Controversially, wild-type ATRX with IDH mutation and 1p19q co-deletion are the characteristic markers of the oligodendroglial tumors (Eckel-Passow et al 2015). The astrocytomas carries IDH-mutation, ATRX, TP53, and oligodendrogliomas carries codeletion of 1p/19q, CIC1 or FUBP1 mutations. ATRX can be used as a prognostic factor in gliomas and important for the molecular classification of gliomas.

BRAF

BRAF is an important regulator of MAPK signaling and stimulates tumor growth (Schindler et al 2011, Chi et al 2013, Dehiya et al 2014). In many cancer types BRAF acts as a proto-oncogene. BRAF abnormalities were detected in 20–75% of gangliogliomas, 50% of anaplastic gangliogliomas, lower than 10% of pilocytic astrocytomas (Schindler et al 2011). Phillips and colleagues identified genetic landscape of anaplastic pleomorphic xanthoastrocytoma and found combination of CDKN2A biallelic inactivation and BRAF mutations in these tumor samples (Phillips et al 2018). BRAF abnormalities are not prognostic factor in gliomas, it is also associated with specific histologic sites which were important for glioma classifications.

MGMT

O6-methylguanine methyltransferase (MGMT) is a DNA repair enzyme which removes alkyl groups from guanine. The temozolomide and lomustine (CCNU) used in GBM treatment as an alkylating chemotherapeutics. The status of MGMT promotor is more important during this treatment. Methylation of MGMT is associated with improved responses to alkylating agents and also improved progression free (PFS) and overall survival (OS) time (Hegi et al 2005, Stupp et al 2005, Yoshioka et al 2018). Now MGMT methylation is routinely used on treatment evaluation of GBM.

RARβ

In glioblastomas, many genes shows different epigenetic characteristics these genes include hypermethylation of the MGMT, RASSF1A, p16/ INK4A and p15/INK4. These genes are important for prognosis, treatment decision making and survival for GBM patients (Burgess et al 2008, Lei et al 2010). The RARβ is important for the morphogenesis, cell growth and differentiation during embryonic period. The methylation of RARβ gene was demonstrated in intracranial ependymomas during the childhood period and in choroid plexus tumors (Piperi et al 2010). The first study regarding to the RARβ methylation was done by Piperi and colleagues and detected 58.8% of RARβ methylation in grade II-IV tumors. They conclude that there is a positive correlation between RARβ methylation and radio/ chemotherapy response in GBM (Piperi et al 2010). The second study for RARβ gene methylation was published by Atli et al and showed association between RARβ gene methylation and longer survival time and positive correlation for the radio/ chemotherapy treatment response in GBM. They suggested that methylation status of the RARβ gene may be use as a positive prognostic marker in glioblastoma (Atli et al 2016).

RNA Methylation in gliomas

Increased number of studies in the field of cancer epigenetics identified epigenetic based cancer treatment by using DNA and histone modifications in different type of cancer (Eckschlager et al 2018). Recently, RNA methylation is another hot topic in cancer research (Du et al 2018, Wang et al 2018).

After discovery of m6A (N6-Methyladenosine) modification in RNA, researchers showed it has a pivotal role in many biological processes. Until now, 171 RNA modification has been identified in MODOMICs database and 72 of these modifications contains methyl group in their structure but the role of m6A in cancer biology, cancer stem cells does not fully understand

(Boccaletto et al 2017). Recent studies demonstrated that this modification has an important role during the self renewal of glioblastoma stem cells and has a critical role during the tumorogenesesis (Cui et al 2017), differentiation and pluripotency of the cell (Jaffrey and Kharas 2017).

Up to now, METTL3, METTL14, NSun2, FTO, ALKBH5 and YTHDF2 identified as a catalytic sub units of the m6A methyltransferase complex and methylated in different types of cancers (Wang et al 2018). Researchers showed the decreased level of m6a is associated with glioblastoma stem cells (Cui et al 2017, Mochizuki and Okada 2007). Also, Cui et al demonstrated that m6a modification is related with self-renewal of glioblastoma stem cells and critically important for tumor growth and differentiation of tumor cell (Cui et al 2017). Identification and reversible nature of these modifications makes them a potential new therapeutic target in GBM.

Future Directions and Conclusion

Identification of biological background of malignant gliomas helps discovery of novel targets of therapy. The advancement of genomic, epigenomic and proteomic profiling of brain tumors provided significant importance for diagnosis and treatment planning of brain tumors. Identification of molecular characterization of gliomas shows predicative, prognostic, and therapeutic values of different genetic abnormalities. Beginning of the 2000s the accepted diagnostic genetic abnormality is 1p/19q in oligodendroglial tumors but now the IDH mutations are more important for disease evaluation and also MGMT, EGFR, ATRX, TERT, and BRAF are now being used to classify brain tumors and also being used as therapeutic targets. Today the discovery of the m6a modification promises big expectancy in new treatment discovery. Together with genetic abnormalities in gliomas, epigenetic abnormalities were also important during the treatment planning of gliomas. Identification of novel abnormalities will be highlight the targeted treatment planning of gliomas. Epigenetic drugs DNMT inhibitors azacitidine and decitabine are the FDA approved drugs for treatment of myelodysplastic syndromes and acute myeloid leukemia. The usage of HDACi and DNMT in epigenetic based cancer treatment represents a new hope in glioma treatment.

Conflict of interest

All authors certify that they have NO affiliations with or involvement in any organization or entity with any financial or non-financial interest in the subject matter or materials discussed in this manuscript.

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TABLE LEGENDS

Table1. Signaling pathways and their importance for glial cells

Table 2. List of affected genes in gliomas

Table 3. IDH mutation and associated genetic abnormalities in gliomas.

Table 4. The important diagnostic and prognostic epigenetic markers for glial tumor

Table1. Signaling pathways and their importance for glial cells

Signal Pathways	**The importance of the pathways in Glial cells**
EGF	Neural stem cell proliferation and survival
PDGF	Glial development Modulating astrocyte migration Stimulate migration of oligodendrocyte progenitor cells
Ras/MAPK (MAP kinase) PI3-K (phosphoinositide 3-kinase) PLC-C (Phospholipase C) JAK-STAT (–signal transducers and activators of transcription)	Regulate cellular proliferation Cell scatter and migration Cytokine stimulation
RB pathway	Cellular differentiation
HIF pathway	Promoting proangiogecity and invasion
WIP/YAP/TAZ pathway	Promote tumor growth and tumor-initiating cell phenotype

Table 2. List of affected genes in gliomas

Genes	**Oligodendroglioma**	**Astrocytoma and GBM**	**GBM with intermediate prognosis**	**GBM with poor prognosis**	**Gangliogliomas**
TERT mutation	+	+	-	+	-
IDH mutation	+	+	-	-	-
1p/19q co-deletion	+	-	-	-	-
MGMT methylation	+	+	+	-	-
BRAF mutation	-	-	-	-	+

Related mutations	CIC, FUBP1, NOTCH, PIK3CA, PIK3R1, loss 4, hemizygous loss of CDKN2A/B	FOXG1,ATRX, TP53, gain 7q, 8q24 (MYC), 4q12, 8q24, 12q14, hemi-zygous loss of CDKN2A/B, co-amplified CDK4 and GLI1	EGFR, PTEN, NF1, gain 7, 19, amplification EGFR, homozygous deletion CDKN2A/B	EGFR, PTEN, NF1, RB1, PIK-3CA, PIK3R1, gain 7, 19, amplification EGFR and MDM2, homo-zygous deletion CDKN2A/B, co-amplified CDK4 and GLI1	

Table 3. IDH mutation and associated genetic abnormalities in gliomas.

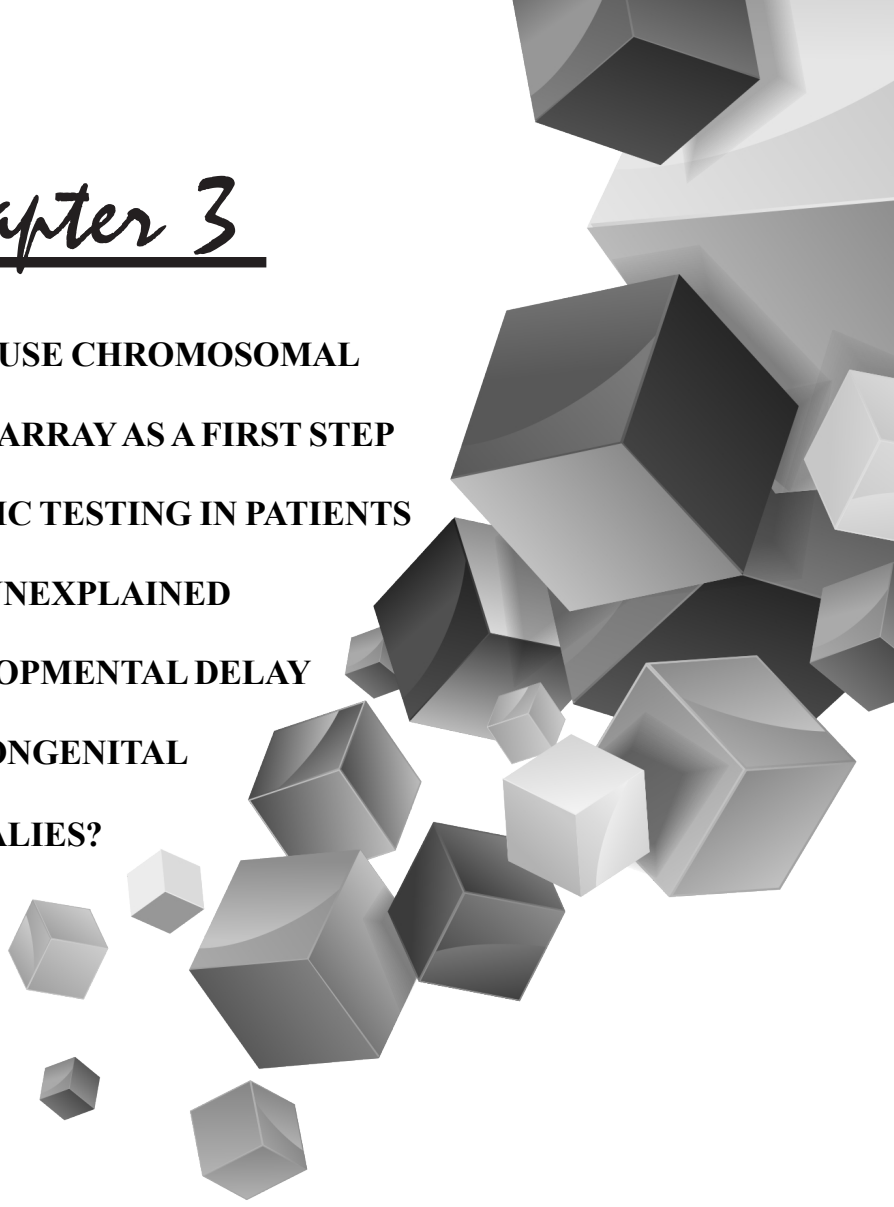
IDH Mutation Status	Co-associated abnormalities	Glioma type
IDH+	TP 53 mutation	Astrocytomas
IDH+	1p/19q co-deletion	Oligodendroglioma
IDH+	PTEN, EGFR, CDKN2A/ CKKN2B mutations	Primary GBM

Table 4. The important diagnostic and prognostic epigenetic markers for glial tumor

Gene	Tumor type	Importance	Diagnostic methods
MGMT	Glioblastoma	Response of alkylating chemotherapeutics	Immunohistochemistry Methylation specific PCR Methylation specific-HRM Bisulfite sequencing Pyrosequencing
RARβ	Glioblastoma	Longer survival time and response for the radio/ chemotherapy treatment	Immunohistochemistry Methylation specific PCR Methylation specific-HRM Bisulfite sequencing Pyrosequencing
SPINT2	Glioblastoma cell line (U87)	Unknown in GBM patients	Methylation specific PCR Methylation specific-HRM Bisulfite sequencing

Chapter 3

DO WE USE CHROMOSOMAL MICROARRAY AS A FIRST STEP GENETIC TESTING IN PATIENTS WITH UNEXPLAINED DEVELOPMENTAL DELAY AND CONGENITAL ANOMALIES?



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Introduction

The failure of achievement of developmental milestones within the expected age range was called as a developmental delay (DD). Developmental delay (DD) is related with variable problems on central nervous system which causes dysfunction, and observed in 3% of the general population.[1] According to the American Association on Intellectual and Developmental Disabilities, DD phenotype includes gross or fine motor skills, speech and language, cognition, personal-social and activities of daily living.[2]

Approximately 40% patients of DD have a genetic etiology, which includes chromosomal abnormalities (trisomies, microduplications, microdeletions), monogenic causes (e.g., FRAX) (3,4,5) or great spectrum of copy-number variants (CNVs), single gene sequence variants or small insertions, duplications, deletions in DNA or defects in the epigenetic mechanisms [6,7,8].

The International Collaboration for Clinical Genomics (ICCG), International Standard for Cytogenomic Array (ISCA) Consortium, has offered CMA as the first-tier diagnostic test for DD.[9] The American College of Medical Genetics (ACMG) has also published guidelines of CMA using for DD cases.[10,11] Chromosome microarray analysis (CMA) is widely accepted diagnostic test for DD of unknown etiologies.[12] Advancement of technology like whole exome sequencing (WES) helps identification of rare heterozygous *de novo* mutations in children with undiagnosed developmental disorders. [13]

DD appears isolated or other congenital malformations, neurological features and behavioral problems. [3,5] DD is phenotypically and genetically wide-ranging and a specific diagnosis is not applied in some cases.[3] The diagnosis of DD is very important because of potential treatment options, to provide prognostic information, gives chance to access special education, gives chance for prenatal diagnosis or pre-implantation genetic diagnosis for future pregnancies. [14] CMA is the first-tier diagnostic test for patients of DD. [3,12,14,15]

In this study, we were aimed to determine diagnostic yield of recurrent and non-recurrent or pathogenic/ likely pathogenic chromosomal abnormalities on a cohort of 53 Turkish cases with unexplained DD/ congenital anomaly DD. We show the array-CGH is an applicable clinical testing in unexplained DD/congenital anomaly.

Material and Methods

A total of 53 cases with unexplained DD/congenital anomaly were diagnosed at the Department of Medical Genetics (Trakya University, Edirne,

Turkey), including 30 girls and 23 boys with median age of 5 (0–32 years). Before genetic analysis being performed, parents signed an informed consent.

Genomic DNA samples were obtained from peripheral blood using MagNaPure system (Roche Ltd., Basel, Switzerland). The quality/quantity were assessed by Qubit® 1.0 (ThermoFisher Scientific)and NanoDrop® ND-1000 (ThermoFisher Scientific, Waltham, MA, USA).

In order to identify chromosomal rearrangements CMA was performed using DNA microarray platforms (180 K): Agilent 4x180K ISCA CGH+SNP Array (Agilent Technologies, Santa Clara, CA, USA) and Cytosure ISCA 4X180K UPD array (Oxford Gene Technology, Oxfordshire, UK).

Human Genomic DNA reference was used to match with samples (Agilent Technologies or Promega, Madison, WI, USA). DNA Microarray Scanner (Agilent Technologies) was used for scanning of microarray slides. Agilent Feature Extraction software 12.0.2.2 and Agilent CytoGenomics 4.0 were used for data evaluation.

Results

Total of 53 cases with unexplained DD/congenital anomaly were examined at the Department of Medical Genetics (Trakya University, Edirne, Turkey). Considering 38 patients with normal karyotype, we detected deletions and duplications in 28 % patients (15/53). Figure 1 and 2 is an example of a deletion/duplication finding of array-CGH (Patient number 31 - 35). Clinical characteristics, age at testing and aCGH results of all of the cases has been shown in Table 1.

The abnormal group included 15 patients which recurrent microdeletion and microduplication syndromes had been identified. Microdeletions are: 22q11.2, 7p22.1-p21.3, 22q11.2, 14q31.1, 22q11.21, 22q11.1-q11.21, 16p11.2, 17q24.1-24.3, 16q24.3, 2q37.2q37, 17p13.2 and microduplications are: 19q13.41, 1q21.3, Xp22.12-p21.3, 7q11.23.

Online database analysis of aCGH results

We used; DGV (Database of Genomic Variants), DECIPHER (Database of Chromosomal Imbalance and Phenotype in Humans Using Ensembl Resources), OMIM (Online Mendelian Inheritance in Man), PubMed, ClinVar, International Standards for Cytogenomic Arrays Consortium (ISCA, https://www.iscaconsortium.org/) and the UCSC Genome Browser for evaluation of aCGH data. All genomic coordinates are based on assembly of Genome Reference Consortium build 37 (GRCh37)/UCSC hg19.

Discussion

DNA microarray helps for identification of submicroscopic rearrangements in pathogenesis of DD. [12] High sensitivity, accuracy and diagnostic yield make them as a first-tier clinical diagnostic test. [12,16] Several studies showed that submicroscopic chromosomal rearrangements can be identified in 15–20% children with DD. [12,14,17,18] An increasing number of smaller pathogenic CNVs are expected to be discovered as there is a tendency for an increasing number of laboratories to use higher resolution CMA platforms.. [19]

Wayhelova and colleagues examined 542 children with ASD, ID/DD and MCA by array-CGH. They detected large regions or non-polymorphic CNVs of CNN-LOH with potential clinical relevance in 32.7% of cases (177/542). They identified 15q11.2 microdeletion in three cases, 16p11.2 microdeletion in one case, 15q11.2-q13.1 duplication in one case and two cases of 22q11.21 microduplication. [20]

Shin and colleagues performed multiplex ligation-dependent probe amplification (MLPA) analysis and CMA in 96 patients with unexplained DD, ASD or MR. They identified pathogenic CNVs in 15 patients by CMA. The diagnostic yield of CMA was reported as a 15.6%, which was the higher from previous reports including chromosomal aneuploidies (12.2%). [21]

Our results were higher than all of the previous results[12,20] we detected deletions and duplications in 28 % patients and the frequency of deletion was higher than duplications (60%- 40%).

Wang et al analyzed 489 patients with DD/ID and they identified 126 cases (25.8%) of pathogenic CNVs by CMA. Microdeletion/ microduplication syndromes were identified 79 out of 126 cases and 76 cases were classified as different syndromes, and 3 cases were classified as rarer syndromes, including Xq28 microduplication syndrome, 15q24 microdeletion syndrome and Lowe syndrome [22]. Shaw-Smith et al. tested 66 patients with DD, and detected pathogenic CNVs in 21% of cases. [23] Lee et al. analyzed 177 DD/ID patients by using array-CGH examination and they diagnosed pathogenic CNV in 27.7% (49/177) of cases. Pathogenic CNVs in loss (71.8%, 51/71) was more than that in gain (40.0%, 12/30) had been detected by Lee and colleagues. [24] Lee and colleagues applied genome-wide microarray analysis in 649 cases and found pathogenic CNVs in 110 cases, which included 100 deletions and 31 duplications of 270 kb to 30 Mb. They reported their diagnostic yield as a 16.9% and they reported the common pathogenic CNVs which were 4p16.3 deletion (Wolf–Hirschhorn syndrome, seven cases), 1p36 deletion (8 patients), 6q26 deletion (5 patients), 10q26 deletion (4 patients), 17p11.2 deletion

(Smith–Magenis syndrome, 6 patients), and 22q11.2 deletion (four cases) [25]. Jang et al applied CMA for 617 cases and 122 patients (19.8%) had abnormal CMA results which includes 16p11.2 microdeletion syndrome, Prader–Willi syndrome, 15q11-q13 duplication, Duchenne muscular dystrophy and Down syndrome[26]. In our cohort, we identified abnormal result in 28 % of the patients. This ratio was higher than all of the previous results. This shows us aCGH is an effective technique for evaluation of DD in Turkish cases. Testing for chromosomal abnormalities by using aCGH in individuals with DD in the clinical setting will have a high diagnostic yield. Additionally, the usage of microarray technology is important for the genotype-phenotype correlation, identification of candidate genes or disease related CNVs. The genetic content, size and type are important determinants for pathogenicity of CNVs.

Genotype-phenotype correlation and identification of candidate genes or disease related CNVs is more important condition during the evaluation of DD. For instance; ISCA Database, showed that CTNS gene was associated with morphological phenotypes with developmental delay and additional significant developmental delay.

In our cohort, we identified CTNS deletion in a 2 years old baby which was DD and lack of the walking observed. Another example is related 1 year old boy which DD was observed and cording to the aCGH, 7q11.23 duplication was identified. Which is duplication 7q11.23 has been reported as a related with developmental delay and delay for crawling and walking in genetics home reference. In 17 years old boy with was referred due to the DD and MR, than; duplication of 19q13.41 has been identified. According to the ISCA 19q13.41 duplication was associated with DD and psychiatric abnormalities which are used for explanation for clinics of our case. All these examples are highlighted the importance of aCGH usage during the identification of DD etiology in cases.

Conclusion

Our findings suggest the necessity of array-CGH as a routine diagnostic test for DD in Turkey and determined a detection rate of 28 %. This makes array-CGH is a powerful diagnostic tool and should be the first genetic test for patients with unexplained DD. Clarification of the genetic abnormalities detected by array CGH helps identification of the possible candidate gene regions/genes underlying the DD pathogenesis. Although array-CGH could not detected balanced translocations, point mutation, low-level mosaicism and inversions, the diagnosis rate in clinical application was higher than conventional karyotyping analysis. In addition, complex rearrangement type and the CNV loss region, large region, has a high potential of having clinical pathological significance.

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Ethical statement

All procedures performed in the study involving human participants were in accordance with the ethics committee of the Trakya University Faculty of Medicine (2014/29) and followed Declaration of Helsinki.

Conflict of interests

The authors declare that they have no conflict of interest.

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Figure Legends

Figure 1: CMA image showing a 4.241-Mb deletion at 17q24.1-24.3

Figure 2: CMA image showing 6.495-Mb duplication at Xp22.12-p21.3

Table Legends

Table 1: General overview of cases.

Table 2: Clinical characteristics and online database analysis of aCGH results

Mini-abstract

Array-CGH as a routine diagnostic test for DD in Turkey and determined a detection rate of 28 %. This makes array-CGH is a powerful diagnostic tool and should be the first genetic test for patients with unexplained DD.

Table 1: *General overview of cases.*

Patient No	Age at testing	Clinical characteristics	aCGH results
1.	1	DD, speech problem	Del 22q11.2 (19,756,992 – 21,440,514) x1
2.	2	Lac of walking, DD	NORMAL
3.	5	Neuromotor DD, ASD	NORMAL
4.	4	DD,MR	NORMAL
5.	1	Microcephaly, flatted nasal bridge, DD	NORMAL
6.	6	DD	NORMAL
7.	17	DD	Dup 19q13.41 (52,321,568 – 52,615,691) x3
8.	16	DD, limb abnormality	NORMAL
9.	3	Microcephaly, DD	NORMAL
10.	2	Craniosynostosis, DD	NORMAL
11.	1	DD	NORMAL
12.	3	DD	7p22.1-p21.3(6870884 – 7456754)x3
13.	8	DD	Del 22q11.2
14.	0	DD, dysmorphic features	NORMAL
15.	1	DD	14q31.1 (80.381.425 – 80.730.691)x3
16.	2	DD	NORMAL
17.	1	DD, teratology of Fallot	22q11.21 (18.894.835 – 21,440,514)x1
18.	1	DD, hypotonia	NORMAL
19.	13	DD, ID	Del 22q11.1-q11.21 (17,084,955 – 19,659,894)x1
20.	1	DD, hypotonia	NORMAL
21.	3	DD, dysmorphic features	NORMAL
22.	8	DD, atypical autism, Rett Syndrome?	Del 16p11.2 (29,656,684 – 30,190,568) x1
23.	1	DD	NORMAL
24.	1	DD, dysmorphic features, hearing loss, ASD	NORMAL
25.	6	DD	NORMAL

26.	1	DD, IUGR, MPS	NORMAL
27.	7	DD, ID, epilepsy	NORMAL
28.	17	DD, ID	NORMAL
29.	4	DD, hypotonia	Dup 1q21.3 (152,954,890 – 153,149,003) x3
30.	1	Prenatal & postnatal DD, hypotonia, dysmorphic features	NORMAL
31.	32	DD, neuromotor DD, Atrial myxoma,	Del 17q24.1-24.3 (64,022,600 – 68,264,351)x1
32.	3	DD, fanconi Anemia	NORMAL
33.	9	DD, epilepsy	Del 16q24.3 (89,824,688 – 89,874,708) x1
34.	1	DD, hypotonia	Del 2q37.2q37.3 (236,789,722 – 243,040,276)x1
35.	4	DD, neuromotor DD, trigonocephaly	Dup Xp22.12-p21.3 (19,855,485 – 26,351,374)x3
36.	6	DD	NORMAL
37.	2	DD, atypical ASD	NORMAL
38.	1	DD, SLOS	NORMAL
39.	9	DD	NORMAL
40.	2	DD, short stature, dysmorphic features	NORMAL
41.	2	DD, microcephaly	NORMAL
42.	24	DD in previous baby (ex)	NORMAL
43.	28	DD in previous baby (ex)	NORMAL
44.	5	DD, hypotonia, epilepsy	NORMAL
45.	1	DD, hypotonia	NORMAL
46.	1	DD	Dup 7q11.23(72,726,572 – 74,133,332) x3
47.	2	DD, epilepsy	NORMAL
48.	6	White matter abnormality, DD	NORMAL
49.	2	DD, lack of walking	Del 17p13.2 (3,540,391 – 3,561,146)x1
50.	1	DD, epilepsy, right eye strabismus	NORMAL
51.	3	DD	NORMAL

52.	5	DD, ID, prominent ear	NORMAL
53.	6	DD, epilepsy	NORMAL

Table 2: *Clinical characteristics and online database analysis of aCGH results*

Age at testing	Clinical characteristics	aCGH results del	aCGH results dup	Pathogenicity	Size (Mb)	OMIM morbid gene (OMIM)
1	DD, ID	Del 22q11.2		Pathogenic	2.545	*DGCR6, PRODH, DGCR5, DGCR9, DGCR10, DGCR2, DGCR11, DGCR14, TSSK2, GSC2, SLC25A1, CLTCL1, HIRA, MRPL40, C22orf39, UFD1L, CDC45, CLDN5, LOC150185, SEPT5, SEPT5-GP1BB, GP1BB, TBX1, GNB1L, C22orf29, TXNRD2, COMT, ARVCF, C22orf25, MIR185, DGCR8, MIR3618, MIR1306, TRMT2A, RANBP1, ZDHHC8, LOC150197, RTN4R, MIR1286, DGCR6L, PI4KAP1, RIMBP3, ZNF74, SCARF2, KLHL22, MED15, POM121L4P, TMEM191A, PI4KA, SERPIND1, SNAP29, CRKL, AIFM3, LZTR1, THAP7, FLJ39582, MGC16703, P2RX6, SLC7A4, P2RX6P, LOC400891*
17	DD, ID		Dup 19q13.41	Pathogenic	0.294	*FPR3, ZNF577, ZNF649, ZNF613, ZNF350, ZNF615, ZNF614, ZNF432, ZNF841*
3	DD		7p22.1-p21.3 (6870884-7456754)x3	Pathogenic	0.585	*C1GALT1,COL28A1,MIR3683,LOC100121257*
8	DD, ID	Del 22q11.2		Pathogenic	2.610	*DGCR6, PRODH, DGCR5, DGCR9, DGCR10, DGCR2, DGCR11, DGCR14, TSSK2, GSC2, LINC01311, SLC25A1, CLTCL1, HIRA, MRPL40, C22orf39, UFD1L, CDC45, CLDN5, LINC00895, SEPT5, SEPT5-GP1BB, GP1BB, TBX1, GNB1L, C22orf29, TXNRD2, COMT, MIR4761, ARVCF, TANGO2, MIR185, DGCR8, MIR3618, MIR1306, TRMT2A, MIR6816, RANBP1, ZDHHC8, CCDC188, LOC284865, LINC00896, RTN4R, MIR1286, DGCR6L, TMEM191B, PI4KAP1, RIMBP3, ZNF74, SCARF2, KLHL22, MED15, POM121L4P, TMEM191A, PI4KA, SERPIND1, SNAP29, CRKL, LOC101928891, AIFM3, LZTR1, THAP7, THAP7-AS1, TUBA3FP, P2RX6, SLC7A4, MIR649, P2RX6P, LRRC74B, BCRP2*
1	DD		14q31.1 (80.381.425-80.730.691)x3	Pathogenic	0.349	*DIO2, DIO2-AS1*

1	DD, teratology of Fallot	22q11.21 (18.894.835-21,440,514)x1		Pathogenic	2.710	*DGCR6, PRODH, DGCR2, TSSK2, GSC2, SLC25A1, CLTCL1, HIRA, MRPL40, C22orf39, UFD1L, CDC45, CLDN5, LOC150185, SEPT5, SEPT5-GP1BB,GP1BB, TBX1, GNB1L, C22orf29, TXNRD2, COMT, ARVCF, C22orf25, MIR185, DGCR8, MIR3618, MIR1306, TRMT2A, RANBP1, ZDH-HC8, LOC150197, RTN4R, MIR1286, DGCR6L, PI4KAP1, RIMBP3, ZNF74, SCARF2, KLHL22, MED15, POM121L4P, TMEM191A, PI4KA, SERPIND1, SNAP29, CRKL, AIFM3 , LZTR1, THAP7, FLJ39582, MGC16703, P2RX6, SLC7A4, P2RX6P, LOC400891*
13	DD,ID	Del 22q11.1-q11.21		Pathogenic	2.574	*XKR3,IL17RA, CECR1, SLC25A18, ATP6V1E1, BID, MICAL3, MIR648, PEX26, TUBA8, USP18, DGCR6, PRODH, DGCR2, TSSK2, GSC2, SLC25A1, CLTCL1, HIRA, MRPL40, UFD1L, CLDN51*
8	DD, atypical autism, Rett Syndrome?	Del 16p11.2		Pathogenic	0.533	*SPN,QPRT,KIF22,MAZ,PR-RT2,PAGR1,MVP*
4	DD, hypotonia		Dup 1q21.3	Pathogenic	0.194	*SPRR1A, SPRR3,SPRR1B,SPRR2A,SPR-R2B*
32	DD, neuromotor DD , atrial myxoma	Del 17q24.1-24.3		Pathogenic	4.241	*APOH, PRKCA, CACNG5,CACNG1, HELZ, PSMD12, PITPNC1, NOL11, BPTF,KPNA2,AMZ2,ARSG,PRK-AR1A,FAM20A,ABCA8,ABCA9,AB-CA6,ABCA10, ABCA5,KCNJ2,CAP112*
9	DD, epilepsy	Del 16q24.3		Pathogenic	0.050	*FANCA*
1	DD, hypotonia	Del 2q37.2q37.3		Pathogenic	6.00	*GBX2,ACKR3,COPS8,COL6A3,M-LPH,RAB17,LRRFIP1, RAMP1,SCLY,-FAM132B,HES6,PER2, TRAF3IP1, AS-B1,TWIST2,HDAC4,NDUFA10,GPC1, CAPN10,KIF1A, AGXT, PDCD1*
4	DD, neuromotor DD, trigonocephaly		Dup Xp22.12-p21.3	Pathogenic	6.495	*SH3KBP1,RPS6KA3, CNKSR2, SMPX, MBTPS2*
1	DD		Dup 7q11.23	Pathogenic	1.406	*TRIM50, FKBP6, FZD9, BAZ1B, BCL7B, TBL2, MLX1A*
2	DD, lack of walking	Del 17p13.2		Pathogenic	0.020	*CTNS*

Chapter 4

CURRENT APPROACH TO CHOLESTEROL

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INTRODUCTION

Cholesterol is a combination of a steroid and alcohol and it is as a stearol, carry in blood plasma. Cholesterol is found in the cell membranes of animals' body tissues. It is also found in lower amounts in plants cells. Since cholesterol was found in gallstones for the first time in 1754, the name of this substance was derived from the Greek-word chole- (bile) and steros (solid) from the -ol suffix in chemistry. Cholesterol is found especially in animal foods, but only a small part of the cholesterol in the body is from food; most are synthesized by the body. If cholesterol is found in the blood more than normal, it accumulates in the vessels, leading to vascular stiffening (atherosclerosis). Sometimes it combines with bile pigments and plays a role in the formation of gallstones.

Cholesterol is involved in many biochemical reactions, it is known that there is a parallel relationship between blood cholesterol levels and heart diseases, especially due to the way lipoproteins carry cholesterol. The body produces hormones (cortisol, reproductive hormones), vitamin D and bile acids that digest fats using cholesterol. For these processes, it is sufficient to have very little cholesterol in the blood. If there is too much cholesterol in the blood, it accumulates in the blood vessels, causing stiffness and narrowing (atherosclerosis or arteriosclerosis). Cholesterol, white blood cells, blood clots, and calcium are substance that accumulates in the vascular wall in atherosclerosis. Atherosclerosis; Also known as vascular stiffness and vascular calcification among people. When talking about the damages of high blood cholesterol, "bad cholesterol" is the level of cholesterol carried by low density lipoprotein (Low Density Lipoproteins-LDL). Cholesterol carried by high density lipoprotein (High Density Lipoproteins-HDL) is called "good cholesterol".

Cholesterol is essential for the structure and maintenance of cell membranes. Cholesterol-containing membranes retain their fluidity over a wider temperature range. Cholesterol is used in the synthesis of bile, which helps digest fats. In addition, its role in the metabolism of fat-soluble vitamins (such as A, D, E and K) is important.

Cholesterol has an anti-hemolytic effect against, bacterial toxins, snake venom, bile salts and other hemolytic substances. It takes part in the synthesis of steroid hormones such as aldosterone, testosterone, estrogen and progesterone and cortisol. Other research shows that cholesterol plays a role in synapses between nerve cells and the functions of immune system cells. As a result of its effect on the structure of the cell membrane, it also affects cell signal transmission and ion and proton permeability in membranes (1).

In this review, it is aimed to give information about cholesterol. Review contains that what is the cholesterol, how to be harmful to the society, its types, the synthesis stages in living things, its control in the body, what high and low cholesterol levels can reveal, its benefits and harms.

Cholesterol

Cholesterol is a steroid of animal origin. It was first isolated from human gallstones in 1775, it is abundant in human bile. Chemical formula of cholesterol; $C_{27}H_{46}O$ (Figure 1).

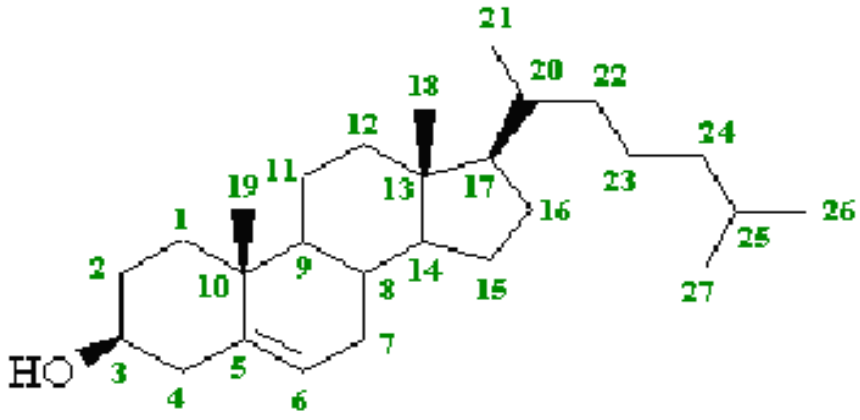

Fig 1. *Chemical structure of cholesterol*

Cholesterol is a white crystalline, tasteless and odorless substance. Cholesterol dissolves in organic solvents, hot alcohol, oils and fats. Cholesterol has very little electrical conductivity. Cholesterol is made in the liver and small intestine and transported in the blood by carriers called VLDL, LDL and HDL, to be delivered to other tissues. VLDL and LDL carry cholesterol from the liver to the tissues, HDL carry from the blood to the liver. Most of the cholesterol in plasma is in its esterified form. A fatty acid is attached to the OH group on the third carbon of cholesterol by ester bond. Esterification makes the structure more hydrophobic. Thus, cholesterol is transported either together with proteins as a component of a lipoprotein molecule or dissolved by the phospholipid or bile salts in bile.

Cholesterol is synthesized by almost all tissues in humans. All cells must have cholesterol in their structure. Thanks to the molecular structure of cholesterol it impossible to dissolve in water. The water-repellent cholesterol in the cell walls protects the inner cell environment from external influences. Most cholesterol is found in nerve tissue that should be least affected by external influences.

Vitamin D, which has vital functions such as controlling bone development in the body, proper function of the nervous system, growth, mineral absorption, insulin production, and strengthening the immune system, is produced from cholesterol. Bile salts, which allow the digestion of fat taken with food, are also produced from cholesterol (2).

Syntehesis of Cholesterol

Most of the cholesterol in the body is synthesized by the organism. 20-25% of daily production takes place in the liver. Also; The amount of synthesis in the small intestine, adrenal glands and reproductive organs is higher compared to other tissues. A person weighing about 70 kg has a total of 35 g of cholesterol in his body. Daily production amount is 1 g and the amount taken through food is 200-300 mg. Half of the 1,200-1,300 mg entering the intestines (via bile and food) passes into the blood (3). Cholesterol is synthesized in most cells and tissues by the reaction chain called mevalonate pathway initiated by HMG-CoA reductase enzyme (4).

Cholesterol biosynthesis can be divided into 6 stages:

1. Conversion of Acetyl CoA to HMG CoA (3-Hydroxy-3-methylglutaryl CoA)

2. Synthesis of mevalonate, a compound with 6 Carbon, from HMG CoA (NADPH is used) (It is the rate limiting step).

3. Formation of isoprenoid units (isopentenyl pyrophosphate) with the loss of CO2 from mevalonate (Mg, ATP is used).

4. Condensation of six isoprenoid units to make squalene, an intermediate product.

5. Conversion of squalene to lanostero, the ancestor steroid (NADPH, FADH2 and O2 are used, and lanosterol is the first steroid compound to appear in cholesterol synthesis.

6. Formation of cholesterol (using NADPH) through several further steps of lanosterol, including the loss of three methyl groups.

Cholesterol biosynthesis occurs outside the mitochondria and the precursor is acetyl CoA.

Transport in the body

Chylomicrons transport cholesterol and triglycerides from the small intestine to the liver. Some of this cholesterol is obtained through food, some of it originates from the bile that the body synthesizes and secretes from the liver. Chylomicrons leave some of the lipids they carry to tissues in the body and are then taken up by the liver. During breaks when there are no chylomicrons left, the main source of cholesterol is the liver.

Very low density lipoproteins (VLDL) are secreted into the blood to transport cholesterol and other lipids produced in the liver to other tissues in the body. As the triglycerides and cholesterol found in VLDL are transferred to cells, the structure and density of VLDL changes, transforming first to ILD and then to LDL. At the end of this process, LDL containing the remaining cholesterol is taken back by the liver.

When rises the amount of LDL in the blood causes these lipoproteins to accumulate in the walls of arterial vessels, which is known as the first stage of atherosclerosis (5). High density lipoproteins (HDL), on the other hand, carry cholesterol synthesized in body cells to the liver to be excreted from the body (Figure 2).

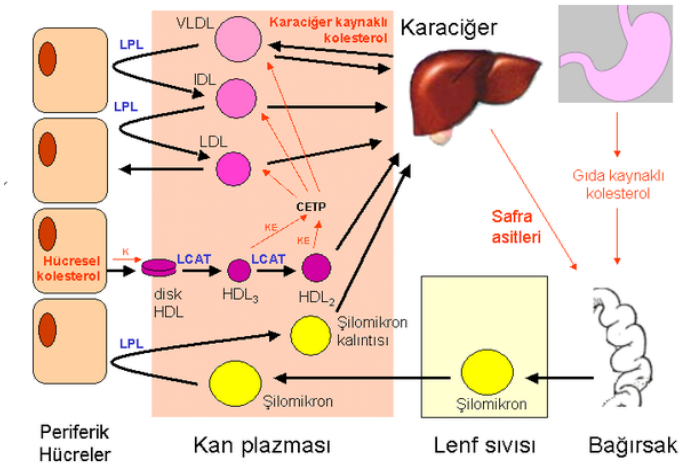

Fig 2. *Transport of cholesterol in the body. The thick black arrows show the formation, transformation, and exit of lipoproteins that transport cholesterol and other lipids from the bloodstream. Red thin arrows show the movements of cholesterol or cholesterol derivatives. K, cholesterol; KE, cholesteryl ester; LPL, lipoprotein lipase; LCAT, lecithin cholesterol acyltranferase; CETP, cholesteryl ester transfer protein*

Excretion from the body

Cholesterol is excreted from the liver through bile and some of it is taken back by the small intestine. In the gallbladder, due to its high concentration, it may crystallize, leading to the formation of gallstones (although more rarely, gallstones composed of lecithin or bilirubin may also be seen) (5).

Roles of Cholesterol

Cholesterol helps the liver make bile acids. Bile acids are necessary for the digestion of fat and the removal of waste materials from the body.

Cholesterol is an important part of the myelin sheath. In the absence of myelin sheath, focusing becomes difficult and memory weakens, and there may be memory losses (6).

The body uses cholesterol to prevent free radical damage that causes cardiovascular disease and cancer. The reason of cholesterol levels increase as we age is due to increased free radical activity.

Cholesterol is needed for proper function of serotonin receptors in the brain. A low-cholesterol diet can cause aggression and suicide attempts. Cholesterol controls cell membrane fluidity and permeability. Since cholesterol is the general restorative material of the body, it is abundant in both damaged vessels and all injured tissues (7).

What is Allostasis?

Allostaz; It means a disorder in the work of the body. The work of the body is under the control of the brain. Disruption of this balance reveals symptoms of allostasis. This term, which refers to the condition of the body whose normal functioning is impaired, is the main cause of many diseases. Abnormalities in blood values, including high cholesterol, are signs of allostasis.

The main cause of vascular structure disorder is allostasis effect. Veins have difficulty adapting to the required conditions. Hypertension or hypotension situation occurs as a result of this adaptation problem. As a result of the work of the veins, the production and effect of some protective substances are reduced. This situation causes the veins to deteriorate easily. Protection of structures called free radicals disrupts the structure of the weakened inner layer of the vessel (8).

Allostasis Symptoms

- Blood pressure (blood pressure) increases.
- Heart rate increases.
- Respiratory rate and capacity increase.
- Storage sugar is burned.
- Blood sugar increases.
- Intestinal motility decreases.
- LDL cholesterol level increases in the blood.
- Hemoglobin sugar level increases.
- 12-hour urine cortisol level increases.
- Adrenaline and noradrenaline levels increase in 12-hour urine.

- DHEA sulphate level in the blood decreases.

- HDL cholesterol level in the blood decreases.

Blood pressure abnormalities, inability to remove free radicals from the environment, and high cholesterol these are develop with the effect of allostasis, are indicators of a problem in body functioning. As results of these abnormality, the body is not well managed and suitable foods are not taken. Allostasis, which occurs before the symptoms begin, is a reversible condition. Therefore, treatments should be based on allostasis (Figure 3).

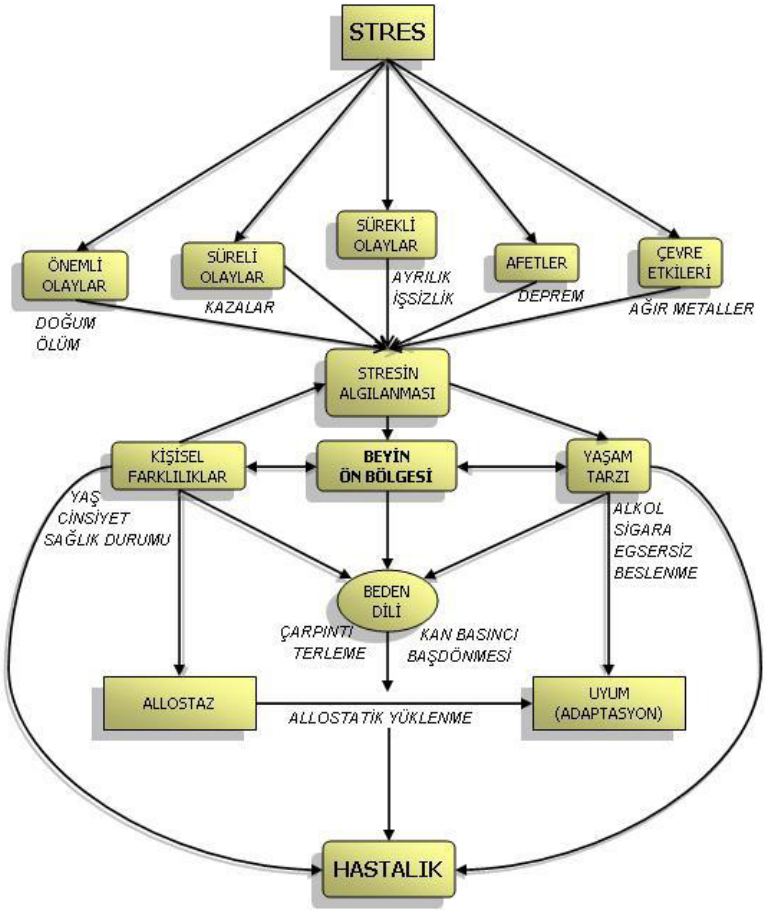

Fig 3. *Way of formation of allostasis and diseases caused by stress*

Why Does Cholesterol Increase?

The cause of high cholesterol is allostatic loading. The reason for the increase in cholesterol in the blood; The allostase effect is due to the fact

that the affected liver helps the situation by increasing cholesterol synthesis, which is the building block. Since the cause of cholesterol raising is not treated, the vessels continue to be blocked.

High cholesterol is not a disease but an indicator of a problem with the body's work. True cholesterol disease is very rare, it is genetic and its levels are above 1000 mg dl. Cholesterol-lowering drugs only lower cholesterol, but the causes that increase cholesterol are not eliminated. The aim should not be to lower cholesterol, but to treat the cause that raises cholesterol (9).

The Importance of Cholesterol Level in the Blood

When the amount of cholesterol in the bloodstream increases, excess cholesterol starts to accumulate in the walls of the vessels that feed the heart. Cholesterol accumulates in the vessel wall and causes hardening of the vascular structure called atherosclerosis or arteriosclerosis. Cholesterol deposits gradually narrow the coronary artery and prevent the blood and oxygen needed for the heart's nourishment from reaching the heart. Taking much less oxygen than it needs, the heart muscle weakens and chest pain occurs. Heart attack or even death may occur if clot formation is added to this narrowing in the coronary vessel, which has narrowed and deteriorated due to cholesterol. Accumulation of cholesterol in kidney vessels can cause high blood pressure and kidney failure.

Accumulation of cholesterol in the vascular wall is a slowly developing event. If the blood cholesterol level can be lowered, cholesterol accumulation can be slowed down, stopped, and even deposits can be reduced, thus reducing the risk of heart disease and death (10).

Because cholesterol is carried in the blood in packages called lipoproteins, lipoprotein types can affect the risk of heart disease. Low density lipoproteins (LDL) carry most of the cholesterol in the blood. Cholesterol and fat carried in LDL are the main source of dangerous deposits that clog arteries. The higher the proportion of LDL in the bloodstream, the higher the risk of developing heart disease. High density lipoproteins (HDL) carry some of the cholesterol in the blood, but this cholesterol is transported to the liver and removed from the body through the vessels. If the good cholesterol level is low, the risk of developing heart disease will be higher (11).

High blood cholesterol levels as well as low levels bring serious problems. Recently, intensive studies have been carried out on the relationship between low cholesterol and psychiatric disorders. The relationship of low or therapeutically lowered serum cholesterol with impulsive and aggressive behavior and suicide attempts has been an

issue for a long time. In fact, it has come to the fore when it was seen that prophylactic cholesterol-lowering treatments provoked aggressive behaviors in individuals with atherosclerotic disease that such a relationship may exist (12). The decrease in cholesterol level decreases the fluidity of neuron membranes and decreases serotonin receptor sensitivity. Both presynaptic; as well as reducing 5-HT neurotransmission in postsynaptic regions, and between this decreased central serotonin activity and aggression and suicidal behavior; It has been suggested that there is a significant relationship, especially in patients with personality disorder (12-14).

The Role of Bile Acids in Cholesterol Balance

Most of the cholesterol breakdown in the body occurs through bile acid synthesis in the liver. In humans, approximately 500 mg of cholesterol is converted into bile acids every day and removed in bile. This removal of excess cholesterol from the body is probably important for all animals, especially when they are fed excessive cholesterol. Also; It has also been determined that bile acids, by participating in cholesterol metabolism, function like hormones that alter the transcription of the rate-determining enzyme in cholesterol biosynthesis (15). The most important way followed for the elimination of cholesterol from the body is secretion into bile. Free cholesterol is almost insoluble in aqueous solutions, but becomes soluble in bile by means of lipids such as bile acids and lecithin (16).

Traditional Nutrition and Heart Diseases

The blood cholesterol level of an average of half of the patients hospitalized in the intensive care unit due to heart disease and paralyzed patients in the neurology clinic is either normal or low (17). Blood cholesterol levels of 50% of patients diagnosed with a heart attack in the emergency services in the USA were found to be normal (18).

In a study, it was determined that a serious rate of cardiovascular plugs was found in 1/3 of middle age group people who had no complaints and did not have any disease risk group (19). In the middle age group, due to the decrease in estrogen and testosterone hormones, the liver increases the secretion of cholesterol into the blood and increases hormone production indirectly. Therefore, the increase in cholesterol seen in the middle age group is a normal event. A calcium plaque of 10 microns in diameter in the heart vessels can break off and combine with blood elements, causing a heart attack (20). This is the primary problem seen in heart attacks leading to sudden death (21). Dr. John Abramson from Harvard University, found that there was no indication that cholesterol-lowering drugs reduced deaths from heart attacks (22). In another study, it was determined that with the

decrease of cholesterol, brain and nerve diseases may occur depending on the cholesterol deficiency in the structure of the brain cells (23).

The incidence of coronary heart diseases is low in Japanese who are dependent on traditional diet. However, the incidence of coronary heart disease increases as a result of changes in dietary habits when they immigrate to America (24). The death rate from coronary heart disease in Turkey is in first place with 43% of all deaths. Approximately 17 million people in the world and 130,000 people in the Turkey die from coronary heart diseases every year. According to the results of all researches; deaths from caused heart attacks do not decrease with lowering cholesterol.

Does a Diet Rich in Cholesterol and Saturated Fat Raise Blood Cholesterol Level?

Cholesterol is not essential and is synthesized in our body. 2000-2500 mg of cholesterol is made in our body per day. The effect of dietary cholesterol on blood cholesterol level is 5-10%. Cholesterol and saturated fats are not related to each other. Cholesterol is not made from a saturated fat or any other fat. Endogenous cholesterol is made from acetyl CoA, a glycolysis product. Many scientists unfortunately convinced the masses that a diet rich in saturated fat and cholesterol is a risk factor for coronary heart diseases. However, the consumption of foods rich in saturated fat and cholesterol has not increased since the 1900s, but rather decreased.

In the publications in which scientific researches conducted with high-fat nutrition methods in the last 10 years were compiled, it was observed that high-fat diet not increased cholesterol levels, but on the contrary, returned cholesterol levels to normal.

Arherosclerosis - Heart Diseases

If there is too much cholesterol in the blood, it accumulates in the blood vessels and causes the blood vessels to harden and narrow (arteriosclerosis). Existing vascular damages are tried to be repaired with a sticky and oxidized lipoprotein called lipoprotein (a) that resembles LDL. The patch formed by lipoprotein (a) is called atheroma plaque. Atheroma plaque saves people from death in the short term but it kills people in the long run. Lipoprotein (a) has lysine and proline amino acid receptors. If lysine and proline amino acids are added to the diet, the lysine and proline receptors of lipoprotein (a) become saturated and its adhesive effect is lost. Thus, the atheroma plaque begins to melt.

In the last century, 35-40% of taken daily calories come from fat in the USA. Although there was no increase or even decrease in the amount of fat removed during this time, there was a tremendous increase in coronary heart disease (25). 60% of the diet of the Masai in Kenya is fat,

and coronary heart diseases have not been observed. Although Canadian and Alaskan Indians and Eskimos diets include 80% fat, coronary heart disease was not observed in them either (26).

Blood Cholesterol Level - Infection

Cholesterol helps kill microbes by stimulating cytotoxic T-lymphocytes. It provides phagocytosis together with enzymes and other substances in the cholesterol cell membrane. Numerous experimental studies have shown that lipid and cholesterol deficiencies increase the severity of infectious diseases (26).

Blood Cholesterol Level -Febrile Neutropenia

Seventeen patients with febrile neutropenia due to cancer treatment were studied, six of whom died. The blood cholesterol levels of the patients who died were not increased. The blood cholesterol levels of the 11 surviving patients were found to be higher than those who died (26).

Blood Cholesterol Level - Rate of Infectious Diseases Coming to the Hospital in a 15-year study conducted on 120,000 people, it was observed that the number of those with low blood cholesterol levels and those admitted to the hospital for infectious diseases was much more than high cholesterol (26).

Blood Cholesterol Level - HIV

In a study conducted on 2446 men for 14 years, it was found that low cholesterol levels had a higher risk of AIDS. In another study, AIDS patients with low blood cholesterol levels have been found to more death rate than high cholesterol patiens (25), in addition to 20 autopsy cases and in 200 cases selected in medical libraries, the relationship between the degree of arterial atherosclerosis and cholesterol level was investigated by Mathur (25). According to this research, there was no correlation between the blood cholesterol level of the deceased and the amount and density of atherosclerotic plaque in the arteries. They found that high or low cholesterol levels have no effect on the growth of atherosclerotic plaque, which is the most important cause of cap diseases.

RESULT

The pharmaceutical industry has created a great “cholesterol phobia” all over the world in a short time. Cholesterol has been introduced to the public and, unfortunately, to doctors as well, with various marketing tactics as the only cause of deadly diseases such as heart attack and stroke. Annual sales of cholesterol-lowering drugs alone exceed $ 25 billion. In fact, high cholesterol is only one of the risk factors such as smoking, sedentary lifestyle, unbalanced diet, obesity, high blood pressure, diabetes and stress that can increase the likelihood of heart attack. High cholesterol alone is never a disease.

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Chapter 5

ENHANCING IMMUNITY LEVEL BY USING PHYTOGENIC FEED ADDITIVES IN ANIMAL DIETS-A REVIEW

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1. Introduction

The phytogenic feed additives (PFAs), also known as botanicals or plant-based compounds, and their products and added to animal feed to boost growth and production of food producing animals. The important plant-based chemicals of PFAs i.e. polyphenols, composition and concentration of these compounds depends on extraction technique, environmental condition, harvesting season, storage conditions, geography, plants and part of the plant being used for this purpose. This term includes a variety of herbs or spices which are utilized in solid, dried or powder form in crude or concentrated form. Apart from these procedures to extract the bioactive ingredient, PFAs are divided into essentials oil (extracted by cold extraction, steam, alcoholic distillation) and oleoresins (extracted by non aqueous solvents) (Windisch, Schedle, Plitzner, & Kroismayr, 2008); (Applegate, Klose, Steiner, Ganner, & Schatzmayr, 2010); (Wielen, Urlings, & Knapen, 2000). The mode of action of PFA is still unknown but is largely similar to the antioxidant, antimicrobial, antibiotic, anti-inflammatory characteristics of the bioactive ingredients being extracted from PFAs. When mixed with animal feed, it alters the gut microbiota, reduce the microbial toxins and restore the intestinal integrity and immune status of animal which leads to optimum growth and production. PFAs impart the immunomodulatory effects e.g. increases the proliferation of lymphocytes, increases the exposure of cytokines and high antibody titer (Kim, Lillehoj, Lee, Jang & Bravo, 2010); (Taylor, 2010); (Liu. 2004); (Pourhossein, 2015); Zhang & Kim, 2010).

Since the time when ban was imposed on using synthetic antibiotics for optimum production in livestock, growth and enhancing immunity level, search for alternative phytogenic and plant-based feedstuffs, having no side effects and considered safe to be used in animal feed, has gained a huge attraction. The main reason of this paradigm shift was antibiotic resistance, antimicrobial resistance and a possible drug residual effect in human beings. So it was a matter of public health concern which made it more sensitive and a ban was put to curtail the residual effects of antibiotics which were used in food producing animal. These phytogenic feed additives enhance the immunity level of animals and make them strong enough to bear the stress. Apart from this immunomodulation, the phytogenics also improve health status and consequently increase the production of animals (Giannenas et al., 2010). Flavonoids is a group of polyphenolic compounds found in plants and it includes two naturally occurring compounds genistein and hesperidin found in soybean and citrus, respectively, and put positive effects on health and egg production of poultry birds (S.Ting, Yeh, & Lien, 2011). In some other research trials soybean isoflavones and flavonoids rich alfalfa extract were found to be beneficial on growth and

production, B and T lymphocyte proliferation in broiler poultry birds (Dong, 2007); (Jiang et al., 2007). In another study it was reported that flavonoids and their extracts had some immunomodulatory effects as well as also affected surface area, villus length of small intestine and improved the overall gut health of poultry birds (Giannenas., 2010); (Hassanpour, Moghaddam, Yazdani, & Bashi, 2010); (Wallace, 2010).

In a study trial it was observed that *Morinda citrifolia* plant juice extracts increased the proliferation of CD4+ and CD8+ T cell of neonates and it positively modulated the immunity level of animals (Brooks et al., 2009). In a research study performed by (Sunder, Rb, Kundu, & Sakthivel, 2007) the immunomodulatory effects of *Morinda citrifolia* was observed in poultry birds when fresh juice of the plant was supplemented @ 5% mixed in water and it boosted both the humoral and cellular immunity level in broiler chickens. It was also observed that humoral immunity response was statistically significant ($p<0.05$) in the treated birds as compared to that of birds of control group and the peak response was seen in 1st week of post infection.

The pathogenic organisms and other environmental stressor can destroy the animal tissues and cells and if this destruction keeps on going, a time will come when amount of reactive oxygen species (ROS) is increased which causes lipid peroxidation and oxidative damage of cell and it challenges the immunity status of animal. If there is no antioxidant to neutralize ROS it triggers the inflammation in which various types of cells are sent to the inflammation site after the cytokines and chemokines are secreted. It is normal physiological response of body towards pathogenic microbes entering the body and to fight against the infection (M. T. Lee, Lin, Yu, & Lee, 2017); (Blackwell, Blackwell, Holden, Christman, & Christman, 1996); (Cuzzocrea, Riley, Caputi, & Salvemini, 2001). GIT is the first site where food is degraded and absorbed and pathogens reach there by destroying the mucus membranes. So the protection of GIT is of great importance to ensure the optimum health and production of animals and it also boosts the immunity level of the animal (Cuzzocrea et al., 2001). The animals react to the external stressors by decreasing feed intake which decreases body weight; these stressors whether are of exogenous or endogenous nature depress the immune response, damage the gut integrity and leads to low growth and production of animals that poses economic losses every year. The antibiotics are used for growth promotion as well as to enhance the immunity level of the animals but due to the detrimental effects of antibiotics; ban was imposed, in 2006 by European Union, on using antibiotics in livestock animals for growthand development. So an alternative approach is made by using phytogenic feed stuff in feeds of livestock for the purpose of growth promotion, optimum production

as well as enhancing the immunity level of animals (Cuzzocrea, 2001); (McLamb, Gibson, Overman, Stahl, & Moeser, 2013).

There are reports that essential oils from thymol, eugenol, curcumin, piperin, carvacrol and cinnamaldehyde are supposed to reduce proliferation of *Clostridium perfringens* in broiler (Jamroz, 2003; Mitsch, 2004; McReynoldsetal., 2009). Apart from the good impacts on microbes living in gut, the essential oils in PFA stimulate the secretion and activities of digestive enzymes i.e. trypsin, lipase and amylase (William and Losa, 2001; Lee, 2003; Wenk, 2003; Jang, 2004; Jamroz, 2005; Jang, 2007) and increase the villus height (Cardoso, 2012). A higher fecal moisture concentration and shorter digesta in the PFA supplemented group is responsible of greater secretion of the small intestinal mucosa.

2.Effect of phytochemicals on immune response

The active constituents of plants, phytochemicals, are broadly categorized into two main groups: terpenes and terpenoids. These compounds are extracted from different parts of plant by adopting different procedures however the powder form is most widely and commonly used (Hanieh, 2010); (Lin, 2011).

Oregano is a part of botanical family of *Labiate* which includes plant species *Origanum vulgare*, *O. onites*, etc containing a very important bioactive compound carvacrol and these are commonly used as feed additives in animals. A study was conducted by (Mohiti-Asli & Ghanaatparast-Rashti, 2017) in which oregano essential oil (OEO) was supplemented @ 300 ppm along with basal diet of broiler chickens and resulted in production of higher antibody titer; more specifically high titer of immunoglobulin G (IgG). It is documented, high doses (500, 1000 ppm) of OEO resulted in enhanced immunity level in the broiler birds which were vaccinated with new castle disease and avian influenza virus (Galal, El-Araby, Hassanin, & Omar, 2015). In swine higher thymus lymphocytic immune cell concentration was observed on 14^{th} day of lactation when OEO was supplemented @ 250 ppm in the diet which resulted in positive immunomodulatory effects (Galal et al., 2015). The concentration of coccidian oocysts in excreta was lowered, improved growth and a better immune response was seen when the broiler diet was mixed with OEO @ 300 ppm or mixture of carvacrol and thymol was added @ 300 ppm (Alp, 2012).

In monogastric models, abundant research has been done on the effects of dietary oregano plant materials. Several positive effects of dietary oregano plant materials have been reported on performance, gut morphology, health, immunity, and product quality in quails (Cetingul et al. 2010, Rahman et al. 2018)

A research trial was performed by (Najafi, S. 2014) in which cinnamon was supplemented in broiler diet @ 0.4% and 0.8% (doses of 100 and 200 ppm essential cinnamon oil) led to increased body weight from 1-6 week, improved FCR, increased hemoglobin and leukocytes concentration in blood of broiler chicken (AL-KASSIE, 2009). On the other hand positively modulated immune response was observed in 21 days old broilers chicks which were offered 5 g/L of drinking water (Sadeghi, Karimi, Padidar Jahromi, Azizi, & Daneshmand, 2012).

Phytogenic material	**Species in which can be used**	**Supplemented form**	**Dose rate**	**Immune response**	**Reference**
Echinacea purpurea L.	Broiler,	Ariel part of plant, powder	5-10 g/kg diet	Higher antibody titer against ND	(Landy & Ghalamkari, 2011)
	Laying hens	Juice	0.25 ml.kg BW	Higher WBC cells	(Böhmer, Salisch, Paulicks, & Roth, 2009)
Oregano	Broilers	Essential oil	300 ppm in diet	Higher IgG titer	(Mohiti-Asli & Ghanaatparast-Rashti, 2017)
Cinnamon	Broilers	Powder	4 and 8 g/kg diet	High lymphocytes	Najafi, S. 2014.
Turmeric	Broiler	Rhizome powder	2.5, 5 and 7.5 g/kg diet	Higher IgA. IgG and IgM titer	(Emadi & Kermanshahi, 2007)
Thyme	Broiler	Oil extract	100 and 200 ppm in diet	Higher WBC cell	(AL-KASSIE, 2009)

The PFAs' are in the form of herbs, spices, EOs', extracts, bioactive compounds which have an overall positive effect on growth promotion, optimum production and enhancing the immunity level up to extent to make them resistant to disease (Windisch, 2008); (Wallace, 2010). The chickens were immunized and infected with *Eimeria tenella* the phytonutrient supplemented feed was given to poultry birds and it increased body weight, higher antibody titer and an increased lymphocytes proliferation as compared to the birds who were not fed the supplemented diets (S. H. Lee et al., 2011). Caprylic acid, an organic acid, when it was supplemented to broiler chickens; it lowered the degree of infection caused by *Salmonella enterica*. This acid down-regulated the gene of bacterium responsible for invasion of epithelial cells and ultimately it ensured the

immunomodulation in broiler poultry birds (Kollanoor-Johny, 2012).

Prebiotics are macromolecules defined as "nonviable feed components which are beneficial for host after the gut's microbial modulation" (FAO, 2007). These are either taken from plant or synthesized by the microbes. The mannanoligosaccharide (MOS), obtained from outer cellular layer of *Saccharomyces cerevisiae*, is used as prebiotic supplement in broiler chicken diets and is reported to enhance immunity level (Janardhana, 2009); (Shanmugasundaram & Selvaraj, 2011).

3.Challenges of using phytogenic compound in animal feed

The complex composition, sometimes it becomes difficult to systematically and comprehensively evaluate the phytochemicals to use them in feedstuff. There can be some challenges Gültepe et al. (2019) observed some positive effects of lemon juice as a water supplement on egg production during the late phase production cycle of laying hens. Furthermore, Çetingül et al.(2019) reported that supplementation of PM with drinking water to laying hens may affect some quality parameters in eggs after 30 days of storage. regarding how to use these compounds in feed due to possible side effects (toxic, unpleasant odor), regularity and legal affairs and their possible interaction (good or bad) with rest of the other ingredients of feedstuff (Lambert RJ. 2001); (Friedman, Henika, & Mandrell, 2002); (Yuan, 2014). There is a dire need to have a state-of-the-art and well developed analytical method to quantify the phytochemicals before being added to the animal feedstuff. The phytoneutrients are natural and organic substitute to the synthetic antibiotics and are considered safe by FDA USA but an authentic and complete assessment protocol, encircling all the procedures to analytically ensure and quantify the bioactive compounds before being used in animal feed, is still needed (FDA, 2006).

4.Mode of Action of Phytogenic Feed Additives

The phytogenic feed additives have a multiplex mechanism of mode of action by imparting a good and health effects on animals in improving their performance, immune status, reinvigorating them to survive in hard conditions and overall health (Hippenstiel, 2011; Mathe 2009; Windisch, 2008).

The mode of action of essential oils on cells depends upon the presence or absence of functional groups. For instance, carvacrol and thymol terpenoids have same antimicrobial effects but have different mode of action on gram-negative and gram-positive bacteria. The phenolic terpenoids are composed of hydroxyl group and acts as antimicrobial (Ultee, 2002; Lambert, 2001). (Hellander, 1998) documented that the bioactive

compounds i.e. carvacrol and thymol neutralize the cellular membrane and release lipopolysaccharides. Furthermore it is also reported that the essential oils can destroy the cellular membrane and can go inside the bacterial and when these are inside the cell, they are supposed to expedite and trigger the blockage of cytosolic proteins resulting in hampering the normal physiological process of bacterium.

Mode of action of some phytogenic feed additives

Sr. No.	Phytogenic feed additive	Mode of action	Reference
1	Ginger (*Zingiber officinale)*	Protein-digesting enzyme, improves digestion, antimicrobial, and kills parasites and their eggs	George et al. (2015)
2	Cinnamon (Cinnamomum *cassia*)	Antimicrobial and appetizing	Kumar et al. (2014) and Qotbi (2016)
3	Thyme (*Thymus vulgare*)	Antioxidant and antimicrobial	Haselmeyer et al. (2014) and ALsafa and AL-Faragi (2017)

5.Relevant literature

A research study was performed on 720, 1 day old Arbor Acres broiler chicks by adding two flavonoid compounds genistein and hesperidin found in soybean and citrus in the diets. Objective was to check effects of these flavonoids on immunity and intestinal morphology of poultry birds. On 16th, 18th and 20th day half of birds were injected *Escherichia coli* intraperitoneally @ 250 µg/kg of body weight to induce immunological response. The samples were collected on 21st, 42nd day of trial and it was observed that the compounds enhanced the immunity level by improving phagocytosis and due to which statistically significant immunomodulatory effect ($p<0.05$) was seen. Other parameters were also improved but no effect was seen on feed intake, body weight gain and FCR of broilers. In conclusion it was observed that the phytogenic feed additives boosted the immunity level and improved gut health of broiler chickens (Kamboh & Zhu, 2013).

Pistacia terebinthus seeds at 20 and 40 g kg−1 supplementation levels could be used to extend the shelf life of eggs without any adverse effect on egg quality (Gultepe 2018).

An experiment was performed on 336, one day broiler birds (Ross 308) chicks to observe comparative impact of *Echinacea purpurea* (EP) with standard antibiotic on growth promotion, carcass quality and immune level. The chicks were divided into 7 different treatment groups. 1st group

was control and only fed the basal diet, 2nd group was given basal diet + antibiotic (4.5 mg flavophospholipol/kg diet), 3rd group was fed basal diet + powder of dried aerial parts of EP (5 g/kg diet), 4th group was given basal diet + powder of dried aerial part of EP (10 g/kg diet), 5th group was supplemented with basal diet + powder of dried aerial part of EP (0.25 g/kg diet), 6th group was fed basal diet + powder of dried aerial part of EP @ 5 g/kg diet but EP was supplemented continuously for 3 days followed by break of 11 days and last group was given basal diet + powder of dried aerial part of EP @ 10 g/kg diet but EP was supplemented continuously for 3 days followed by break of 11 days. At 28th and 31st day the blood sampling was done to analyze the immune status. The overall results showed that supplementation of EP @ 5 g/kg diet had statistically significantly better ($p<0.05$) daily feed intake, daily weight gain and higher antibody titer against Newcastle virus and sheep red blood cell (SRBC) as compared to rest of other treatments groups (Landy & Ghalamkari, 2011).

A study trial was conducted in Ferdowsi University of Mashhad, Iran, on 200, 1 day old Ross broiler chicken to check the immunomodulatory effect of turmeric rhizome powder (TRP). The chicks were randomlt allocated 4 main treatment groups and 5 replicates which were composed of 10 birds each and these groups were supplemented with corn-soybean meal having TRP at the concentration of 0%, 0.25%, 0.50% and 0.75%. On 14th day one bird from each replicate was infected with 0.2 ml of 5% SRBC while the blood sampling was done at 21st and 42nd day. The results showed that supplementation of TRP significantly increased IgA, IgM, and IgG as well as TRP also significantly decreased monocytes ratio. In a conclusion it was reported that TRP had positively modulated the immunity response in broiler chickens (Emadi & Kermanshahi, 2007).

The supplementation of herbal oil has been in practice since so many years as a part of ethnoveterinary practices in animal nutrition; in fact it is a way to boost immunity and strategically control the viral diseases. An experiment trial was performed on 120 broiler chicks which were randomly divided into 4 groups. Group A was not vaccinated being control group while other 3 groups were vaccinated with inactivated avian influenza and live Lasota vaccine. Oregano essential oil (OEO) was orally administered @ 0.005 and 0.01% to group C and group D respectively. Results showed that the oral supplementation of OEO had positive effects on performance of birds and it also positively modulated the humoral and innate immunity in birds (Galal et al., 2015).

(Rahman et al. 2017) study indicated that supplementation of *Mentha Piperita* oil and its juice in the laying hen's diet had no significant effect on egg quality traits during storage for 15 and 30 days at 4°C.

The immunomodulatory effects of proanthocyanidins rich extract (PAE) from *Pinus radiata* bark, proanthocyanidins are natural compounds present in fruits, flowers, seeds, barks, and vegetables, were observed in specific pathogen free (SPF) White Leghorn chickens. The proliferation of mononuclear cells was increased in birds which were treated @ 20 mg/kg PAE for 2 weeks. On the other hand proliferation of splenocytes and bursal cells was also increased in the birds treated @ 5, 10 and 20 mg/kg PAE for the period of 5 weeks. The thymocytes cell proliferation was increased in birds which were treated @ 5 and 10 mg/kg PAE for the period of 5 weeks. The PAE enhanced the expression of T helper 1 cytokines (interferone-γ) and lowered the expression of T helper 2 cytokine (interleukins-6). So it was concluded that the PAE had an effective immunomodulatory effects on SPF white leghorn chicken (Kang & Mun, 2019).

In an experiment the chicks were orally injected the virulent oocysts of *Eimeria tenella* and were supplemented the mixture of two phytobiotics, VAC (carvacrol, capsicum oleoresin and cinnamaldehyde) and MC (turmeric oleoresin and capsicum oleoresin) to observe the immunomodulatory response after immunization with Eimeria profilin protein. After being orally infected with *Eimeria oocysts* and immunized with profilin protein, the chicks were given VAC or MC supplemented diets which increased BWG, higher antibody titer and increased proliferation of lymphocytes as compared to control group. Prior to the oral infection, the MC fed immunized chickens expressed reduced interferon-γ (IFN-γ) and interleukin-6 (IL-6). After the chickens were infected they showed increased levels of IFN-γ and IL-6. On the other hand, decreased IL-17F and TNFSF15 was shown only in infected chickens when VAC supplemented diet was fed. In conclusion the VAC or MC supplemented diets showed immunomodulatory impact against avian coccidiosis (S. H. Lee et al., 2011).

An experiment was performed to comparatively analyze two commercially available oregano essential oils (OEO) mixed in broilers diets to check growth productivity and immune response of 200, 1 day old Ross 308 broiler chickens. The dietary protocol included: (1) control group, no phytobiotics, (2) mixture of phytonutrients @ 150 ppm, (3) OEO @ 300 ppm, (4) OEO @ 500 ppm. The results showed that higher antibody titer particularly the IgG ($p<0.05$) was seen in the broiler which were fed OEO @ 300 ppm as compared to the control group (Mohiti-Asli & Ghanaatparast-Rashti, 2017).

6.Conclusion

The immunological system of animals is the first line of defense whenever the animals are subjected to heat, toxin, external, internal, environmental, pathological stress, etc. In this regard the defense system

plays a pivotal role in protecting the body from these stresses and it ensures optimum health, growth and production of animal. The animal obtains energy from type of feed and the function of immune system depends on the quality of diet being fed to the animals. It also depends on how much the feed is natural and organic. The phytogenic feed additives are natural, organic and are aligned with the physiology of animal; these plant based bioactive compounds are safe for animals, do not have side effects and are also safe for the end consumers, the human beings. Since majority of the phytogenic feed stuffs have the properties of antioxidant, anti-inflammatory, so these react accordingly to modulate and boost the immunity level. The antibody titer is increased, the concentrations of leukocytes, T lymphocytes, B lymphocytes, immunoglobulins are increased which modulate and enhance the immunity level and consequently the animals become strong enough to bear the detrimental situation. In this way the GIT of animal is protected which ensures favorable site for probiotics, good health, growth, optimum production and better immunity level.

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Chapter 6

AUTISM TREATMENT

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Introduction

Autism is one of the most serious developmental disorders that occur in early childhood and threatens the normal development of the child and his adaptation to the social environment. As a complex social problem, the autism stimulates the interest of many experts and scientists around the world, and especially its complexity requires research into the etiology, characteristics, opportunities for education, improvement in the treatment and rehabilitation of these children. Autism is a developmental disorder that both fascinates and frustrates the scientific and clinical community (Trajkovski, 2004).

Autism and other pervasive developmental disorders are phenomenologically related to neuropsychiatric disorders. These conditions are characterized by delays and deviations in many areas of development, and their occurrence is typical in the first months of life (Volkmar and Klin, 2005). Autism is a disability in early childhood with characteristic social disorders, communication disorders and rigid ritualistic interests (Trajkovski, 2011). Autism is neuro-behavioral disability characterized with social-communication deficit, as well as stereotyped and repetitive behavior. Most of the persons with autism spectrum disorders need professional support all life (Glumbic et al, 2013). First epidemiological study of autism, implemented in the sixties, concluded that the autism is a rare disorder with prevalence of four to five respondents with autism on 10.000 persons from the general population (Lotter, 1966).

In the last years exponential increase is shown in the number of children at the autism spectrum, which leads to epidemiological rate worldwide. Actual data shows that autism is very common disorder that shows in one of 54 children (Autism Speaks, 2020). With the increased number of people with autism the need for new treatment and appropriate protocols increased too. Many scientific researches show that there are some treatments available for autism that can decrease the symptoms and improve their everyday life.

The children with autism treatment should be intensive, continuous and multidisciplinary. Michael Router (1985), eminent British psychiatrist, singles out five main goals in the autism spectrum treatment: development support, education support, reducing rigidity and stereotypes, elimination of non-specific maladapted behaviors and relieving family unhappiness and grief. All this, usually has to be combinate with behavioral treatment, special education, logopedic treatment, pharma therapy and family support.

In the last years, the biomedical approach treatment including the gluten and casein free diet is recommended by some professionals while

others prefer the Nemecheck Protocol in order to treat the autism spectrum better. On the other hand, there are so many professionals that support the hyperbaric oxygen therapy, too. Since the spectrum is getting larger and the response to treatment is different from one child with autism to another, we are still not able to discuss for only one treatment approach.

Gluten-free/ Casein-free diet

A gluten-free/casein-free diet is one of the alternative treatments for children with autism. Foods containing gluten and casein are removed from daily food intake. Among the benefits parent's report changes in speech and behavior. Some reliable data shows that some children with autism are showing intolerance to foods that are containing gluten and casein. The reaction to this intolerance shows with many behavioral and physical problems such as headache, stomach pain, nausea, oversensitivity, sleeping problems, hyperactivity, aggression, hypersensitivity to sounds, fatigue, depression, intestinal problems, ear infections, and other problems. To improve this symptomatology, the foods that contain gluten and casein should be avoided (Trajkovski, 2011).

Several studies are showing that this diet can be used to improve the symptomatology of autism spectrum disorders in some children. Cade and his co-workers (2000) conducted a study with 270 individuals. Of these, 120 have been diagnosed with schizophrenia, and 149 have been diagnosed with autism. For the purpose of the study, the children have been assessed independently by the parents, doctors, and teachers for the presence of autism symptoms using the Likert scale.

Assessments have been given initially at the start of the study, then implemented again after one month of using the treatment, and later they continued every three months for a total of one year. In order to determine the absorption of peptides contained in wheat products (gluten) and milk (casein), blood samples have been examined and associated with IgA and IgG antibodies to these products. Gluten- and casein-free diet treatment has been followed by reports showing improvement in 81% of children for the first 3 months (Cade et al., 2000).

In a comparative study, Arnold et al. (2003) have evaluated amino acids in 26 children with autism on a regular diet, 10 on casein and gluten-free diet, and 26 children with developmental disorders who have been used as a control group. In summary, children with autism more likely to have essential amino acid deficiency compared to the control group. The results of this study suggest that children with autism are at high risk for deficiency of amino acids and they may benefit from this structured diet. The authors have noted that the main drawback of the study was the small sample (Arnold et al., 2003).

Knivsberg and colleagues conducted a randomized, 20 subject, blind study, to measure the response to a gluten-free and casein-free diet in children with urinary peptide disorders that belong to the autism spectrum. The children in the control group have been matched for age, autism symptoms severity, and cognitive level with those in the experimental group. Results from the observation have shown changes in both groups, still, the experimental group has shown more statistically significant changes. There has been a significant improvement in behavior, nonverbal cognitive level, and problems in motoric at the experimental group of children with autism (Knivsberg et al., 2002).

Another study has reported the results of a 24-month, randomized, controlled, two-stage study. The first stage involved 72 Danish children (aged 4 to 10 years and 11 months) who have been on a diet (A) and a group (B) of children who have not been on a diet. The ADOS and GARS scales have been used to assess autism behavior, and the VABS has been used to assess developmental level. Participants have been tested at the beginning, at 8 and at 12 months. According to the protocol, data have been available for 26 children on a diet and 29 control subjects, available at 12 months. During this period there has been a significant improvement in the group that has been on the diet, according to measurements with ADOS and GARS. In the second stage, data has been obtained for 18 children from A group and 17 children from B group in a period of 24 months. Multiple analysis based on comparisons between groups and within the groups themselves have provided some evidence of clinical improvement. These results have shown that dietary intervention can positively affect the development of some children with autism spectrum disorders (Whiteley et al., 2010).

All of these results from previous studies are interesting in terms of the hypothesis of the effects of the casein and gluten-free diet on the behavior and cognition of people with autism, but they are still limited to a small sample of respondents. There is a need for a rigorous, controlled clinical trial that evaluates the physiological and behavioral effects (Trajkovski, 2011).

A recent and past study review, suggests there is a lack of scientific evidence to say whether the gluten-free and casein-free diet can be helpful or not. Unfortunately, in the case of casein and gluten sources elimination, it is difficult to conduct randomized clinical trials, especially in children with autism.

Hyperbaric oxygen therapy

A higher concentration of oxygen delivered in a chamber or tube containing higher than sea-level atmospheric pressure is provided during

Hyperbaric oxygen therapy (HBOT). Recently, hyperbaric oxygen therapy (HBOT) has increased in popularity as a treatment for autism. (Singh, 1999). It has been postulated that patients with autism might benefit from Hyperbaric oxygen due to the potential increase in cerebral perfusion that occurs during this treatment. Inhalation of 100% oxygen might result in arterial partial pressure elevation of oxygen, which increases oxygen delivery to the brain (Calvert JW, Cahill J, Zhang JH, 2007).

Oxygen therapy aims to increase the level of oxygen in the bloodstream. It is performed in hyperbaric chambers, small rooms in which there is a possibility of changing the atmospheric pressure, which enables better absorption of oxygen. In doing so, concentrators are used to isolate the oxygen molecules from the air, creating an air that is composed of 100% oxygen. Increased oxygen levels increase the efficiency of red blood cells and blood vessels. Hyperbaric oxygen therapy is used in many countries around the world. The results achieved with this type of therapy vary from case to case but many parents of children with autism testify to the positive experiences. Hyperbaric oxygen therapy raises the level of oxygen in the brain tissue which increases the circulation, speeds up the metabolism in the brain tissue, and regenerates the tissues that suffer from hypoxia (Trajkovski, 2011).

In children with autism, according to some studies that use techniques for accurate examination of the brain (SPECT Scan), especially in many of them there is a reduction in aggressive behavior, reduction or disappearance of anger attacks, and in all children, it is observed improving communication skills, especially eye contact. It has also been noted that they have adapted easily at school, following verbal instructions from teachers, meaning the ability to understand has been increased in students with autism (Figure 1). In a group of 6 children with autism, betterment in autism behavior ($p < 0.02$) have been observed using a hyperbaric treatment of 1.3 atmospheres (atm) during a 40-hour treatment (Rossignol and Rossignol, 2006).

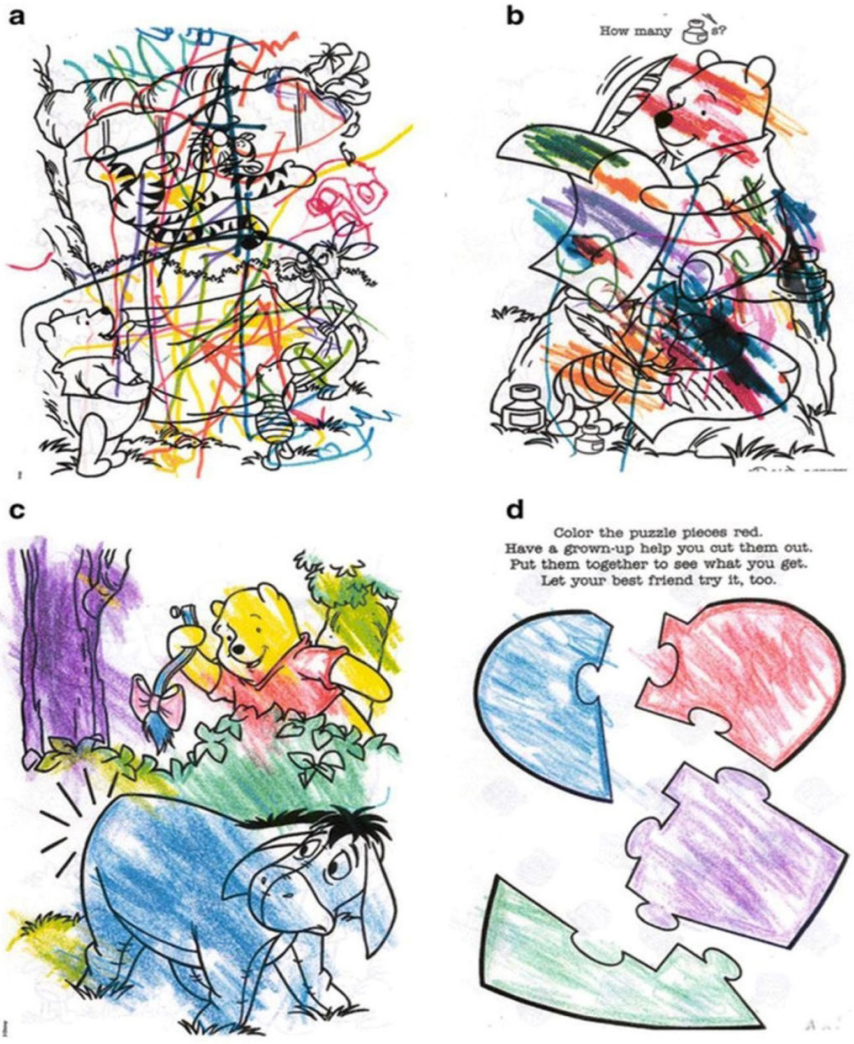

Figure 1. Pages that 17 year old girl with autism colored: (a) before starting with Hyperbaric Oxygen Therapy; (b) after one week, she has begun to create stretches of color to fill up the space; (c) 3 weeks later, she has been used correct colors; and (d) after 5 weeks, she has begun to respect borders and boundaries. After 6 months, her coloring skills have stayed stable. (Rossignol, 2012).

An open-label trial of 18 children with autism described noticeable improvements in motivation, communication skills, and cognition ($p < 0.05$) after the implementation of Hyperbaric oxygen therapy (with 12 children receiving 1.3 atm for 45 minutes during a total of 40 treatments, and 6 others receiving 1.5 atm for 45 minutes each), (Rossignol et al, 2007).

In a recent controlled study, randomized and double-blind, with a sample of 62 children with autism, the implications of hyperbaric treatment have been examined with a 24% oxygen and a pressure of 1.3 atm and compared with a group used as placebo (21% oxygen and 1.3 atm). Children that took 1.3 atm have had significant betterment according to physicians and parents in many areas including overall functioning (Figure 2), receptive speech, social communication, and eye contact ($p < 0.05$ for each) in a comparison with the placebo group (Rossignol et al, 2009).

Figure 2. A 6-year-old boy handwrite (a) before and (b) after 1.3 atm, 40 Hyperbaric Oxygen therapy sessions (Rossignol, 2012).

There have been many theories as to the cause of Autism Spectrum Syndrome such as aberrant cerebral bloodstream to the brain areas. Kinaci et al. (2009) have undergone a retrospective examination of 108 children with Autism spectrum disorders (23 of them have been females and 85 males) among the ages 3-12. They have implemented special questionnaires of Autism Treatment Evaluation Checklist (ATEC) endorsed by parents (for 54 children) and therapists or clinicians (for the other 54 children).

They have used 1.5 atm for 60 minutes every day, a total of 50 sessions Hyperbaric Oxygen Therapy (HBOT).

Autism Treatment Evaluation Checklist (ATEC) implemented by clinicians or therapists, specified that children have shown 79% improvement in Speech, Language, and Interaction, 85,5% improvement in Social relations, 87% improvement at Sensory and Cognitive Awareness and 75,2% improvement at Wellness and Physical Behavior. This study evidenced improvements in behavioral, physical, and brain perfusion, after applying the Hyperbaric oxygen therapy (Kinaci, 2009).

Markley made one of the most detailed researches about the hyperbaric treatment. He has examined the benefits of hyperbaric oxygen therapy on fifteen key behaviors mutually assessed in children with ASD. The study has been implemented on 20 children (4 girls and 16 boys at the age from 3 to 7 years old), tested with a DSM-IV diagnosis of Autistic Disorder, verified with Autism Diagnostic Observation Schedule (ADOS) and Autism Diagnostic Interview-Revised (ADI-R). Experienced clinicians for testing autism have undergone Pre and Post Hyperbaric Oxygen Therapy testing. All of the children in this research had been diagnosed for at least a year or longer before being included in the study. All participants have been completed 20 hours of hyperbaric oxygen therapy in a period of 6 weeks. 30 days after the final HBOT session, the researchers have conducted post-study testing (Lerman et al., 2008).

The results of Markley's research showed: 40% of the children in this study have been moved from the most severe Autism into the less severe "Autistic Spectrum" category. 20% of all of the children have shown improvement to a point that they haven't been on the autistic spectrum in the area of communications and verbal skills, anymore; 83% of the children have showed measurable improvements in resistance to change or insistence on sameness; 20% of the children had shown emotional outbursts in the pre-testing, 50% of them have shown reduction in behaviors such as laughing, crying for no apparent reason and other emotional outbursts; Post-therapy testing has noted that 93% of children have showed significant improvement in difficulty mixing with others improving their ability to communicate with peers in school, better interaction with siblings, and be more comfortable during recreational sport activities and other situations where they have been exposed to significant number of strangers; 77% of the children with autism have experienced measurable improvements in reducing the number of tantrums according to the post-therapy testing; 66% of the children have shown measurable improvements in their willing to comfort other children or to be cuddled; as 40% of the children have had little or didn't keep any eye-contact in the pre testing, 87% of them bettered their eye-contact after the therapy; 83% of the children were responsive to usual teaching methods in the post-testing showing that the child was more ready to be integrated into more common classroom setting; 62.5% of the children have changed their scoring related to odd play such as spinning objects or self, unusual attachment to

objects etc.; as noted in the post-testing 60% of the children have experienced significant measurable improvements in over or under sensitivity to smells, textures, tastes, sounds, fear or pain; 88% of the children in the study became more adapted to verbal cues, with better response overall; 50% of the children reduced the aggressive behavior they have shown in pre-testing such as: skin scratching, biting, hitting, intending to harm others, and alike destructive behaviors; 85% have showed noticeable reduction in imitative behaviors, while 81% have showed reduction in repetitive behaviors following hyperbaric therapy. 70% of the children with autism that took part in this study have shown improvement in their GAF scores in the tests between the before and after therapy. The improvements that the remaining six children have shown have not been enough to change their overall GAF score (Lerman et al., 2008).

However, the positive results of several previous studies, have shown that the use of hyperbaric treatment might be a safe and promising treatment for children with autism. It is also clear that many previous researchers have identified promising results that are strong enough to justify further studies of Hyperbaric Oxygen therapy for patients with autism. The inclusion of HBO as part of standard therapy for patients with ASD should be examined in future studies. Presently, there is not enough evidence to endure the use of HBOT in the treatment of children with ASD in order to recommend it as a form of treatment. Detailed research may show that only specific groups of children with autism can benefit from this therapy (Sakulchit, 2017).

Biomedical treatment

The etiology of autism shows many causes such as genetic changes, teratogenic substances, autoimmune disorders, biochemical and metabolic diseases, viral and bacterial infections, improper use of vaccines, heavy metals, hypoxia, allergic reactions, and others. In order to eliminate or alleviate these causes, researchers emphasize the need to implement biomedical treatment in the treatment of this condition. The essence of medical treatment is the treatment of the condition, unlike biomedical which is aimed at treating the biological individual characteristics of the child with autism. The treatment itself includes several procedures, such as vitamin therapy and therapy with minerals, amino acids, enzymes, detoxification of heavy metals, application of dietary supplements, organic acids, probiotics, and antifungal therapy (Trajkovski, 2011).

Oxytocin is a small peptide hormone composed of nine amino acids that are secreted by the mother during birth and lactation. Some data suggest a possible role for oxytocin in the treatment of autistic symptoms. In one study 29 children with autism and 30 age-matched children without autism were examined for the oxytocin concentrations in their body. Children with autism appeared to have lower levels of oxytocin. While children without autism have had higher levels with age, children with autism didn't show any change (Modahl, 1998).

Hollander et al. performed a placebo-controlled, double-blind study of oxytocin infusion in adults who have been diagnosed with autism or Asperger syndrome. From the 15 participants who received oxytocin infusion in the research, they observed a decrease in repetitive behavior in 13 subjects. In the placebo group which also had 15 participants, only 6 subjects showed a reduction in repetitive behavior. No differences in side effects were found between the oxytocin and placebo groups. In conclusion, the authors suggested that oxytocin treatment might improve social behavior in children with autism (Hollander et al., 2003).

Carnosine has been given for eight weeks in a double-blind placebo-controlled study where 31 children with an autistic spectrum disorder participated. They used 800 mg of L-carnosine every day. The therapy has been well-tolerated in all participants and has shown noticeable improvements in socializing, communication, interaction, and vocabulary, as well as improvements in general autistic behavior compared with minor improvements in the placebo group (Chez et al., 2002).

New biomedical treatments and protocols are showing that the treatment has to be implemented according to every child's individual characteristics. Rivera (2018) created a protocol that starts with a gluten-free and casein-free diet, continues with Clorox dioxide therapy, and ends with hyperbaric oxygen therapy. Although her protocol is medically not approved, she claims that 357 children with autism spectrum disorder have been cured thanks to this protocol.

Hormonal therapy

Several studies have shown that secretin improves the symptoms of autism, arousing interest in secretin as a possible therapy for autism. Children with gastrointestinal problems that belong to the autism spectrum disorders, may receive secretin during diagnostic testing (Trajkovski, 2011).

A 4-year-old patient with autism, Peter Beck, has received secretin during laboratory testing to determine the cause of chronic diarrhea. During his secretin infusion intake that lasted for 3 weeks, his behavior has been changed dramatically. Peter could utter over 100 words, say short sentences, answer questions, and his eye contact was also improved (Whitman and Kolberg, 2004).

Another cross-sectional, placebo-controlled, and double-blind study, found that secretin improved the symptomatology in children with autism that had chronic diarrhea, far more than in children without the condition. Janet Kern et al have examined 19 children with autism by giving to each child one infusion of secretin and one infusion of placebo saline. The examiners described that five boys with chronic diarrhea has experienced a decrease in hyperactivity, irritability, crying, and anxiety when they were treated with secretin and had no placebo response.

In addition, children diagnosed with autism and chronic diarrhea have shown improved language skills and reduced stereotypical behavior. However, it should be noted that deterioration was observed in one child without gastrointestinal symptoms, with escalating behavioral disruptions. In conclusion, the researchers noted that there might be a subtype of children with autism and chronic diarrhea who can benefit from secretin therapy (Kern, 2002).

Vitamin therapy

18 studies evaluating the effects of vitamin B6 in children with autism have shown good results without any side effects. This is more than an important result for efficacy and safety, especially because it is ascertained that other medications given for autism have shown conflicting results together with the risk for side effects (Trajkovski, 2011).

Research for vitamin B6 use has begun in the 1960s. In 1968, the German researcher V.E. Bonisch has described that 12 out of 16 children diagnosed with autism have had a noticeable improvement in behavior when given high doses (100mg- 600 mg) of vitamin B6 daily. The results from this open clinical trial have shown that three of the patients have spoken for the first time after receiving vitamin B6.

In 1964, Dr. Bernard Rimland has begun an extensive research of over 200 children with autism who have been given mega doses of vitamin B6, niacinamide, pantothenic acid, and vitamin C. All vitamins have been stored in a single tablet specially designed for this study. After 4 months, at the end of the study, it has been clear that vitamin B6 had significant importance of all 4 vitamins tested and, in some cases, it has led to outstanding improvement. Vitamin B6 has shown a significant improvement in 30% to 40% of children. Merely some of the children have shown minimal side effects (sounds sensitivity and irritability) but they have disappeared when magnesium has been given. Magnesium eliminates side effects, and it often improves communication skills and behavior.

Another two studies by US research groups, implemented by Thomas Gualtieri et al., (University of North Carolina) and George Ellman (Sonoma State Hospital- California), have found beneficial results in autism spectrum disorder patients (Rimland, 2002).

Recent data show that there are over 20 studies of vitamin B6 in addition to magnesium in the treatment of autism. Twelve of them have been double-blind, placebo-controlled studies. Almost all studies have found that 45-50% of children and adults with autism have benefited from taking these drugs. Vitamin B6 participates in over 100 enzymatic reactions. The reason why many children and adults benefit from high doses of vitamin B6 remains unknown, but a possible explanation would be that they have a reduced ability to convert vitamin B6 to its active form and defective enzymes to build key neurotransmitters that are usually having the large need for the active form of vitamin B6 (Adams, George, and Audhya, 2006).

Vitamin C use has been examined in a study that had shown relief of symptoms in school children with autism. The authors hypothesized that the action has been shown as a consequence of the presumed vitamin C dopaminergic effects. However, these study results haven't been repeated, yet (Dolske et al., 1993).

Chelation therapy

Several studies have identified that some children with Autism Spectrum Disorders exhibit clinical and behavioral improvements with chelation as a process of removal of heavy metals with medication.

In one publication, 2 children diagnosed with Autism Spectrum Disorder have had lead toxicity. Both children have received chelation and that way their blood lead concentrations have been reduced. A subsequent evaluation has shown that the children did not meet the criteria for Autism Spectrum Disorders any longer (Lidsky and Schneider, 2005).

The Autism Research Institute has looked at the impact of various treatments on children with autism spectrum disorders, such as nutritional supplements and medicines, and when evaluating the chelation benefit, 74% of parents have described improvements in their child's behavior. Only 3% of parents have rated their child as having worsening chelation behavior (ARI, 2008).

Another study, which has examined 479 parents of children with autism, showed that 32 children tried chelation, 50% of parents have reported that their children had improved behavior and 6% had to worsen (Goin-Kochel, Mackintosh and Myers, 2009).

It should be pointed out that placebo-controlled chelation studies have not been performed on individuals with an autism spectrum disorder. However, despite the obvious limitations, researchers' findings imply that chelation may be an effective form of treatment for some autism spectrum disorder individuals having a heavy metal load. Further research should include well-designed controlled chelation studies with relevant clinical monitoring, together with objective screening to detect children with autism spectrum disorders who have enhanced heavy metal concentrations (Rossignol, 2009).

Immunologic therapy

The idea that autism is an autoimmune disorder is confirmed by the good response of patients by giving immunomodulatory medications. Depending on the nature of the immune abnormality, the aim of therapy should be to normalize the immune response, rather than to induce immune suppression or stimulation. Immunotherapy should always be performed in consultation with a physician. it is used using some immunological interventions: steroid therapy, intravenous immunoglobulins, oral tolerance to autoantigens, and plasmapheresis (Trajkovski, 2011).

There is one study in autism that has shown an improvement in autistic symptoms when children are treated with the adrenocorticotropic hormone. These results have suggested that steroids are potentially useful in improving the clinical symptoms of autism. However, the effectiveness of steroids has not yet been fully evaluated in autism (Singh, 1997).

Intravenous immunoglobulin therapy has been used to treat children with autism. Low doses and high doses of intravenous immunoglobulins have been used. Singh and co-workers find that high doses are better than low doses. Clinically children show improvement in speech, communication, social interaction, and attention. Despite the success, the researchers concluded that this treatment is not suitable for every child with autism (Singh, 1999).

Nemecheck protocol

The Nemecheck Protocol is a new way of treating children with autism and developmental disorders discovered by Dr. Patrick M.Nemecheck. According to him, this protocol helps a variety of childhood issues and disorders that surprisingly share the similar origins of an overgrowth of intestinal bacteria and multiple mechanisms that fuel inflammation. Childhood developmental disorders (developmental delay, ADD, ADHD) and childhood mood disorders (anxiety, chronic depression, OCD) are primarily the consequence of excessive brain inflammation from intestinal bacteria. Autism is the consequence of excessive brain inflammation in addition to a toxic encephalopathy from the overproduction of propionic acid from intestinal bacterial overgrowth. Therefore, the Protocol is implemented in two steps: rebalancing the intestinal tract and reduction of brain inflammation (Nemecheck, 2017).

In a recent study, Dr.Nemecheck claims that autism spectrum disorder can be reversed by normalizing the gut microbiome and with reducing pro-inflammatory cytokine production. In his research, the patients have been treated with a combination of dietary supplements or antibiotics to correct small intestine bacterial overgrowth and consumed omega-3 (known as fish oil) and omega-9 (known as olive oil) to counter inflammatory dietary omega-6 fatty acids. The observations in his study have been made with clinical follow-up and parents' reports. In the results, they noticed that young children have become more aware of their surroundings, making more eye contact, and initiating social interactions.

Their results also have shown that many children have begun speaking within 2-8 weeks and the improvement in all developmental areas has been re-initiated. Over 12-24 months, the results have shown that autistic behaviors faded, developmental deficiencies resolved, and a variety of features of autonomic function greatly improved. Within this time frame, most children under the age of seven have become indistinguishable from their peers (Nemecheck and Moore, 2020).

Conclusion

There have always been attempts to find appropriate therapy and treatment for autism, but unfortunately, most of them have not been confirmed for treating this condition. Many studies have shown positive effects and reduced the symptoms of autism spectrum disorders, however, some of them have shown negative effects on a small sample.

Given the size of the spectrum, it is impossible to find a single therapy that would be appropriate for all children with autism spectrum disorders and it remains unclear which therapy would be appropriate for each individual child. This is the reason why the parents of children with autism are going from one autism specialist to another in order to find the treatment that will work best for their child.

On the other hand, the need to find appropriate therapy and treatment is more than necessary. Especially considering that the number of children with Autism spectrum disorders is increasing every year. With years, children with autism have only been sent to special education without any medical examination for their condition. If we notice that some children show high percentages of improvement from some treatments until complete cure, as some protocols claim, then it is important to accept the need for a unified medical approach that will improve the child's clinical picture before starting with the education process.

Reducing many of the symptoms of autism in some therapies suggests that autism should be treated as a medical problem. There is a great need for additional tests and studies of possible treatments in order to confirm or decline their effectiveness.

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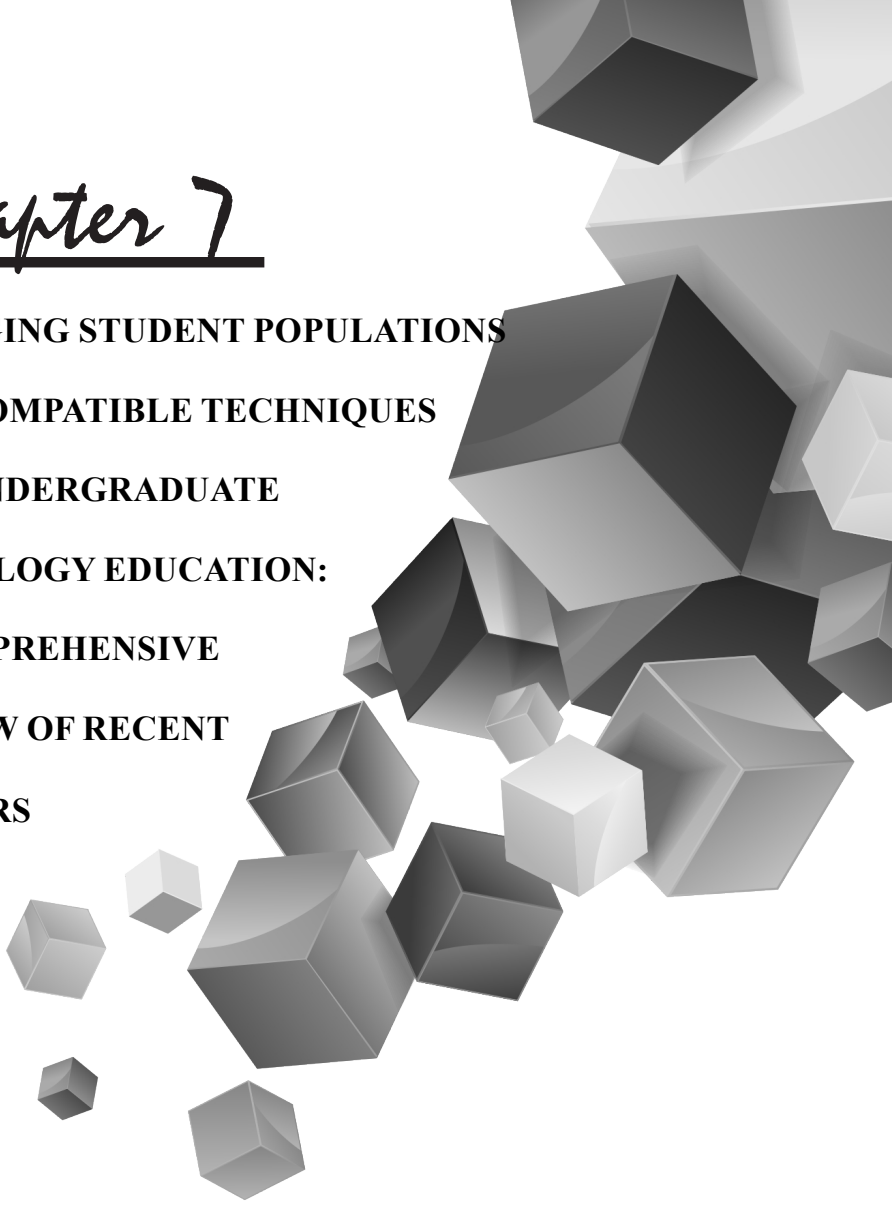
Chapter 7

CHANGING STUDENT POPULATIONS AND COMPATIBLE TECHNIQUES FOR UNDERGRADUATE PATHOLOGY EDUCATION: A COMPREHENSIVE REVIEW OF RECENT 10 YEARS



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Introduction

In medical schools, pathology education usually starts in the 2nd grade and continues in the 3rd grade, which is a period of transition from basic sciences to clinical internship training. Pathology is a science that enables students, who understand normal anatomy, histology, physiology and biochemistry, to learn the etiology and pathogenesis of the diseases at the molecular, microscopic and macroscopic level and thus, to understand and interpret the symptoms, diagnosis and treatments more easily (Buja, 2019; Domizio, 2006; Gencer & Dere, 2019; Khonglah et al., 2019). It is imperative that the generation which is still studying in medical schools shows significant differences from the past ones, and also, has to be ready for the dissimilar world of the future. Therefore, medical education should be arranged in accordance with the needs of the current generation. In this review, through a detailed search of national and international literature, current approaches and recommendations in the field of undergraduate pathology education are examined and new studies are presented. Firstly, in the light of publications, required modern doctor profile and the ways to reach this profile are explained. Then, the place of pathology in this new education model is discussed. At last, how the doctors of future will be able to be equipped with the desired pathology knowledge and skills as well as how the education can be assessed and evaluated are explained in the light of literature.

What kind of youth occupies the desks in medical schools?

The generation who fill the medical universities today and will start working in the near future, is the generation Z formed by those born between 1996 and 2012 (J.E. Hunt, Lick, R. Hunt, 2018; Nicholas, n.d.; Plochoki, 2019). They, with major differences from the previous ones, are defined as young people with high self-confidence, self-worth and even almost narcissism. On the other hand, they have high stress and anxiety levels so that, nearly half of medical students experience burnout, 11% attempt suicide and 82% show signs of psychological distress (Plochoki, 2019). Though, entrepreneurship, volunteerism, tolerance to differences, spirit of togetherness are more pronounced in this generation from the earlier ones, probably, the most striking difference is their birth into a digital world. Smartphones, connection with short internet messages, e-shopping, Youtube, on-line music and movies, distance education and all kinds of e-sharing are the everyday parts of their lives, and contrary to what is believed, actually, they are highly communicative (Nicholas, n.d.; Plochoki, 2019; Savas, 2019). Accordingly, in a study with 1300 students aged 13-18, 95% stated that teamwork improved them. Also, 64% wanted to use websites, 50% DVDs, 46% smart boards and digital books, and 45% online videos among the educational tools in the classroom (Nicholas, n.d.).

Although, today's medical students are highly intelligent, they are reluctant to read long articles; prefer a structured but interactive learning experience; and are more successful in short courses supported with video and similar materials. The number of students who prefer distance and recorded courses is also high (Buja, 2019; Hunt et al., 2018). It is clear that for generation Z, an educational environment will be required in which technology will be used intensively; communication and feedback will take an important place, and where traditional didactic lessons will be replaced by active learning methods.

How should the doctor profile be in the new age?

In today's world, where digitalization has entered all spheres of life, it is imperative that newly grown doctors have to be individuals who keep up with this age. Doctors of the digital age should be experts who can easily access unlimited sources of information, and pick up the most accurate answers to their questions, in the most practical way. Future doctors need to be able to think, make decisions and act in the most quick way. In this process, in an "information pollution", solution-oriented approaches are essential that can question, analyze and synthesize, can combine information and achieve the results. Future doctors have to be prone to teamwork in order to succeed in an ocean of science that is gradually expanding and becoming more complex. Additionally, they should value people, be helpful, reliable, respectful to nature, aware of the responsibility of their job, have to lead, be curious, enthusiastic to learn, open to development and tireless. In short, doctors of the new age must be equipped with more knowledge as well as different skills in order to maintain and improve public health (Buja, 2019; Khay-Guan, 2019).

How should the doctors of the new age be trained?

Medical education has been a master-apprentice relationship that has been carried out by transferring the knowledge and skills of experienced physicians, face-to-face, to the new ones. Until the 1950s, a good instructor was a person with a wide knowledge and skills who transferred all, without any hesitation. In the second half of the 1900s, with the winds of change in the world, the adequacy of such a method of education began to be discussed and new approaches emerged. The core education programs were created and the learning goals and the courses they had to take to reach this target were determined. The courses, in which each subject was presented in full detail, were replaced by integrated education which was a cluster of lessons grouped according to systems (Khonglah et al., 2019; YOK, 2020). Methods such as problem-based, case-based or team-based learning started to take place in education life. Today, attempts are ongoing on switching to interactive education models where students are

more active and access information not readily, but by their own effort. These new education methods are combined with the old, didactic style while creating new curricula. This modern education system is expected to be suitable for the doctor profile of the near future (Elharram et al., 2017; Gao et al., 2019; Nagesh, Giurca, Lishman, 2018; Nivala et al., 2013; Onan, Usubutun, Sezar, 2019; Osman&Kutty, 2013; YOK, 2020).

How has the place of pathology changed in medical education models?

In the last 100 years, there has been a change in the education of pathology in medical faculties, from being an assistant discipline to becoming a major player and gradually moving towards the edge of the field. The question, "how much should pathology be taught to medical students?" is still being discussed today; some argue that knowing the pathological bases does not change the morbidity and mortality rates and is unnecessary, while others think that scientific basics should be known for evidence-based practice (Domizio, 2006).

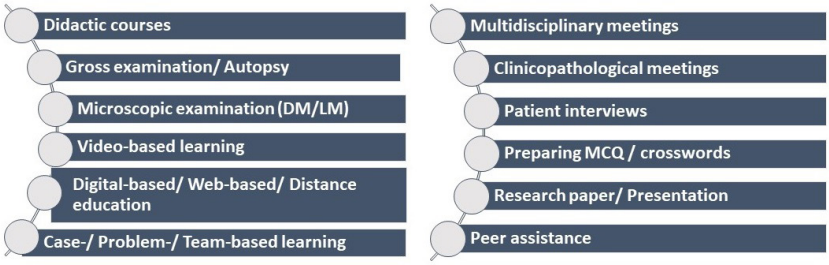
In the early 1900s, surgeons were the ones who taught pathology and examined surgical specimens. Over time, pathologists began to take part in both courses and autopsies. The Todd report, in 1968, recommended general pathology to be included in the compulsory part ot the the preclinical period. After 1980s, it became important not only to teach the basics of medicine, but also to encourage students to think and learn throughout life. So, new methods have become necessary for critical decision-making and implementation of have been learned. Self-directed learning and problem-based learning became popular. In many medical schools, till the late 80s, system-based approach was adopted. While the importance of the lessons was decreasing, seminar based studies and learning in small groups became more and more important. In this process, the time allocated to pathology in the medical faculty curriculum began to be diminished and supported by self-learning packages. Autopsy trainings, which were popular in previous generations, gradually decreased; As a result, it was difficult for students to see macroscopic pathology and to understand clinicopathological correlation.

As a consequence of serious discussions on medical education, 1910 North America Flexner report, 1993 United Kingdom "Tomorrow's Doctors" and 2010 North America Carnegie Report were published (Buja 2019; Sadofsky et al., 2014). In these reports, it was emphasized that the medical curriculum, which should be "student centered", should be organized around a core of basic knowledge and skills. This curriculum would also include several study modules students chose based on their interests. The traditional distinction between preclinical and clinical

years would disappear and the system-based approach would replace the discipline-based curriculum. The aim was to train patient-centered physicians with better communication and practical skills. These changes were also reflected in pathology education. According to a study conducted in the United Kingdom in 2001, the duration of pathology education decreased by up to 53%, the teaching of morphological changes caused by the disease decreased considerably, and epidemiology and public health gained more importance.

Pathology gradually began to be taught in an integrated manner. Didactic lessons were replaced by self-learning, and problem-based learning (PBL) while the facilitator took the place of the trainer (Buja, 2019). Instead of entering the laboratory classes for microcopy examination, students started computer-aided education and web-based learning. Meanwhile, pathology museums disappeared, and autopsy numbers dramatically declined (Buja, 2019; Buja, Barth, Krueger, Brodsky&Hunter, 2019). Thus, the opportunity of today's medical students to make a face-to-face and real-time macroscopic examination has diminished, and increasingly, many are able to see pathology specimens only via digital-based applications. Today's medical students are in an environment which is student-centered instead of educator-centered; integrated instead of discipline-based; prone to choose courses according to their interests; active and problem-based instead of passive; and open to computer-based methods (Buja,2019; Domizio, 2006). Now, as an advantage of diversified pathology learning methods, students can learn better [Figure 1] (Gencer et al., 2019; Kar et al., 2012; Khan et al., 2019; Onan et al., 2019; Venkatesh, 2011).

Figure 1. Current methods that can be used in undergraduate pathology education



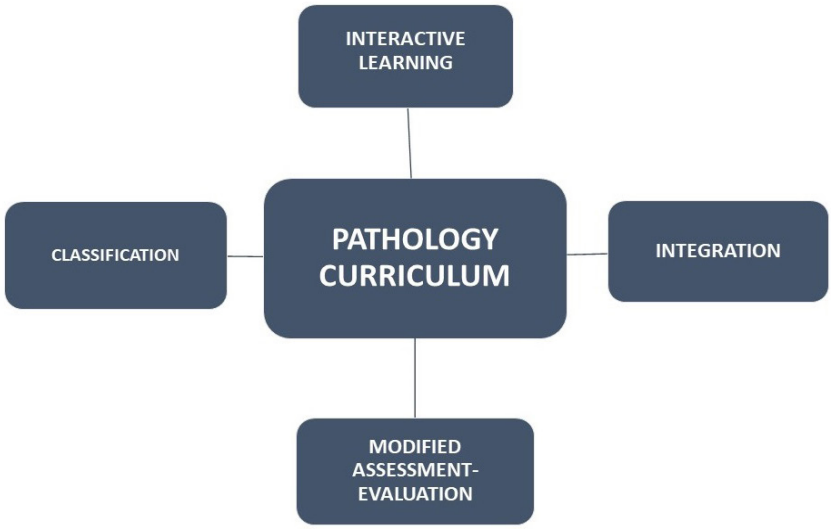
DM: Digital microscopy; LM: Light microscopy; MCQ: Multiple choice question.

According to publications examined in this review, new approaches in medicine and pathology education are accepted both in our country and globally (Al Nemer, 2019; Benoy, Shah&Bhatt, 2018; Gencer, 2019; Khongiah et al, 2019; YOK, 2020). However, traditional methods are not completely avoided and in practice, each country and every faculty prefer

to combine new methods with traditional ones at unique rates.

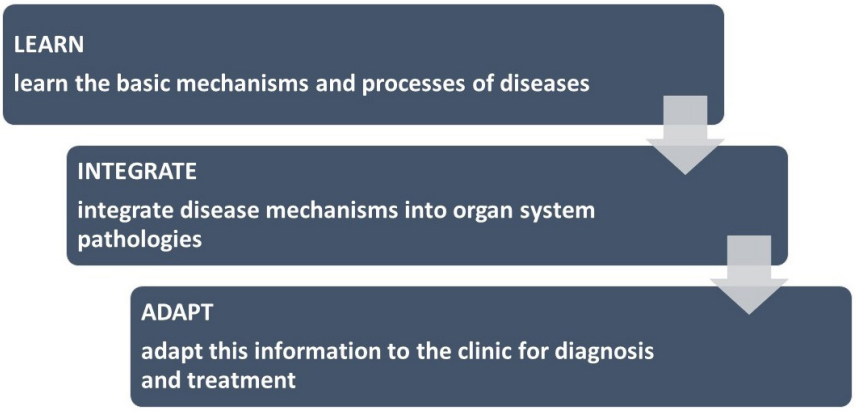
Pathology curricula are recommended to be overhauled according to the following four topics: 1) It should be classified as things should be known, desired to be known and nice to be known; 2) It should be integrated with clinical sciences; 3) Along with didactic lessons, problem-based learning and other interactive education methods should be applied; and 4) Student assessment methods should be modified giving less weight to morphology and more to clinical interpretations [Figure 2] (Khonglah et al, 2019; YOK, 2020).

Figure 2. Topics to consider for improving the pathology curriculum



According to the current approach, the competencies that medical students should gain in pathology lessons are; to learn the basic mechanisms and processes of diseases, integrate disease mechanisms into organ system pathologies and adapt this information to the clinic for diagnosis and treatment [Figure 3]. On every competency basis; learning goals and optional sub-goals should be determined and learning, integration and use of knowledge should be measured and evaluated (Sadofsky, 2014; YOK, 2020). What is desired from a medical student is not to function like a pathologist, but to understand pathology.

Figure 3. Competencies expected from medical students in pathology lessons



Examples of current education models related to pathology

Among the articles published in the last 10 years, 94 articles are considered worth discussing in this review. Twenty-five (26.6%) of them which included a review article (Buja et al.,2019), 2 editorials (Henson&Grimley, 2015; Minhas, Enogieru, Mitchell&Mata, 2017), 4 debate articles (Buja et al., 2019; Cavanagh, Vanstone, Ritz, 2019; Gao et al., 2019; Y.Y.Li, K.Li, Yao, Xu&Cai, 2015), 3 recommendations (Magid et al, 2015; Knollman-Rirschel, Suarez, Gilliland, Conran&Pock, 2017; Sadofsky et al., 2014), 5 personal experiences (Brooks, Paus, Corliss&Ranheim, 2016; Dahlstrom, 2012; Humpreys et al. 2020; Osman&Kutty,2013; Venkatesh 2011), a letter to editor (Benoy et al.,2018), and 9 research articles (Gencer et al., 2019; Herrmann et al., 2015; Kar et al., 2012; Khonglah et al., 2019; Marsdin&Biswas, 2013; Mirzazad et al.,2017; Nivala et al., 2013; Omidifar, Keshtkari, Dehghani&Shokripo,2017; Saco, Bombi, Garcia, Ramirez&Ordi, 2016) are grouped separately. It is noteworthy that between 2010- 2020 various educators around the world has thought hard on how to update education, and subsequently developed suggestions and shared experiences. Total 56 (59.6%) articles convey the studies in which various new methods have been discussed, some alone, and others in addition to the traditional methods [Table 1].

Table 1. Distribution of articles published between 2010-2020 on current learning methods in undergraduate pathology education (n=56)

Method	**Number of publications**	**Researchers**
Integrated learning	7	Atta et al.2018; Elharram et al., 2017; Haspel et al.,2012; Herbert et al., 2017; Magid et al.,2012; Nagesh et al.,2018; Onan et al.,2019
Problem based and case based learning	7	Datta&Ray,2016; Dominick, McDaniel, Tilton, Flory&Kondrashov, 2018; Emerald, Han & Oo, 2016; Kapoor, 2015; King. Sharma, Jackson& Fiebelkorn, 2019; Srinivas, Kotwal & Tekian, n.d; Villatoro, Lackritz&Chan, 2020
Team based learning	3	Alwahab et al.,2018; Anwar,Shaikh, Dash&Khurshid, 2012; Carbo et al.,2012
Digital/web based learning and distance education	8	Craig, McGee, Mahoney&Roth, 2014; Kanthan, 2011; K. Kayser, Ogilvie R, Borkenfeld S&G. Kayser, 2011; H. Nautiyal, Pathak, R. Nautiyal, Sachdev & Pandey, 2018; Peacock & Grande, 2016; Ripoll, Oparka, Campbell & Erolin, 2017; Sarıoğlu, 2016; Williams, Lee, Oien&Treanor, 2018
Mobil based learning/code mediated	1	Mogali et al., 2019
Video based learning	2	Fatima, Ghias, Jabeen&Sabzwari, 2019; Jacquier et al., 2019
Macroscopy training with 3-D systems	1	Mahmoud & Bennett, 2015

Comparison between digital microscopy and light microscopy based training	13	David et al., 2018; Foad, 2017; Helle et al., 2011; Lee et al.,2019; Nauhria&Ramdass, 2019; Ordi et al., 2015; Sağol et al., 2015; Samal &Prakash, 2019; Simok et al.,2019; Sivamali, Murthy, Gupta& Wooley, 2011; Vhriterhire, Orkuma, Jegede, Omotosho&Adekwu, 2016;
Cytopathology training	1	Van Es, Kumar, Pryor, Salisbury & Velan, 2016
Autopsy/cadaver mediated learning	6	Bamber et al., 2014; Geldenhuys et al., 2016; Gopalan, Dissabandara, Nirthanan, Forwood&Lam, 2016; Rae et al., 2018; Talmon, 2010; Wood, Struthers, Whiten, Jackson & Herrington, 2010
Preparation of multiple choice questions	3	Bekkink, Donders, Kooloos, deWaal&Ruiter, 2015; Grainger, Dai, Osborne&Kenwright, 2018; Herrera, Lucena&Quiroga, 2019;
Peer assisted learning	3	Grover, Sood & Chaudhary, 2017; Leong et al.,2017; Tayler, Hall, Carr, Stephens&Border, 2015;
Preparation of crossword puzzles	1	Htwe, Sabaridah, Rajyaguru & Mazida, 2012
Total number of studies	**56**	

In publications from almost all over the world, the experience in transition to integrated education has been presented (Humpreys et al., 2020; Nagesh et al., 2018; Onan et al., 2019; Osman et al., 2013; YOK, 2020). Also, it is mentoned that while time spent for theoretical courses diminished, time allocated to case discussions, problem-based learning, group studies, peer-assisted learning and other education models has increased (Alwahab et al., 2018; Gao et al., 2019; Grover et al., 2017; Nagesh et al., 2018; Osman et al., 2013; Villatoro et al., 2019).

An important part of the research includes the studies comparing digital microscopy and traditional light microscopy in education (13/56

publications, 23.2%) (David et al., 2018; Foad, 2017; Helle et al., 2011; Lee et al., 2019; Nauhria&Ramdass, 2019; Ordi et al., 2015; Sagol et al, 2015; Samal&Prakash, 2019; Simok et al., 2019; Sivamalai, Murthy, Gupta& Woolley, 2011; Solberg, 2012; Vhriterhire, Orkuma, Jegede, Omotosho&Adekwu, 2016; Wilson et al., 2016). Digital pathology has been concluded to have positive effects on learning in all studies. Due to results, almost all students learned faster; satisfied with access from outside the school, annotations, good quality of slides and enjoyed the technology. It has been reported that cytopathology training can also benefit from virtual microscopy (Van Es, Kumar, Pryor, Salisbury&Velan, 2016).

Numerous articles were published about digital / web-based learning and distance education after 2010 (Alwahab et al., 2018; Craig et al, 2014; Kanthan, 2011; Nautiyal et al., 2018; Onan et al.,2019; Peacock, 2016; Ripoll et al., 2017). Nautiyal et al compared formalin- fixed surgical specimens with digital-based pictures and videos in macroscopic education, and stated that the image deteriorated with time in the formalin-fixed group; while digital based materials were better understood (Nautiyal et al., 2018). Articles have been published on combining online and face-to-face education (blended learning) (Sadofsky et al., 2014), animations in distance education (Ripoll et al., 2017), digital game-based education (Kanthan, 2011), patient simulation software (Craig et al., 2014), and learning through online app platforms (Peacock et al., 2016). It was found that learning was positively influenced with high quality macroscopy specimens marked with Quick Response (QR) codes (Mogali et al., 2019) or with 3D-printed autopsy or macroscopy specimens (Mahmoud et al., 2015). Interestingly, in the last 10 years, 11% of the studies on education methods focused on the importance of autopsy and cadaver in pathology education (Bamber et al., 2014; Geldenhuys et al., 2016; Gopalan et al., 2016; Rae et al., 2018; Talmon, 2010; Wood et al., 2010), and despite the decreasing number of cadavers / autopsies, all emphasized their great benefit.

Preparation of multiple choice questions (Bekkink et al., 2015; Grainger et al., 2018; Herrera et al., 2019) or crossword puzzles (Htwe et al., 2012) were thought to improve students' analytical thinking skills. In studies on this subject, positive (especially in immunopathology, (Herrera et al., 2019) or in male students, (Bekkink et al., 2015)) or neutral (Grainger et al., 2018) results were reported.

Examples of current assessment-evaluation management about pathology

After 2010, studies conducted to investigate assessment and evaluation methods in pathology constituted only 7.4% (7/94 studies) of the publications

presented here [Table 2]. In four studies, web-based experiences were presented. There are very few publications that convey the experiences of the objective structured practice exam (OSPE) (Gonneppanavar&Dakka, 2013; Htwe, Ismail&Low, 2014) and the objective structured clinical exam (OSCE) (Gupta, Dewan&Singh, 2010). However, many studies investigating new educational methods have measured the outcome of the methods with various assessment-evaluation methods (Alwahab et al., 2018; Craig et al., 2014; Dominick et al., 2018; Foad, 2017; Grover et al., 2017; Htwe et al., 2014; Nauhria et al., 2019; Onan et al., 2019). Since the main purpose in those articles was to analyse the learning method, they are not discussed under this section. Assessment and evaluation are important components of education. They show the success of the method as well as the missing parts. Also, evaluation method can directly affect the student's motivation and learning level (Htwe et al., 2014; Khan et al., 2019). However, more emphasis is needed on this area.

In one of the relevant publications (Kahn et al., 2019), application of the pathology microscopy and macroscopy practice exam in the form of a digital power point slide show is reported to be more objective, structured and time-saving compared to traditional desktop exams. In a letter to an editor on OSPE (Gonneppanavar et al., 2016), 10 preclinical, radiological, and gross-microscopy stations were prepared for the exam, and 80% of students stated that OSPE included a wide range of topics; but more than 80% students needed more education and orientation. In the other study, the efficiency of the method using high resolution pictures was compared with the traditional method using real macroscopy and microscopy preparations during OSPE and the students were more successful in the first method ($p<0.05$) (Htwe et al., 2014). There is only one publication (letter to editor) related to OSCE, which explains that it is possible to arrange an OSCE-type pathology exam (Gupta et al., 2010).

Table 2. Publications published between 2010-2020 on methods used during assessment and evaluation in undergraduate pathology education (n=7)

Method	**Number of publications**	**Researchers**
Objective structured practice exam (OSPE)	2	Htwe et al., 2014; Goneppanvar et al.,2016
Objective structured clinical exam (OSCE)	1	Gupta et al., 2010
Web based measurement-evaluation	3	Alyusuf, 2013; Hu et al.,2016; Patil, Gosavi, Bannur & Ratnakar, 2015
Video based evaluation of pathology practice	1	Khan et al., 2019
Total number of studies	7	

Other suggestions

After 2010, there are also original, interesting and up-to-date studies that can not be classified under other groups. For example, Bramstedt et al. indicated that proper clinical observation requires more than a glance; it requires attention to details. So, visual arts such as cinema and painting facilitates the understanding of medical pathologies (Bramstedt, 2016). Pinnock et al. suggested the way to integrate artificial intelligence into medical education and how and where to use it during clinical reasoning (Pinnock, McDonald, Ritchie & Durning 2020). In the last 10 years, 3 studies have been published which pointed out the significance of accelerated repetition of pathology during the Internship (Howel, Wahl, Ryan, Gandour-Edwars&Green, 2019; Naritoku, Vasovic, Steinberg, Prystowsky&Powell, 2014; Smith, Collins&Hall, 2019). These publications stated that in the last year of the medical school, a rapid repetition of pathology-specific medical information, basic skills and processes for anatomical pathology and laboratory medicine would be beneficial and would increase the inclination towards pathology specialization.

Students' perceptions

In a study with 1018 German medical students, students preferred virtual microscopy (89%), and wanted the pathology curriculum to be supported with autopsies (87%), seminars (79%) and digital-based learning tools (Herrmann et al., 2015). A study from Saudia Arabia put forward the problem that the majority of the students were not aware of the role of pathologists or did not consider pathology as a future career (Al Nemer, 2020). In another study, environmental / external factors and internal factors that affect the learning were questioned; the educator's willingness and ability to teach were the most important external factors, while the student's desire to learn and interest in pathology course were reported as the most important internal factors (Mirzazad et al, 2017). Thus, increasing the equipment of the educator on new education methods seems to facilitate the achievement of learning objectives by increasing student interest.

Core attributes of a medical educator

Finally; according to John Hopkins University School of Medicine, a medical educator should; Embrace science and instill this pasion to learners; demonstrate integrity and throughness and expect this from learners; be a role model for honesty, integrity and kindness and fair, equitable and respectful treatment of others; instill in learners an appreciation for the importance of individual variability in human biology, genetics, behaviour and envirnment; foster a positive learning environment that is diverse, respectful, inclusive and collegial; develop the next generation; always

strive for excellence and aspire to continually do better; teach and serve as a role model for the wise use of society's resources; help learners understand and appreciate the value of colloboration across disciplines and demonstrate a focus on the public good (Buja, 2019). Undoubtedly, the medical educator has a huge responsibility in shaping the medical future.

Conclusion

In undergraduate pathology education, the use of new learning models along with didactic education will make it easier for today's medical students to learn and become ready for the developing world. Distance education models should be developed with digital and web based applications. Although the importance of macroscopy and autopsy education is accepted, new options such as video-based, 3D or digital solutions can also be adapted. The advantage of interactive methods such as case discussions, working in teams, preparing questions or crosswords is approved. Studies on diversification of measurement/evaluation options in pathology are required.

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Chapter 8

OZONE TREATMENT AND HYPERBARIC OXYGEN TREATMENT ON FIBROMYALGIA

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1. Fibromyalgia

Fibromyalgia (FM) is a complex disease which identified with usually chronic pain. Last times, other symptoms such as fatigue, cognitive dysfunction, and sleep disturbances were added FM definition. All FM symptoms can be seen on other diseases thus diagnosis is so quite difficult on FM. If patients can get diagnosis to FM, their laboratory tests have to be normalities and eliminated other diseases. Because of these, for FM diagnosis are improved some criterion. Firstly, these criterias were improved by the American College of Rheumatology (ACR) in 1900. 20 years later, ACR were updated criterions according to new improvements. Last criterions is involved an index and a questionnaire. In this way, FM is assessed not only physical but also other parameters like anxiety and depression (Chinn et al, 2016).

Chronic widespread pain is main symptom of FM. Pain is usually localized shoulder, arms, lower back, and hips. While mostly patients complain to continuous pain, pain can be time to time for some patients. Also pain level can be effected some conditions like as weather, mood, and stress. Losing sleep quality is another symptom of FM. Decreasing quality of sleep can cause to both losing functions and increase to pain and other symptoms (Borchers and Gershwin, 2015). Fatigue is the third symptom of FM. According to reports, at least 75% of FM patients were complained fatigue. Although fatigue is not assessment all causes on FM, it is still affected importantly FM patients life quality. Depression, anxiety, cognitive complaints can be involved symptoms of FM. In fact, many researchers reported that depression and anxiety has correlations between FM.

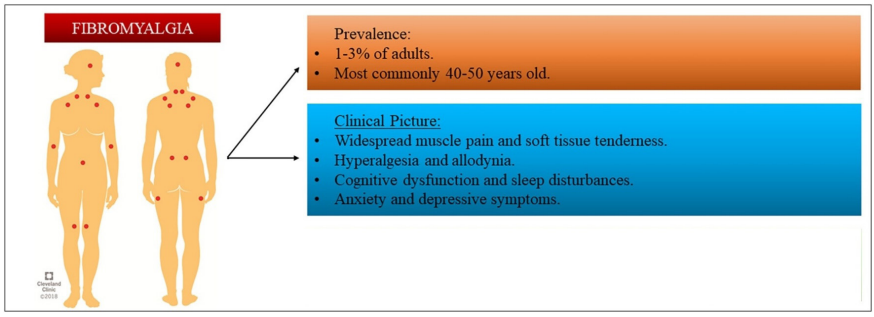

Figure 1. *Prevalence and clinical picture of Fibromyalgia (El-Shewy et al., 2019).*

Pathogenesis of FM is still not clear but sensory and pain play a role in mechanism. This unknown mechanism connected with central nerve system and non-central mechanisms (Chinn et al., 2016). Genetic and epigenetic factors are affected on these mechanisms (Borchers and Gershwin, 2015)

In treatment of FM, it purpose is take to control of symptoms. In accordance with this purpose, medical and complementary treatment can be applied (Oh et al., 2010). Even, some patients use extracts like as Ganoderma lucidum which called immortality mushroom (Mateo et al., 2015; Eroğlu and Beytut, 2018). Sometimes, they can use supplement such as Vitamins C (ascorbic acid) and E (Nazıroğlu et al., 2010; Ozturk et al., 2017). As a result, pharmacologic and non-pharmacologic treatments can be combined for FM.

2. Ozone Treatment

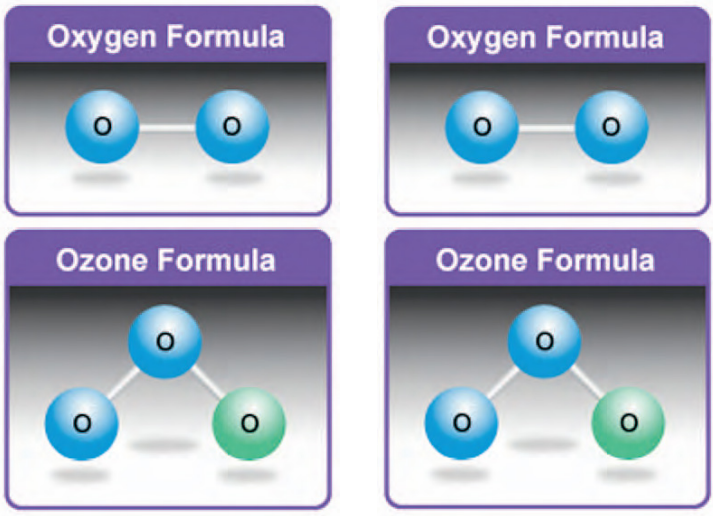

Figure 2. *Oxygen and Ozone Formula (Nogales et al., 2008).*

Ozone is tri-atomic oxygen form which was discovered in 1840. It is a soluble gases. Also, ozone places free radical category of oxygen (de Souza et al., 2018).

In the nature ozone occur two pathways. Firstly, oxygen molecules connected by thunderstorms. In thunderstorms, there are electrical discharges and these discharges provide energy for bond. Second way is caused from sun. The sun releases ultraviolet rays and this occur energy which cause of connected oxygen atoms (Nogales et al., 2008).

Ozone has potential toxic material. On the other hand, if ozone is used suitable doses, it can be good treatment method for some disease (Ozturk et al., 2018). When ozone mention for using treatments, it's a mix which contains O_2 and O_3 (Adali et al., 2019). The mixture which is certain ratios (95% Oxygen and 5% Ozone), it is not exist in nature. Therefore some special systems are using for occurring medical ozone (Nogales et

al., 2008; Bilge et al., 2019).

Ozone mixture is affected by some diversely such as antioxidant activity and nitrite oxide pathways. On the grounds that effect, ozone is using for treating many disease. Vascular disease affecting circulation, wound healing, viral diseases, such as hepatitis and AIDS and autoimmune diseases are some samples about these (Adali et al., 2019). Last years, ozone treatment is becoming more common and more popular due to antioxidant effects of ozone (Eroğlu et al., 2020a). Even, there are 260 different pathologies which are treating by ozone's antioxidant effects (Nogales et al., 2008).

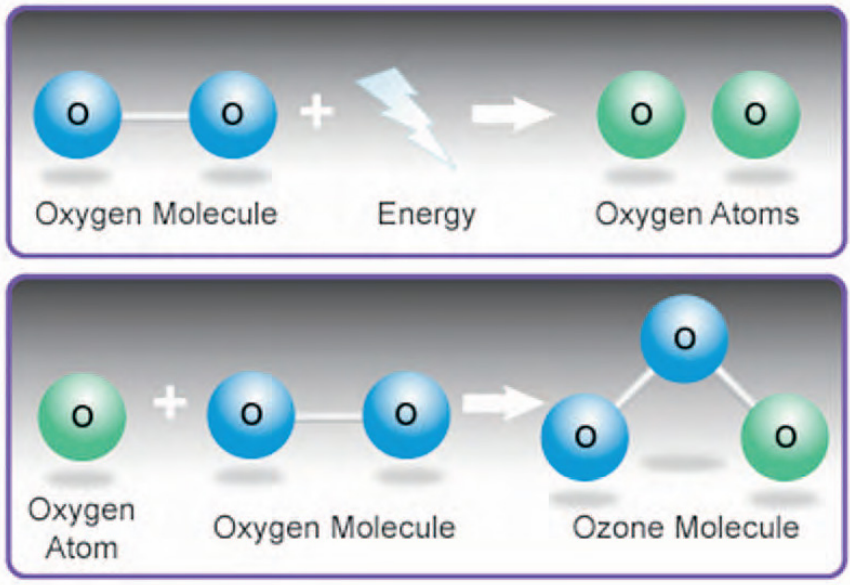

Figure 3. *Production of Ozone (Nogales et al., 2008)*

If ozone is a free radical, how to be effects like an antioxidant? This question was explained with too many researches. Firstly, ozone creates an oxidative stress to dissolve in the aqueous component of plasma. This stress causes to creating water, hydrogen peroxide (H_2O_2) and reactive oxygen species (ROS) because of ozone's reaction of polyunsaturated fatty acids. Later, existing ROS causes some a series of reactions. The transcriptional factor mediating nuclear factor-erythroid 2-related factor 2 (Nrf2) becomes active, then it causes activating the transcription of antioxidant response elements (ARE). Upgrading ARE transcription increases some enzymes levels like superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione S-transferase (GST), catalase (CAT), hemeoxygenase-1 (HO-1), NADPH-quinone-oxidoreductase (NQO-1), heat shock proteins (HSP).

By that way, ozone impacts on tissues as an antioxidant (Smith et al., 2017; Eroğlu et al., 2020b).

Last researches are revealed to that ozone therapy could have anti-inflammatory effects in additions to antioxidant effects (Yu et al., 2017). Anti-inflammatory effects are eventuated two phases. While pro-inflammatory mediators are released in first phase, second phase is result of antioxidant activity (Bilge et al., 2019). Also, Chen et al. (2016), is associated anti-inflammatory effect with down regulation of TRL4 on their study. The other effects of ozone are antibacterial, antiviral, and antifungal. These effects are lead to using ozone in dentistry processes (Almaz and Sönmez, 2015).

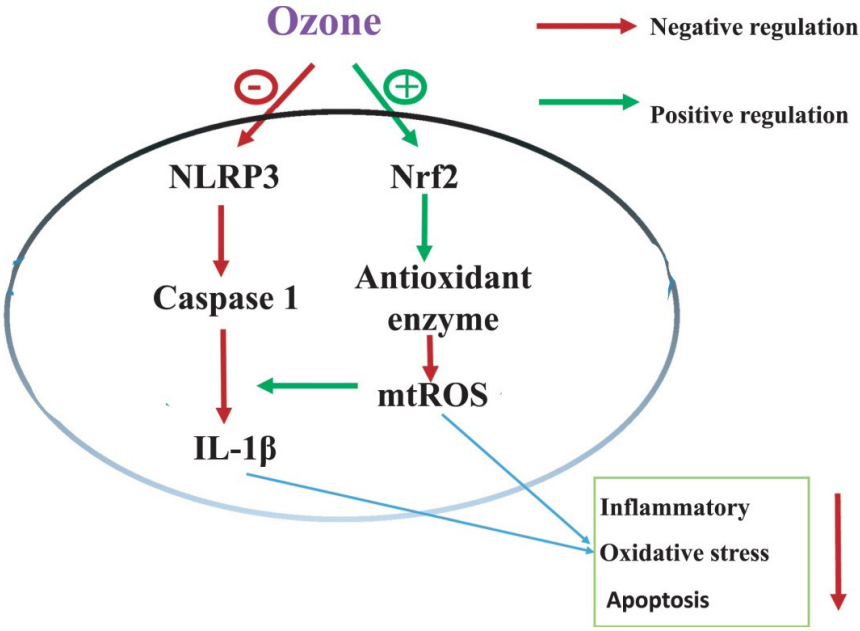

Figure 4. *A schematic representation effects of Ozone (Wang et al., 2018)*

Ozone can apply by systemic, rectal insufflations, minor autohemotherapy or topical. Rectal insufflations are the first and the oldest shape of ozone treatment. Easily applicable, affordable price and minor side effects are advantages' of rectal insufflations. Therefore, rectal insufflations method still uses with the other methods. Also, it is especially preferred on children.

Secondly, systemic application of ozone arises from major autohemotherapy (MAH). MAH usually is preferred complementary medical for oncology, rheumatology and the other parts of medicine. MAH is applied patients by their venous blood. Mainly, blood enriches with ozone then retransfers to body.

Minor autohemotherapy is the third method for ozone applications. A few intravenous blood is reinjected intramuscularly after enriching ozone.

Last method is topical applications. This method is especially used would healing and applications do with ozone-treated water or ozone cream (Viebahn-Hänsler et al., 2012; Eroğlu, 2019b).

Fibromyalgia (FM) is common syndrome which characterized chronic musculoskeletal pain and tender points. Although pathogenesis of FM is still not clear, it is known that multifactorial and complex (Marques et al., 2017). One of the factors causing FM is considered that oxidative stress. Because of this reason, ozone treatments can be used in FM (Hidalgo-Tallón et al., 2013; Balestrero et al., 2017).

A study, which made 30 women with FM, was considered as complementary treatment of ozone (Vélez, 2014). Another study including 40 patients with FM, was found significant increasing patient symptoms after ozone treatment. Researchers were accepted its preliminary and were designed more larger study (Tirelli et al., 2018). While 65 patients were participated on study, Tirelli et al (2019) were supported that ozone could be treatment for FM (Tirelli et al., 2019).

An open-label pilot study with O_3 therapy by rectal insufflations for the treatment of FM, was resulted that ozone can usefulness for physical symptoms and depression (Hidalgo-Tallón et al., 2013). Moreno-Fernández et al. (2019), was reported that ozone treatment was reduced of tender points and Fibromyalgia Impact Questionnaire score which decreases shown to healing symptoms of FM.

Although there are a few study effects of ozone on FM, ozone treatment seems to be an option of patients with FM. On the other hand, there need to more studies for understanding effects of ozone on FM.

3. Hyperbaric Oxygen Treatment

Hyperbaric Oxygen Treatment (HBOT) is a treatment which is applied in a chamber. In the chamber, patients expose specific pressure and 100% oxygen. Pressure has to greater than sea level (1 atmosphere absolute [ATA]) but it can changeable with diseases. The Undersea and Hyperbaric Medical Society specifies that the pressure must be greater than or equal to 1.4 ATA. Pressure is preferred commonly 2 to 3 ATA in clinical applying (Lam et al., 2017).

According to the Undersea and Hyperbaric Medical Society list, HBOT can use lots of disease such as air or gas embolism, carbon dioxide poisoning, decompression sickness, compromised grafts and flaps. While researches are being continued, list is become longer (Moen and Stuhr, 2012).

From a physiological point of view, HBOT is enhanced oxygen levels without depended on haemoglobin. This caused of much more oxygen is stayed on tissues. Also, HBOT indicated protective effects on tissues. It realized increasing reactive oxygen free. At the same time preventing lipid peroxidation is protected lipid membranes. Moreover, HBOT is a play role against to bacteria and anaerobes (Eroğlu, 2019a).

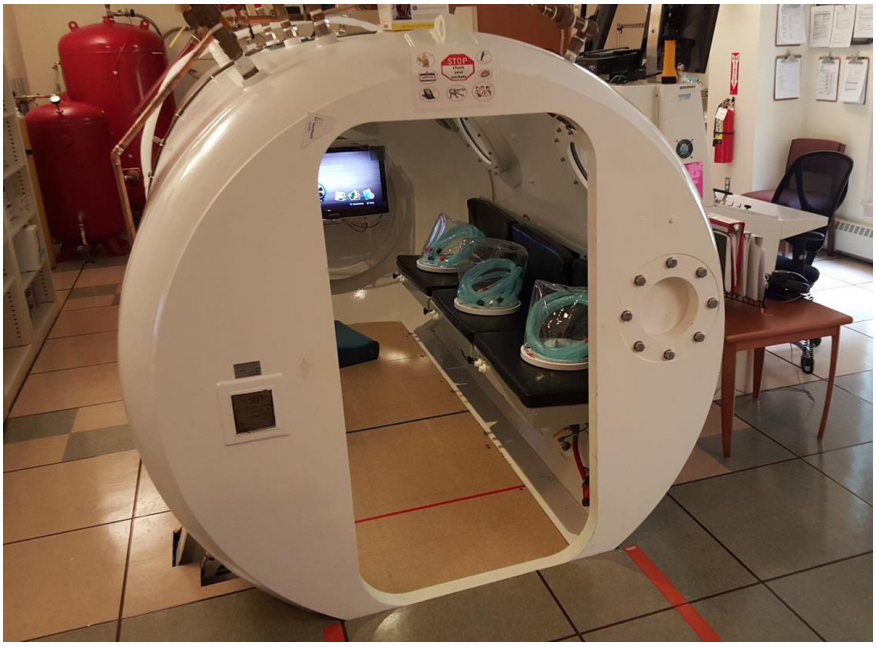

Figure 5. *Hyperbaric Oxygen Chamber (Lam et al., 2017).*

Hyperbaric Oxygen treatment can be tried modalities for these patients because of has not certain treatment for FM. Also, according to Efrati et al. (2015), HBOT is a useable method for FM, due to having abnormalities in brain of these patients. Therefore, in the study about HBOT and FM, is found that HBOT was successfully in terms of symptoms. Yildiz et al. (2004), was offered that HBOT is a good options for increase to in the same study. Another study was supported and reported that symptoms on FM, except depression and sleep, were significantly improved with HBOT (Atzeni et al., 2019). A study, searched FM pathogenesis and effect to HBOT, was suggest that HBOT was modulated T helper 1 activation. Additionally, HBOT was increased level of sera serotonin so patients with FM could obtain better life (Guggino et al., 2019).

A review which was analyzed researches about HBOT-FM in a study, effects of HBOT's on FM was schematized like figure 6 (El-Shewy et al., 2019).

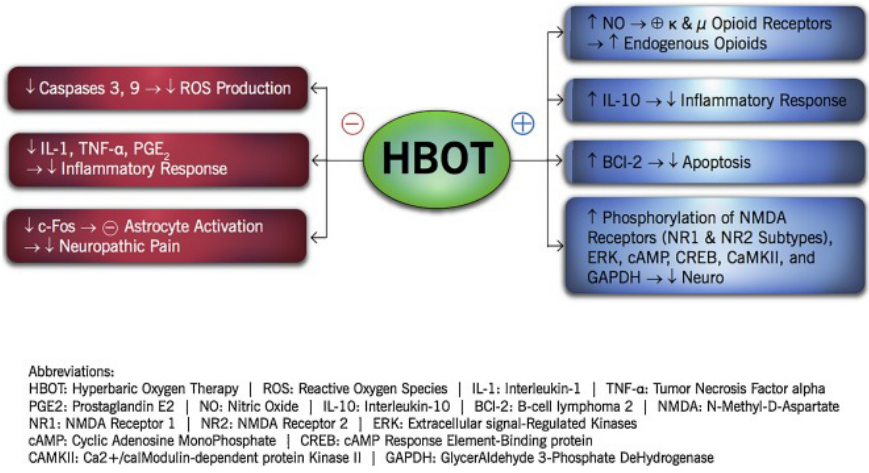

Figure 6. *Effects of Hyperbaric Oxygen Therapy on Fibromyalgia (El-Shewy et al., 2019).*

Conclusion, HBOT is increased life quality of patient, is reduced symptoms and can be tried to patient with FM, even if HBOT on fibromyalgia is a topic which is needed some researches.

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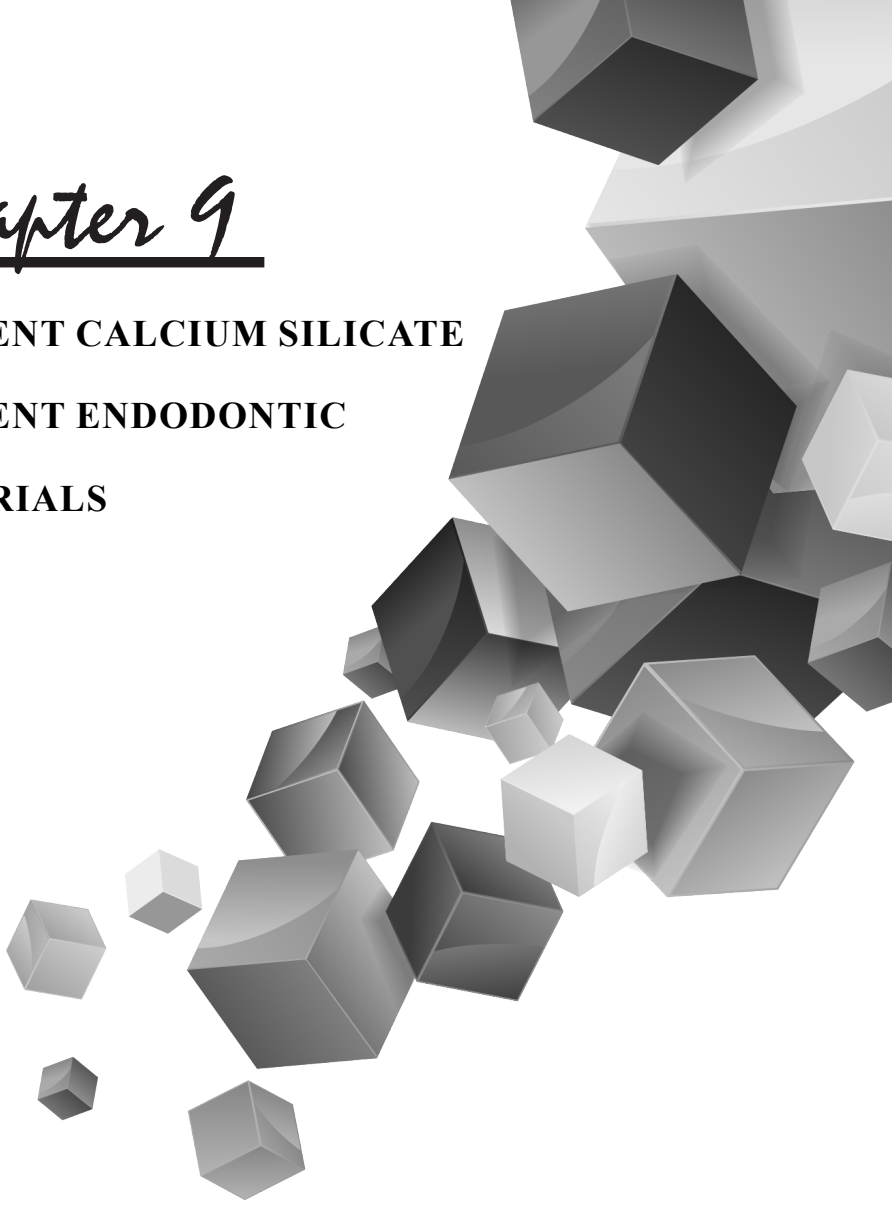
Chapter 9

CURRENT CALCIUM SILICATE CONTENT ENDODONTIC MATERIALS



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Introduction

In modern dentistry, with the production of new dental materials, treatment procedures are changing and the treatment prognosis is improving. Calcium silicate-containing materials that have gained popularity in recent years; They have begun to replace traditional methods due to their high clinical success and advantages. Although a variety of calcium silicate-based products have been launched on the market, some of them particularly stand out (1-3).

Calcium silicate based materials; It has a wide range of uses such as pulp capping, partial pulpotomy, cervical pulpotomy, retrograde filling material, root canal sealer in root canal treatment, repair of iatrogenic perforations, internal resorption treatment, barrier in internal bleaching, revascularization, apexification (4, 5).

Calcium hydroxide's use with current materials is limited due to its weak bonding to dentin, its inability to show long-term resistance to microleakage, dissolving in the presence of moisture and causing porosity (tunnel defects) in the dentin bridge (6-8). For these reasons, bioceramic materials containing calcium silicate, whose hydration results in calcium hydroxide formation, such as Mineral Trioxide Aggregate (MTA), Biodentine, BioAggregate, have become more popular in vital pulp treatments (9, 10).

Mineral Trioxide Aggregate (MTA), which has a high clinical success and is the most widely used bioceramic material both clinically and experimentally, was developed by Mahmud Torabinejad and his friends at Loma Linda University and was first used in dentistry in 1993 (11). In 1998, it was approved by the FDA (U.S. Food and Drug Administration) (12).

ProRoot MTA (Densply, ABD)

As the first commercial preparation, ProRoot was introduced to the market as MTA. The dust content of MTA, which hardens in the presence of moisture, consists of tricalcium silicate, tricalcium aluminate, tricalcium oxide and silicate oxide (SiO2). Bismuth oxide was added to impart radiopacity. It has two types, gray and white. Since the first gray colored formulation of ProRoot MTA produced causes discoloration in teeth, a white version was developed (13). The manufacturer company stated that the working time of the Gray ProRoot MTA is 5 minutes and the hardening time is 4 hours, while the working time of the White ProRoot MTA is 5 minutes and the hardening time is 15 minutes. No difference was found between White MTA and Gray Mta in terms of physical properties and biocompatibility. In terms of content, it has been observed that the ratio

of iron, aluminum and magnesium in Gray MTA is higher than in BMTA (14). It has been reported that as the material hardens, calcium ions move towards the dentinal tubules and increase the calcium ion concentration in this area over time (15).

The pH of MTA was determined as 10.2 right after the preparation; It has been stated that this increases up to 12.5 within three hours following the hardening and remains at this value (13, 16). The high pH of MTA and calcium hydroxide induces the formation of hard tissue in materials and gives the materials antimicrobial properties (16). MTA's compressive strength increases as it hardens; It is stated that the compressive strength is 40 Mpa after mixing the material and 70 Mpa 21 days after mixing (17).

It is stated that the biocompatibility of MTA is associated with several positive biological processes. Some of those; It has minimal cytotoxicity, pulpal and periapical irritation, is not mutagenic, has positive effects on cell adhesion and proliferation activities, increases alkaline phosphatase and osteocalcin levels, stimulates interleukin production (IL-6, IL-8), cement formation and a good quality dentin bridge formation (18).

In addition to all these advantages of MTA, it has been stated that it has disadvantages such as long hardening time, difficulty of manipulation, high cost and causing discoloration on the applied tooth (13). New calcium silicate based material formulations are being developed in order to eliminate these disadvantages (19).

Although various calcium silicate-based products have been launched recently, some of them in particular have found more clinical uses (20).

Mta Angelus (Angelus, Londrina, PR, Brezilya)

MTA Angelus consists of 80% Portland cement and 20% bismuth oxide. The working time of Angelus MTA has been reported as 5 minutes and the setting time as 14.28 ± 0.49 minutes. This period is very short compared to the setting time of WMTA and GMTA(21, 22).

Chemical and physical differences may arise between the first produced ProRoot MTA and the MTAs introduced over time. Although ProRoot MTA and MTa angelUs do not differ much from each other in terms of properties such as pH and calcium ion release, there are significant differences in terms of properties such as setting time and particle size (17, 23). In a study investigating the biological effects of MTA-Angelus, MTA Repair HP and NeoMTAPlus on human dental pulp stem cells, sufficient biological responses were obtained in terms of cell proliferation, morphology, migration and attachment (24).

BioAggregate (Innovative Bioceramix, Vancouver, BC, Kanada)

Bioaggregate; It is the first material marketed in dental markets with the claim that it contains new generation bioactive, ceramic nanoparticles. The difference of Bioaggregate from Gray MTA in terms of content; It is stated that it does not contain aluminum, calcium sulphate, bismuth oxide and contains nano-particulate hydroxyapatite. It has similar content with the white MTA(25).

Powder part of the material; It contains tricalcium silicate, dicalcium silicate, tantalum pentoxide, single base calcium phosphate and amorphous silicon oxide. The liquid part is deionized water. It does not contain calcium aluminate. Tantalum pentoxide was added to the material to provide radioopacity (26). It is easy to manipulate. It has been reported that Bioagregate stimulates sementogenesis and provides a hermetic seal in root canals. It shows its effect by preventing bacterial invasion (27).

In a study comparing the cytotoxic effects of Bioaggregate and MTA, the level of inflammatory reaction and foreign body reaction was found to be lower in Bioaggregate compared to MTA, therefore it was stated that Bioaggregate was a biocompatible dental material (26).

Bioaggregat was determined to cause dentin sclerosis by stimulating the differentiation of cells into odontoblasts in contact with mesenchymal cells. Therefore, Bioaggregat can be used as a capping material (28).

The antimicrobial effects of MTA and Bioaggregate were found to be similar (29).

MTA Repair HP (Angelus, Londrina, Brazil)

MTA Repair HP consists of the classic Mta formulation. However, calcium tungstate was added to provide radiopacity and contains a plasticizing mixing fluid. It is used for the same indications as other calcium silicate based materials. According to the manufacturer, this new formula preserves the original chemical properties of MTA, while its physical properties for manipulation have been improved (30).

MTA Repair HP appears as a short setting time, a fast and effective bioactivated endodontic cement. The short setting time of MTA Repair HP is associated with the high surface area of the precursor powder, high Al content and the absence of sulphate phases (31). The biological response of MTA Repair HP was found to be sufficient in terms of the proliferation, morphology, migration and attachment of stem cells in the dental pulp (32).

Neo Mta Plus (Avalon Biomed Inc., ABD)

Neo MTA Plus is one of the alternative calcium silicate based materials developed to overcome the disadvantages of Mta (such as long hardening time, difficulty of manipulation, coloring on the crown)(33).

NeoMTA Plus is the new powder tricalcium silicate and mixed with a water-based gel that facilitates manipulation and contains tantalum oxide (Ta2O5) as a radioactive agent (34). Tantalum oxide prevents discoloration and has a triggering effect on mineral secreting cells (35).

It becomes usable by mixing powder and gel. The working time is stated to be about 20 minutes at room temperature and when mixed in dense consistency. However, the working time can be extended when mixed by using more gel. It has been reported that the setting time is less than 1 hour at 37 ° C, but can extend up to 5 hours when more gel is used (36). NeoMTA-Plus can be used as a canal sealer or repair cement in endodontics with its calcium release, capacity to prevent bacteria leakage, adequate radiopacity and satisfactory sealing ability (37).

Biodentin (Septodont, Saint-Maur-des- Fosses Cedex, Fransa)

Biodentin is a new calcium silicate-based material developed recently as a response to the disadvantages of MTA. The powder part of Biodentine consists of tricalcium silicate, dicalcium silicate, calcium oxide, calcium carbonate and zirconium oxide providing radiopacity. Tricalcium silicate is the main ingredient, dicalcium silicate is the second main ingredient. The liquid part consists of calcium chloride accelerating hardening, water-soluble polymer and water. Biodentine is a material that can release calcium like other calcium silicate-containing materials and forms hydroxyapatite as a result of contact with synthetic tissue fluids (38). The addition of calcium chloride shortens the early and final setting time and contributes to the early structural strength (39).

Regarding the disadvantages of the material, although it contains zirconium oxide, its radiopacity is lower than MTA Angelus. As the radiopacity decreases gradually over time, it may cause difficulties in long-term radiographic observations (40). Biodentinin, like other calcium silicate-based materials, decomposes into a calcium silicate-based gel and Ca (Oh) 2 in contact with body fluids. It is stated that Ca (Oh) 2 forms a hydroxyapatite-like structure in contact with phosphate ions. These apatite crystals can extend towards the dentinal tubules like a hybrid layer and bacterial invasion can be prevented by the marginal sealing provided by this hybrid zone. Biodentine provides an excellent covering due to its micromechanical bonding without any preparation on the surface (41, 42).

The marginal sealing provided in the interaction of Biodentin with hard and soft tissues in both direct and indirect capping treatment allows the formation of tertiary dentin and remineralization by the pulp. Therefore, it can be concluded that biodentine is the preferred material for indirect and direct pulp treatment (43).

Singh et al. Stated that Biodentine does not have a negative effect on the specific functions of the mesenchymal cells in the pulp in either direct or indirect capping treatments (44). The most important difference of biodentine from other classical calcium silicate cements is that metallic residues are eliminated during the production phase. The material's low level of inflammation is associated with the removal of these metallic debris (45).

In a randomized clinical study, no abnormality was observed after 12 months in direct pulp capping performed with Biodentine in permanent young teeth, while a failure of 13.6% was observed in the ProRoot MTA and CaOh2 group (46). In other studies, it has been shown that Biodentine and MTA are superior to calcium hydroxide in terms of pulp capping (10, 47).

Mineralized tissue formation was observed two days after the application of biodentine to the perforation area in extracted human teeth. In the same study; The mineralized texture formation rates of Biodentin, MTA and KH were compared and the hard tissue formation was first detected in the group where Biodentin was used (48).

When biodent is applied directly to the pulp, it triggers the formation of repair dentin, while no inflammatory pulp response occurs, well-sorted odontoblast or similar cells are observed after 6 weeks (49). It has been observed that biodentine significantly increases TGF-1 secreted from pulp cells to dentine and triggers the synthesis of early forms of repair dentin (50).

A recent study showed that pulp capping materials, including Angelus MTA, CEM and Biodentinin, exhibited less cytotoxic effects on human dental pulp stem cells than TheraCal. CEM and Biodentine are promising alternative materials in clinical situations where human dental pulp stem cells are needed for repair dentin quickly (51).

As a result, a fully formed dentin bridge, lack of inflammatory response, rapid hardening, and a resin-composite-like consistency make biodentine prominent in pulp treatments (52-54).

Theracal lc (Bisco Inc, Schamburg, IL, USA)

TheraCal LC is a calcium silicate based light curing resin modified material designed as pulp capping material.One of the major disadvantages

of biodentine is that it is a water-based chemical and shows poor bonding to the top resin restoration. To overcome this situation, TheraCal LC was produced as pulp capping agent (55).

It consists of a mineral part containing calcium oxide, calcium silicate, stronium, silica, barium sulfate and barium zirconate, and a resin part containing Bis-GMA and PEGDMA. TheraCal LC has the ability to release calcium ions. TheraCal LC from Dycal for 28 days shown to release significantly more calcium ions (56). Camilleri et al. showed that TheraCal LC releases less calcium ions than Biodentine (57) . TheraCal LC acts by the formation of apatite precipitates by Ca + 2 and OH “ions, which it releases as a result of interacting with phosphate ions in biological fluids such as blood, plasma and dentin fluid (58).

The bioavailability of calcium ions released from TheraCal LC is in sufficient concentration to activate dental pulp cells and odontoblates . In TheraCal, it was observed that higher calcium ions were released than ProRoot MTA and Dycal and that the released calcium decreased over time in all materials. TheraCal has a lower resolution than ProRoot MTA and Dycal (56).

Poggio et al. reported that the antibacterial activity of TheraCal on Streptococcus mutans was lower than MTA and higher than Biodentine in their study (59). In a study comparing TheraCal LC with its Biodentine, calcium ions released from TheraCal LC are sufficient to activate dental pulp and odontoblasts. However, it is significantly lower than Biodentine and its reaction rate is slow (56, 57). Therefore, Therecal Ic has a lower remineralization potential compared to biodentin. Manufacturer on data sheet of TheraCal LC of August 2011 while reporting that it contains (5-20%) bis-GMA, the manufacturer stated that it does not contain HEMA in the manufacturer’s information form in March 2012. Similarly, Nilsen and Einar reported that TheraCal LC did not contain bis- GMA in their study with UPLC-MS analysis (60).

In similar studies, they reported that the calcified barrier formation of TheraCal samples was not of good quality, excessive or moderate inflammation and the formed odontoblastic layer were not sufficient (61, 62).

Bioaggregate (Innovative BioCeramix Inc, Vancouver, BC, Kanada)

Bioaggregate is a calcium silicate based root canal repair material consisting of disposable powder (1g package) and liquid (0.38 ml capsule deionized water). Powder part of the material; It contains tricalcium silicate, dicalcium silicate, tantalum pentoxide, mono-base calcium phosphate and

amorphous silicon oxide, while deionized water forms the liquid part. BA does not cause tooth discoloration. Working time is 5 minutes, Curing time is 4-72 hour. When powder and liquid are mixed, a gel-like calcium silicahydrate nanocomposite structure is formed and when applied into the root canal, it provides a hermetic closure (63). Tantalum pentoxide was added to provide radiopacity to the bioagregeat. It is very similar to Mta in content. BA is the first material marketed in dental markets with the claim that it contains nanoparticles. Manufacturers state that the material provides a hermetic seal and stimulates sementogenesis (27, 64). BA and MTA had little or no cytotoxic effects on osteoblast and fibroblast cells (27, 65, 66).

De Deus et al. used primary human mesenchymal cells to evaluate the toxic effects of aggregate BA. According to the results of this study, it has been reported that BA and MTA give similar results and are biocompatible. However, it has been determined that BA gives better results than MTA in the 48th and 72nd hour evaluations. The presence of bismuth and aluminum ions in the structure of MTA at a very high rate It caused toxic effects on osteosarcoma cells and fibroblasts of rats (67, 68).

Bioaggregate causes dentin sclerosis by stimulating mesenchymal cells to odontoblasts. For this reason, it has come to the fore to use BA as a pulp capping material (28, 69).

Endosequence Root Repair Material (Brassaler, Savannah, ABD)

ERRM is a bioceramic material with ready-to-use paste and injector paste forms that facilitate manipulation. It consists mainly of calcium silicate, zirconium oxide, tantalum oxide and calcium phosphate monobasic. Monocalcium phosphate takes part in the formation of hydroxyapatite. Tantalum oxide and zirconium oxide are responsible for the radiopacity. The setting time is between 2-4 hours. ERRM has been suggested to be used in perforation repair, apical surgery, apical plug and pulp capping (70-72). Endosequence; It is a hydrophilic, water-insoluble, radiopaque, aluminum-free material with high pH. Nano-particles in the ERRM It facilitates its penetration into the dentinal tubules (73).

High alkaline pH during the setting reaction provides the material to exhibit antibacterial properties (74). Similarly, in another study, it was stated that ERRM has an antibacterial effect on Enterococcus faecalis and this is due to high pH during hardening (75). The compressive strength of the ERRM is 40-50 MPa and this value is similar to the compressive strength of MTA (76). In studies evaluating the cytotoxicity of ERRM, such as MTA and MTA Angelus It has been reported to have minimal

cytotoxicity (77, 78). Gingival fibroblasts to the ERRM surface it is shown by SEM that it has spread and connected (79)

Calcium Enriched Mixture *(Bionique Dent, Tehran, İran)*

CEM ‘is used in clinical applications similar to MTA. CEM is different from MTA in terms of chemical content. CEM contains phosphorus pentoxide, silicon dioxide, aluminum trioxide, sodium oxide, magnesium oxide and chlorite. After the material hardens, it forms calcium and phosphate rich compounds and calcium hydroxide. Thanks to the calcium chlorite content in the material, the hardening time is reduced (80-82). CEM is made ready to use by mixing with powder and distilled water. The hardening time is less than 1 hour.

CEM was found to be similar to MTA in terms of working time, pH, and physical properties. It has been reported that calcium and phosphate ions released from CEM cement form hydroxyapatite (52, 83). CEM to smaller particle size than MTA has acceptable sealing properties it is thought to be related to this (83, 84).

While its resistance against compressive strength was found similar to Angelus MTA, it was lower than ProRoot MTA and Biodentine (85). In the studies of Parirokh et al., comparing the reactions of subcutaneous tissues to MTA and CEM performed in rats; Unlike MTA, it has been reported that CEM does not induce tissue necrosis. In the examination performed after 60 days, it was reported that the inflammation levels observed in the CEM group were quite low compared to MTA. In addition, the presence of dystrophic calcification in the area adjacent to the materials indicates that the materials have osteoinductive properties (86). Like Mta, CEM has antibacterial effects on E. Faecalis (87). In another study; It reports that CEM has more antibacterial effect on Enterococcus faecalis than MTA, as it is more bacterial inhibitör (88). In the studies of Malekafzali et al; They compared the success of MTA and CEM cement in amputation treatment. As a result of the 12-month follow-up period; It was stated that there was no significant difference between the two groups in terms of clinical and radiographic success (89).

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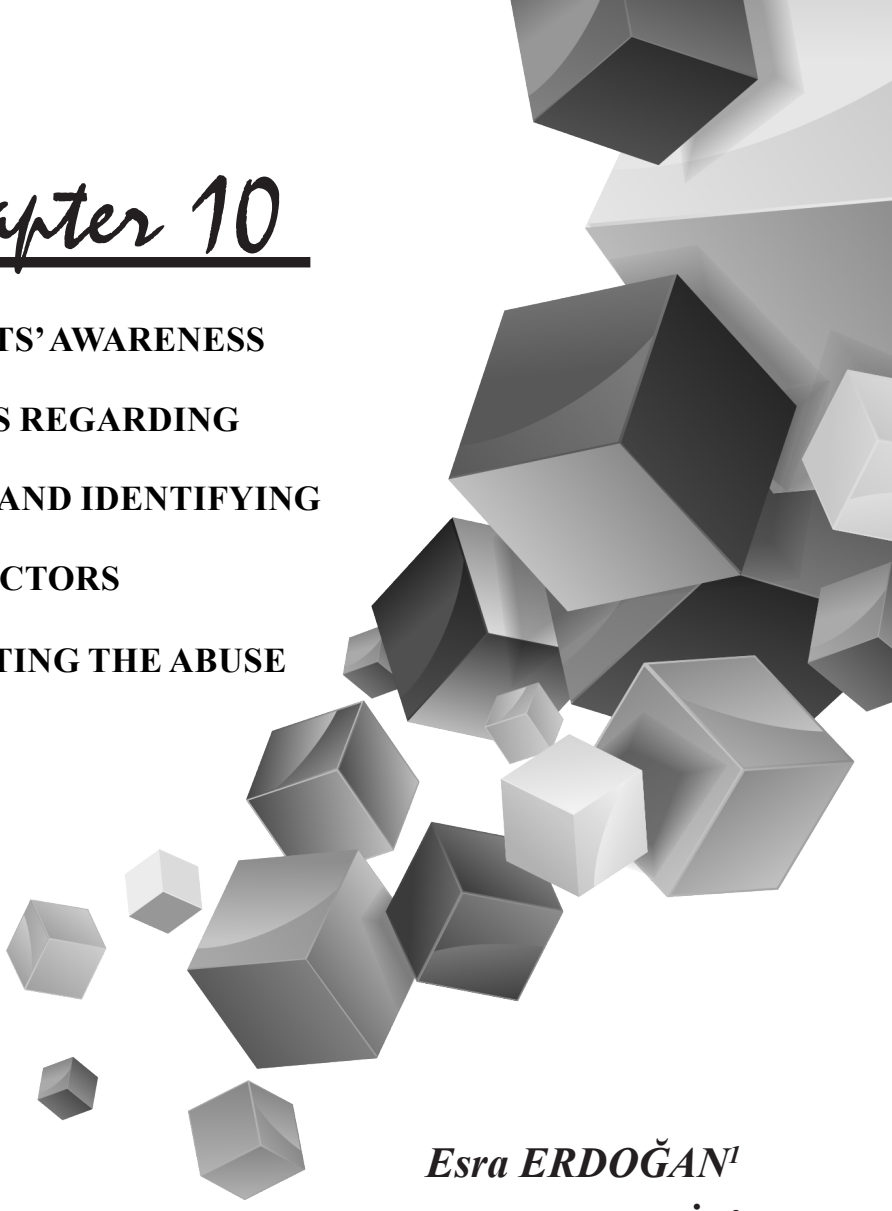
Chapter 10

PARENTS' AWARENESS LEVELS REGARDING ABUSE AND IDENTIFYING THE FACTORS AFFECTING THE ABUSE



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INTRODUCTION

Child abuse is the most difficult type of trauma to detect and treat, as it can be repeated especially by the closest people and has long-term effects that affect the child's life.[1] Generally to children under the age of 18; Physical, emotional and sexual maltreatment that "negatively affect the health and development of the child and leave permanent marks" are called "child abuse".[2,3] The World Health Organization defines child abuse as all kinds of behaviors that adversely affect the health, physical and psychosocial development of the child.[4] As a general definition; sexual abuse is defined as the adult using the child for sexual satisfaction. Sexual abuse is not only in the form of oral, anal, vaginal sexual intercourse or penetration with a substance, but also in the form of behaviors such as kissing, caressing, exhibitionism or even watching sexual material without physical contact.[4]

Children have a right to grow up in an environment where they feel safe. Protection of children's rights is still an unresolved and important problem in developed and developing countries. In ensuring the development and continuity of societies, the value that society attaches to the child is directly proportional to the level of development.[5] In order to prevent child abuse, it is necessary to raise the awareness of parents as the people who are closest to children from the moment they are born, and who take care of them.[3]

There are many risk factors for sexual abuse that can be evaluated socio-demographically. These are social / institutional factors, individual and family factors, and child related factors.[4,6] *Social/institutional risk factors;* Although physical abuse and neglect are very common among people with low economic status, it is also seen in families with medium and high economic levels. In environments with a high socio-economic level, especially family conflict or a new baby in the family are social factors that increase the possibility of abuse. The fact that these families are not noticed in the society, that they have strong acquaintances and assurances are the factors that put the blame on caregivers and make families look innocent. Inadequate health care in terms of abuse and neglect and lack of support for extended families and communities are also among the risk factors. *Risk factors for the child*; Babies born prematurely and with low birth weight, separated from the mother for a long time after birth, children with physical and developmental disabilities, children with mental retardation, chronic physical disease or congenital anomaly are perceived as their own distorted images by families who are particularly narcissistic and prone to abuse.[4,6] Familial factors are the most important among these risk factors. Low socioeconomic level, communication problems between parents, unwanted pregnancy, unaccepted child, divorced parents increase

the risk of sexual abuse. Mental disorders and a history of physical, emotional and sexual abuse in the parents have been reported as a risk factor. Alcohol-substance use in parents is also considered as a risk for sexual abuse. The low education level of the parents increases both the risk of abuse and the risk of developing a mental disorder after abuse.[4,6] The effect of sexual abuse on the child's mental life may vary depending on the individual differences and developmental stages of the child, the type, duration, and severity of the abuse, and the relationship between the child and the abuser.[3,4] In numerous studies in literature, it has been stated that various mental disorders such as anxiety disorders, dissociative experiences, sleep disorders, mood disorders, behavioral and sexual behavior disorders, alcohol and substance use can be seen in children and adolescents from childhood to adulthood.[4]

Abuse creates a traumatic effect on the way children become an individual and causes many physical, psychological and social problems. Especially negative emotions experienced in children who are abused by their parents affect the child's relationship with others and cause the child to imitate what he/she experiences. Since child abuse has a great impact on the personal development and life of the child, it is very important that the parent-child relationship is protective, supportive and nurturing. Article 19 of the Child Rights Convention emphasized this importance as "taking the necessary measures to protect the child against abuse and neglect within the family". One of the most important duties of the family is to inform the authorities after a case of abuse occurs. However, while many parents do not even know where to apply, many are afraid to report such a case for various societal reasons. Parents need to be informed and supported to protect their children from abuse.[3,4]

In a study conducted in Turkey with parents, the prevalence of sexual abuse in children between 7-18 years was found to be 10%, while in the past year it was 3%. In studies where personal reports of adolescents and young people were analyzed retrospectively, it was found that the prevalence of sexual abuse varied between 8% and 26%.[7]

A common misconception in society about child abuse is that sexual abuse occurs in places such as parks, deserted streets, dark places, and empty construction sites. However, the places where sexual abuse occurs are generally safe places such as home and school. [7]

Child abuse and neglect causes many behavioral, emotional, cognitive and somatic problems in the early or long term. Neglect causes social and emotional problems such as hostility, aggression, learning problems, late speech, low self-esteem in children, while physical abuse causes emotional and behavioral disorders such as depression, alcohol problems,

suicide attempts, behavioral disorders, antisocial behaviors, and tendency towards violent crimes.[8] Sexual abuse causes aggression, hostility, substance abuse, sexual dysfunctions, eating disorders, communication disorders, insomnia, memory loss, PTSD, fear, anxiety, anger, depression, and systemic disorders. In addition, it negatively affects their physical, emotional, social and sexual development, quality of life, mental health and life satisfaction.[4]

The rates of child abuse in the records do not reflect the real figures, and the fact that the majority of these cases remain confidential further increases the problem. These facts remain secret for many reasons, such as protecting the family or the abuser, shame, fear of repeating the abuse or stigmatization. With the increasing number of professionals working in the field of child abuse in Turkey, education studies conducted to create awareness on this issue, conferences and seminars showed an increase.

Knowing the abuse awareness levels of the parents and the factors affecting them can guide healthcare professionals in determining the target group to be supported and planning the scope of the support program. Therefore, this study aims to determine the parents' awareness of abuse and the factors affecting the abuse.

The hypotheses of the study

1. Descriptive characteristics of the parents affect the awareness level of abuse.

2. Descriptive characteristics specific to abuse (parents' way of defining child abuse and their attitudes towards abuse) affect the level of awareness of abuse.

METHOD

Research Design and Method

In this study, a "descriptive cross-sectional model" was used. The universe of the study consisted of parents who have children aged 4-6 and applied to the pediatric outpatient clinics of three hospitals, including private and public hospitals in the city center of Samsun (Samsun Maternity and Children's Hospital, Samsun Büyük Anadolu Hospital, Medical Park Samsun Hospital). Parents who met the inclusion criteria of the study and volunteered to participate in the study constituted the sample of the study. Inclusion criteria in the study were "Being married and literate, living together, having a regular income". The exclusion criteria in the study were "being under the age of 18, having chronic and physical illness, being diagnosed with a mental illness, mental retardation,

impaired cognitive abilities, using any substance other than smoking, and the child having a chronic physical and mental illness". The sample of the research was determined according to the Power Analysis and Sample Volume Calculation formula. NCSS (Number Cruncher Statistical System) - Statistical and Power Analysis Software- PASS (Power Analysis and Sample Size) softwares were used in the sample size calculation. The sample size was determined by examining studies similar to this study in terms of sample, intervention and measurement tools in the literature. Considering the regression analysis results for the multiple regression findings related to the abuse potential of the parents as a result of the power analysis, a total of 197 observation values were taken for 95% confidence, 80% test power and 0.067 effect size for R2 = 0.063. As a reference, Pekdoğan's study "Examining the abuse potential of mothers in terms of some variables" was used. [9]

IMPLEMENTATION

The study was conducted between July and December 2018. In this study, it was ensured that all data collection tools were filled in face to face with parents. In order for the parents (mother/father) to fill the forms comfortably, a meeting was held on the polyclinic floor in a suitable environment (with seats and silent) within the facilities of the hospital. Support was received from nurses, doctors and secretaries working in the polyclinic for the guidance of the parents. When the mother and father were contacted at the same time in the polyclinic, the interview was conducted one by one rather than at the same time so that they felt comfortable during the filling out of the forms. During the data collection phase, the researcher informed the parents about the purpose of the research and how to fill in the forms, and this process was completed in about 10-15 minutes. Informed written consent was obtained from the participants and "Personal Information Form" and "Abuse Awareness Scale, Parent Form" were administered to the parents who agreed to participate in the study. In addition, a brochure has been given to parents for information. In the brochure, information about "*the definition of abuse, types of abuse, the effects of abuse on the child, signs of abuse, awareness of abuse, what to do in case of abuse and institutions that can be helped*" are included.

Data Gathering Tools

"Personal Information Form" and "Abuse Awareness Scale-Parent Form" were used to collect the data of the study.

Personal Information Form: It includes questions about the sociodemographic characteristics of the patient, whether he / she has

encountered abuse before, about abuse and about the Child Monitoring Center (CMC).

Abuse Awareness Scale, Parent Form: This form was developed by Pekdoğan (2017) for parents with children aged 4-6 in Turkey. In the validity and reliability of the scale, Cronbach Alpha internal consistency was found to be .98; test-retest correlation coefficient was found to be .94. The scale consists of 18 items on a 5-point Likert scale ranging from 1 (*Strongly disagree*) to 5 (*Strongly agree*). Items 12, 15 and 17 are scored in reverse. The lowest score that can be obtained from the scale is 18 and the highest score is 90. The range of 18-42 points of the scale indicates low level of abuse, 42-66 point range indicates moderate abuse, and 66-90 point range indicates high level of abuse. The higher the score obtained from the parent form, the higher the abuse potential of the parents.[10]

Ethical Issues

Prior to study, written permission was obtained from Gazi University Ethics Committee (Number: 77082166-604.01.02) and the institutions where the research will be conducted, and permission to use the scales in the study was obtained from the owner of the scale by mail. Participation in the study was voluntary and informed written consent was obtained from the participants. A brochure prepared by the researcher was given to the parents who participated in the study immediately after the data collection tools were applied. It is thought that this brochure will support parents in terms of getting to know the abuse, the effects of the abuse on the child, and the institutions that can be helped to combat abuse.

Statistical Analysis

NCSS (Number Cruncher Statistical System) - Statistical and Power Analysis Software- PASS (Power Analysis and Sample Size) software were used in the sample size calculation. IBM SPSS v23 software package was used in data analysis. The Shapiro Wilk test was used to examine the distribution normality. Chi-square (X2) test was used to analyze qualitative data according to groups. Tukey test, one of the post-hoc tests, was used for paired comparisons of multiple groups. Correlations between variables and the Abuse Awareness Scale were evaluated using the Mann Whitney U test and the Kruskal Wallis test. Quantitative data are presented as mean and standard deviation, qualitative data as frequency and percentage, and data that do not show normal distribution are presented as median (min-max). The level of significance was taken as $p < 0.05$.

FINDINGS

This study was conducted with the participation of a total of 197 parents, 139 of which were mothers and 58 fathers. Most of the participants in the study had university or higher education, between the ages of 30-40 and has children in the age group of 6 (Table 1).

Table 1. Descriptive characteristics of the parents (n=197)

Characteristics	N	%
Parent		
Mother	139	70.6
Father	58	29.4
Education Level		
Primary school	20	10.2
Middle-school	50	25.4
University or higher education	127	64.5
Working status		
Working	169	85.8
Not working	28	14.2
Income level		
Sufficient	146	74.1
Insufficient	51	25.9
Substance use [a]		
Yes	56	28.4
No	141	71.6
Parent's age [b]		
<30	38	19.3
30-40	131	66.5
>40	28	14.2
Child's age [c]		
Age of four	64	32.5
Age of five	54	27.4
6 Age of six	79	40.1

a:Smoking ; b:X ±SS (Min.-Max.):36±5.58 (22-58); c: X ±SS (Min.-Max.):5±0.83 (4-6)

Table 2. Awareness Scale Scores according to the descriptive characteristics of the parents

	Abuse Awareness Scale*		
Descriptive characteristics	**Median (min-max)**	**Test statistic**	**p**
Parent			
Mother	54 (33-66)	U=3.624	0.263
Father	52.50 (26-63)		
Education Level			
Primary school	52 (36-63)	χ^2=3.639	0.162
Middle-school	55 (26-63)		
University or higher education	53 (33-66)		
Working status			
Working	53 (26-66)	U=2.881	0.110
Not working	55.50 (36-63)		
Income level			
Sufficient	54 (26-66)	U=3.911	0.590
Insufficient	53 (36-63)		
Substance use			
Yes	54 (36-66)	U=3.646	0.402
No	53 (26-63)		
Parent's age [b]			
<30	54 (43-63)	χ^2= 6.943	**0.031****
30-40	53 (26-66)		
>40	51 (33-60)		
Child's age [c]			
Age of four	53.50 (26-63)	χ^2 = 0,993	0.118
Age of five	54 (36-62)		
6 Age of six	54 (36-66)		

; U: Mann Whitney U test: X ±SS (Min.-Max.): 54±6.08(52-66)*

p<0.05 Significance level, **:p<0.05, There was a statistical difference between the groups according to the chi-square test and the Mann Whitney U test.

The total score average of the Abuse Awareness Scale of the parents participating in the study was 52.83. According to the scoring criteria of the scale, the mean score reveals that the parents have moderate abuse potential since they are between 42-66 points. There was no statistically significant difference between the average scores of the abuse awareness scale according to who the parents are, education level, employment status, income level, and the age of their children (p>0.05). A statistically significant difference was found between the abuse awareness scale mean scores according to the

age of the parents ($p<0.05$). As the age of the parents' decreases, the average score of the abuse awareness scale increases (Table 2).

Table 3. Descriptive features specific to abuse

	n	%
What child abuse means for parents		
Sexual abuse	85	43.2
Child marriage	32	16.2
Forced labor	26	13.2
Physical and sexual abuse	30	15.2
Removal of all rights of the child	24	12.2
Previously witnessing abuse		
Yes	13	6.6
No	184	93.4
The place to apply in case of witnessing abuse		
I dont know	30	15.2
Police	138	70.1
ASPB	14	7.2
CMC	10	5.1
Teacher	5	2.5
What is known about CMC		
I dont know	160	81.2
Unit that protects the rights of the child after abuse	18	9.1
Unit providing psychological support to the abused child	19	9.6
Purpose of the CMC		
I don't know	140	71.1
Preventing child abuse	29	14.7
Protecting children in the judicial process within the MoH *	28	14.2
In which situations should be applied to CMC?		
I do not know	130	66.0
In case of abuse (sexual, physical, psychological)	55	27.9
On suspicion of negligence and abuse	12	6.1
According to which child the questions are answered		
Girl	113	57.4
Boy	84	42.6
Planned pregnancy status		
Planned	167	84.8
Unplanned	30	15.2
Which child		
First	131	66.5
Second	56	28.4
Third	10	5.1

**MoH:Ministry of Health*

Among the parents participating in the study, 43.2% consider child abuse as sexual abuse, and 16.2% as child marriage. Others, on the other hand, consider physical / sexual abuse (15.2%), forced labor (13.2%), and taking away all rights of the child (12.2%) as child abuse. The rate of parents who stated that in case of witnessing any child abuse, they should apply to the police is 70.1%. When asked what is known about CMC, 81.2% of the parents stated that they did not know about CMC and that they heard the name of this institution for the first time. After the researcher explained that CMC was a "child monitoring center", the parents expressed their thoughts about the purpose of this institution and in which cases it should be applied. Regarding what the purpose of CMC is, 71.1% of all parents who participated in the study stated that they did not know, and 28.9% stated that "prevent child abuse" or "protect children in the judicial process within the MoH". Regarding in which situations should be applied to CMC, 66% of the parents stated that they do not know, 27.9% of them stated in cases of sexual/physical/psychological abuse, and 6% in cases of negligence and abuse. When parents asked "Regarding which of your children are you answering questions about abuse?", 57.4% of the parents stated that they have a daughter, 84.8% of them stated that she was a planned child, and 66.5% of stated that was their first child (Table 3).

Table 4: Relationship between Abuse Awareness Scale score and parental age, child age and number of children.

		Scale
Parental Age	r	-0.171
	p	**0.016***
Child age	r	-0.018
	p	0.801
Number of children	r	-0.053
	p	0.459

r: Spearman correlation coefficient, p <0.05 significance level,: p<0.05*

There is a significant negative correlation between the scale score and parental age (p = 0.016). The scale score decreases as the parental age increases. As the age of the parents increases, the level of awareness of abuse increases, in other words, the abuse potential against children decreases. There was no significant relationship between the scale score and child's age and the number of children they have (p>0.05; Table 4).

Table 5: Abuse Awareness Scale Scores according to descriptive characteristics specific to abuse

	Median (min-max)	Test statistic	p
What child abuse means for parents			
Sexual abuse	54 (26 - 63)	χ^2= 5,174	0,270
Child marriage	54 (33 - 62)		
Forced labor	54,5 (36 - 63)		
Physical and sexual abuse	51,5 (41 - 58)		
Removal of all rights of the child	53,5 (36 - 66)		
Previously witnessing abuse			
Yes	53 (39-62)	U= 1,271	0,703
No	54 (26-66)		
The place to apply in case of witnessing abuse			
I dont know	53,5 (26 - 63)	$\chi^2 = 4,686$	0,321
Police	54 (33 - 66)		
ASPB	52 (47 - 58)		
CMC	51 (41 - 58)		
Teacher	51 (44 - 62)		
What is known about CMC			
Unit that protects the rights of the child after abuse	53 (41 - 59)	$\chi^2 = 1,341$	0,720
I dont know	54 (26 - 63)		
Unit providing psychological support to the abused child	54 (41 - 66)		
I have learned now	51 (39 - 63)		
Purpose of the CMC			
I don't know	54 (26 - 63)	$\chi^2 = 4,447$	0,108
Preventing child abuse	54 (41 - 66)		
Protecting children in the judicial process within the MoH	50,5 (39 - 63)		
In which situations should be applied to CMC?			
I do not know	54 (26 - 63)	$\chi^2 = 3,786$	0,151
In case of abuse (sexual, physical, psychological)	52 (36 - 66)		
On suspicion of negligence and abuse	54,5 (44 - 59)		
According to which child the questions are answered			
Girl	53 (36-62)	U=4,877	0,739
Boy	54 (26-66)		
Planned pregnancy status			
Planned	53 (26-66)	U=2,874	0,198
Unplanned	55 (37-62)		
Which child			
First	54 (33 - 66)	$\chi^2 = 0,960$	0,619
Second	52,5 (26 - 63)		
Third	53,5 (48 - 56)		

No statistically significant difference was found between the abuse awareness scale mean scores according to descriptive features specific to abuse (p>0.05; Tablo 5).

DISCUSSION

Child abuse is the exposure of a child in the 0-18 age group to a harmful, non-accidental and preventable behavior by a dependent or not dependent adult.[11,12,13] Studies in the field have focused on the concepts of child abuse, sexual abuse, physical and emotional abuse, and physical and emotional neglect.[11-15] In our study, when the parents' definition of abuse and their awareness of abuse were evaluated in line with these concepts; It was observed that parents defined abuse as sexual abuse, child marriage, forced labor, physical-sexual abuse, taking away all rights of the child. Also, they found to be have a moderate abuse potential. Parents' perception of child abuse not only as physical or sexual abuse but as abuse of any kind of harmful behavior towards the child is important in terms of showing that the society considers the rights of the child as a whole. However, it should not be overlooked that parents have moderate abuse potential regardless the descriptive characteristics of abuse according to the abuse awareness scale scores.

Among the descriptive characteristics of the parents, it was found that only the "parental age" affects the potential of abusing. In the study conducted by Güler et al.[16], it was found that economic distress and the number of children are risk factors for child abuse and neglect.[16] In our study, it was found that the level of awareness of abuse / abuse potential was not affected by the number of children and income level. In another study, it was found that younger mothers abuse and neglect their children more.[17] Similarly, in our study, it was found that the potential of parents to abuse the child increases as the age decreases, but the age of the child and the number of children do not make a difference in this regard. In our study, it was observed that the parent's being a mother or father did not affect the level of abuse awareness. In the study of Yalçın et al.[18] in which mothers and fathers' attitudes towards child abuse were examined, the abuse potential differed significantly according to the parent variable. It was found that fathers' average abuse scores and, accordingly, their abuse potential were higher compared to the mothers.[18]

Child Monitoring Centers (CMC) are important and valuable life centers for children's mental health in case of child abuse. It is very important not only for the child but also for the society that they overcome the post-abuse process in the slightest way. CMC is a child-friendly center established under the Ministry of Health hospitals in order to minimize the secondary trauma of sexually abused children and to ensure that forensic

and medical procedures are carried out in a single center with educated people in this field. Sexual abuse is a crime according to the Turkish Penal Code and there is an obligation to report this crime.[19] There are resources in the literature explaining the functioning, procedures, services and types of child abuse of CMC. [19-24] There are limited number of studies including data on how much of the parents and society know about CMC. This study included questions to evaluate the parents' knowledge about CMC. To the question of having information about CMC, 81.2% of the parents stated that they did not know about CMC, 71.1% did not know the purpose of CMC and 14.7% stated that their aim was to prevent child abuse.

Another important problem in preventing neglect and abuse of children is that the cases encountered are not reported or how to follow the path. Golge et al. [8] stated that the reason for this problem is most of the parents was not known what kind of procedure should be followed. In our study, a question was asked to the parents as "where to apply when witnessing abuse". While 70.1% of the parents answered the police and 5.1% CMC, 15.2% stated that they did not know. This finding suggests that the lack of information about the procedure continues as a current problem. In another study, the application forms to CMC were examined and it was determined that 88% of 848 cases applied through law enforcement.[21] Similarly, in our study, most of the parents answered "I will go to the police" in case of abuse.[25] The fact that the first step of the applications is institutions other than CMC creates a secondary trauma effect for the child and may adversely affect the biopsychosocial integrity of the child.

Limitations

The limitation of this study is the evaluation of the abuse potential of the parents for only the 4-6 age group in parallel with the usage characteristics of the scale.

CONCLUSION

The age of the parents participating in the study affects the potential of abusing children in the 4-6 age group, as the age decreases, the parent's child abuse potential increases and the level of abuse awareness decreases. Parents' being a mother or father, education level, working status, income level, smoking status, number of children and age characteristics do not affect the potential for abuse. Descriptive characteristics specific to abuse, "parents' definition of abuse, witnessing of abuse, the place to be consulted when witnessed, what is known about CMC, the gender of the child, the planned pregnancy status and the number of children" do not affect the abuse potential / abuse awareness level. In our study, the abuse awareness level of the parents, in other words, the abuse potential for

children in the 4-6 age group was found to be moderate. These findings suggest that abuse awareness programs should be conducted to increase parental attitudes and approaches that support the healthy development of the child by decreasing the medium-level abuse potential in parents, especially in younger parents.

Conflict of Interest

The authors declared that there is no conflict of interest.

Thank

The authors thank all participants.

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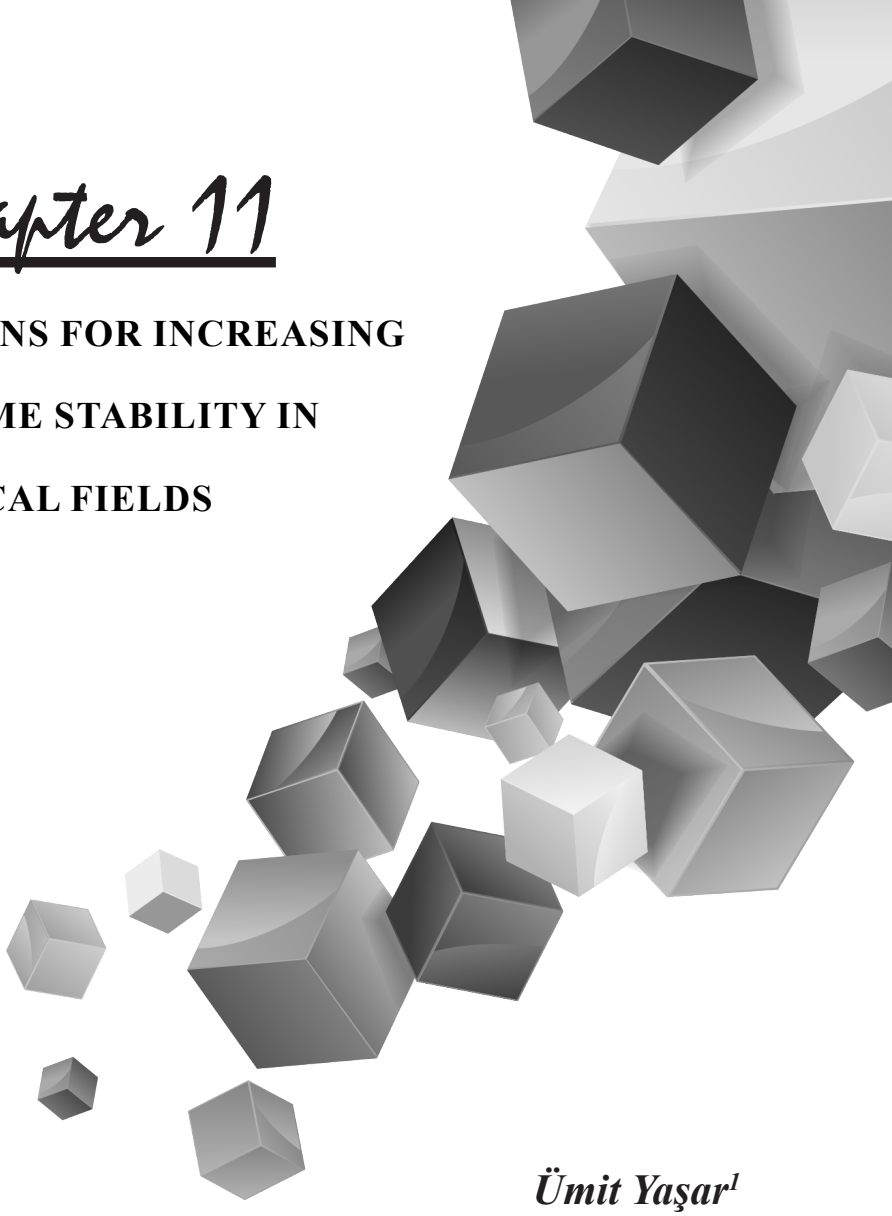
Chapter 11

OPTIONS FOR INCREASING ENZYME STABILITY IN MEDICAL FIELDS



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INTRODUCTION

Enzymes are efficient catalysts designed by nature to work in the physiological environments of living things. Enzymes using as catalysts in drug production and medical ammlications is one of the attractive subject that is open to developed and strengthened, in the 21st century chemistry. It is possible to encounter with enzyme stabilization work particularly in biomedical and medical biochemistry areas. Specificity, selectivity, activity and a very high binding capacity of these molecules in appropriate circumstances such as low pressure, optimum temperature and an aqueous medium, are used the leading cause in medicine, medical, food, energy, and many chemical processes. However, in terms of the catalytic activity of the enzyme that are preferred in various fields and processes, are passed the difficulty of switching prevents loss of activity in harsh conditions (high temperature, pH, etc.). Therefore, both being cost and time loss and may be faced with low yield and undesirable by-products in these fields. In fact, this is an issue related to the stability of enzymes. Therefore, the stability of enzymes and their application in processes requiring the use of extreme environments to which catalysts may be exposed is a key issue.

1. Enzyme stabilization, hypotheses and techniques

Enzymes are protein molecules made up of folded polypeptide chains of amino acids that are necessary to perform a range of biological functions. The order of these amino acids in a protein determines the tertiary structure through molecular geometry and intramolecular chemical interactions. Depending on the amino acid composition, these proteins can contain both acidic and basic functional groups that play an important role in their structure. Conformational changes such as surface changes, aggregation and precipitation can cause less dissolution of enzymes (Chang and Yeung, 2010). The solubility of enzymes can change as a result of their exposure to different residues of the surrounding environment. A few of the salient properties of enzymes are that they have their own chemistry and polarity, and are functional and versatile.

Enzymes are subjected to various denaturation reactions during production, storage and applications(Iyer, 2008). Denaturation is the structure of the tertiary structure of the enzyme in a disordered polypeptide where the functional or structure-stabilizing key residues are no longer adequately aligned (Robic, 2010). This trend can be reversed if the denaturing effect is removed (Burioni et al., 2004). Enzymes must be formulated to fulfill their biological functions and to ensure proper folding (Scholz et al. 1992).

Proteins of hyperthermophilic microorganisms can remain stabilized even under extreme conditions (Ramos et al., 1997). Preserving the stabilization of protein structures even under difficult conditions such as

the boiling point of water is an evolutionary vital feature (Lamosa et al., 2000). The resistance of proteins to temperature denaturation in this type of organism is due to their nature. This suggests that hyperthermophilic organisms have alternative existence strategies for protein stabilization. One hypothesis that tries to explain intercellular protein stabilizations is that thermophiles accumulate large amounts of osmolites and these compounds provide protection against heat in macromolecules of the organism in vivo (Baldwin, 1996). Along with the hypothesis, the idea of using osmolites and proteins in stabilization against temperature emerged and studies on some possible mechanisms on osmolite were also reported (Timasheff, 1998).

In the previous periods, some models and approaches have been made for the resistance of the proteins in the organism at high temperatures. One of them is the hydration model (Scholz et al. 1992).. In this model, it is mentioned that dissolved molecules in solution are excluded by the protein surface and this makes denaturation entropy more advantageous (Arakawa et al., 1990). However, this view was not fully explained and did not comply with general principles. The magnitude of the stabilization effect has been explained by certain solvent-protein interactions (Lee & Lee 1987).

Another approach to stabilization and destabilization has argued that it is caused by the contact between free groups and solvent in the protein structure (Qu Y et al., 1998). In this approach, the effect of stabilization is based on the partially folded states of the main backbone in the underlying protein and the specific interactions of the groups in the side chain. In other words, interactions, the decrease in the intrinsic mobility of the protein, the contraction of the protein structure has been mentioned(Baskakov et al., 1998).

In fact, the reason for the high thermal stability in thermophilic proteins is due to the rigidity of the protein structure. X-ray and NMR structural data on homologous proteins have proven the high degree of packaging and / or strong local interactions of polypeptide chains that cause high rigidity (Jaenicke & Bohm 1998). It has been mentioned that the increase of flexibility in the active centers of enzymes causes the catalytic activity to decrease. Therefore, the design of enzyme immobilization techniques has a very important place in protein stability (Jaenicke et al., 2000). Although a poorly designed immobilization technique does not allow the desired result to be achieved exactly, it may also cause differences in explaining the stabilization that occurs. Complex immobilization techniques performed in this way may not only not develop rigidity fully, but may cause situations in which the support and enzyme interaction cannot be fully explained, resulting in a decrease in stabilization (Cesar et al., 2007).

Despite its excellent catalytic properties, activity, inhibition, substrate selectivity, stabilization and immobilization studies are ongoing to

increase the usability and continuity of some critical enzymes in industrial applications. These studies show that enzymes have a better functionality than the free enzyme by using a simple immobilization technique. In these immobilization studies, it is evaluated that especially covalent modification techniques are useful, simple and effective in stabilization of monomeric and multimeric enzymes (Cesar et al., 2007).

Chemical modifications between macromolecules is a common strategy where enzymes can be more useful in terms of biocatalytic function. In this type of stability studies, natural or synthetic polymers such as dextran, polyethylene glycol and carboxymethylcellulose are generally preferred (Kılınç et al., 2002). It is known that the amino acid residues in the basic structure of enzymes contain reactive groups (lysine, cysteine, aspartic acid, glutamic acid, tyrosine, serine and threonine). Chemical, ionic or chelation reactions between these reactive groups in amino acids can cause soluble or insoluble binding of proteins in the solid support. Immobilization techniques are one of the most important ways used to increase the stability of enzymes. In this technique, the movement of protein molecules by using chemical bonds is restricted and thus stabilized. When the correct orientation is made with these techniques, enzyme activation can be obtained for a longer time compared to free enzymes in solution (Dobson, 2003).

In recent years, various numbers and structures of products produced with nanotechnology have found a wide commercial application. Currently, stabilization studies for the biocatalysis of enzymes continue at full speed (Jungbae 2006). These reported studies mostly focused on enzyme immobilizations involving enzyme adsorption, enzyme encapsulations, various combination combination methods, covalent modifications of enzymes, ionic interactions, hydrophobic interactions, multiple point binding, polymer pore size and volume (Vinogradov et al., 2001). In addition, enzyme stabilization studies using nanostructures with various physical and chemical properties such as nanoparticles, nanofibers, mesoporous materials, single enzyme nanoparticles (SENs) are also reported (Rodrıguez et al., 2011). It is evaluated that stabilization studies of enzymes designed especially with nano structures can achieve serious success against the harsh conditions encountered in the reaction environment(DeSantis et al., 1999), (Quiocho et al., 1964).

With “enzyme modification”, covalent bond and reactions of enzyme molecules are tried to be defined. Functional groups or polymers can be attached to the enzyme surface and stabilization is aimed by changing the properties of enzymes (Jungbae and Jay 2003). By using molecular biology techniques such as protein engineering, directed evolution, or site-directed mutagenesis, the amino acid sequence changes of enzymes are studied in order to obtain products with a very stable structure through intrinsic pathways. In this approach, the main issue

is the change of anhydrous organic solutions and salt compositions in aqueous media. In the studies of "cross-linked enzyme crystals" and "cross-linked enzyme aggregates", the situation is that enzyme crystals are "multipoint" (from many points) (Imre and Endre 2009). Recently, interest in using nanoparticles as carriers in enzyme immobilization studies to increase enzyme stabilization has increased. Nanoparticles provide an ideal solution to contradictory issues in optimizing enzymes that are usually immobilized. r: Minimum diffusion limitation, maximum surface area per unit mass, and high enzyme loading. Theoretical and experimental studies have shown that particle mobility, driven by particle size and solution density, can affect the intrinsic activity of enzyme-linked nanoparticles, and binding of enzymes to surface-activated nanoparticles improves thermal stability, reusability and storage stability(Desantis and Jones 1999). Highly branched polymers are also used in enzyme stabilization. Enzyme conjugation can be applied to the surface of such aromatic polyamides. In studies, it was stated that the half-life of enzymes bound in this way could increase 3 times more than in their natural state. There are also studies to increase stabilization by immobilizing the enzyme encapsulated in nanogel on the dendrimer surface. It has been mentioned that the encapsulated enzymes can maintain 80% of their activity even though they are kept at 90oC for 65 minutes. The natural forms of the same enzymes have a lifespan of 10 minutes at the same temperature. Enzymes coated with nanometer sized polymers (nanogels) play very important roles in pharmacological studies and nano drug studies today (Kabanov et al., 2009). (Fig 1)

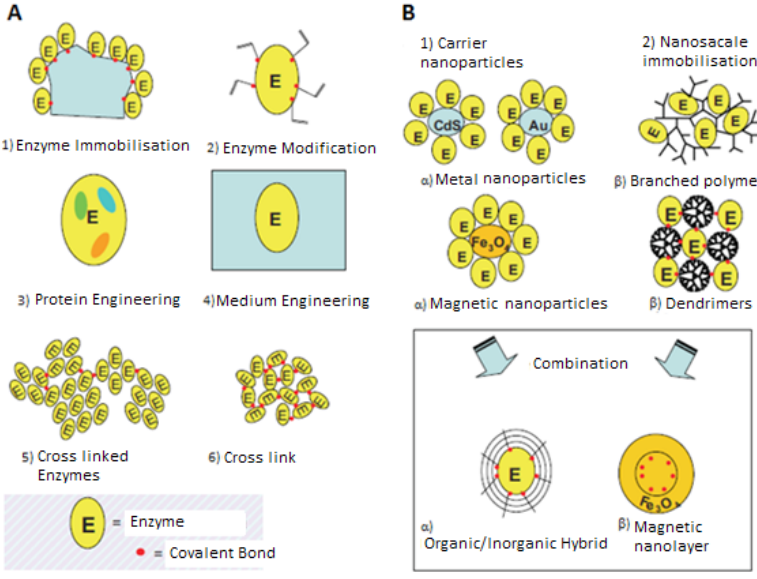

Fig 1. *New techniques and classifications in enzyme stabilization research. (A) classical techniques (1) Immobilization of enzymes on the carrier surface or inner space (2) Modification of the enzyme surface (3) Protein engineering*

(4) Medium engineering reaction (5) and (6) cross-linked enzyme crystals and aggregates. (B) New approaches used by reducing the size of enzyme carriers (1) Use of α and β magnetic nanoparticles as carriers; (2) (α) hiper dallanmış polimer ve hyper branched polymer and (β) encapsulation into dendrimers; (3) (α) polymer network (nanogel) and (β) ingle enzyme encapsulation with magnetic nanolayers (SENs) (Imre and Endre 2009).

1.1 Stabilization of enzymes by covalent modification

In stabilization, it is inevitable to use enzymes in protein structure consisting of large surface area and even many subunits (Iyer and Ananthanarayan 2008). Although successful immobilization studies with subunits have been reported recently (Mateo et al., 2006), it is not sufficient to contain the enzyme subunits in the maximum amount in random immobilization studies(Mateo et al., 2005). Therefore, a second enzyme orientation strategy has been defined recently. The strategy can prevent separation in the enzyme, as well as increase stabilization by making it rigid. This strategy involves activating enzyme groups (such as glyoxyl, epoxy) and binding with activated groups to the large-surface support (Mateo et al., 2007).

In this way, reversible imino bonds are made with the amino groups on the enzyme surface of the aldehyde groups formed on the support obtained. Thus, at alkaline pH values, it is ensured that proteins are immobilized spontaneously to the solid support at many points where lysine amino acids are concentrated. In this case, some amino acid groups in subunit enzymes spontaneously interact with the surface of the solid support. This situation positively affects stabilization (Grazu et al., 2006).

Tetrameric β-galactosidase obtained from E. coli and trimeric glutamate dehydrogenase enzyme stability studies obtained from *T.thermophilus* in some subunit enzyme studies performed by covalent modification method also mentioned that covalent bonding may be useful (Bolivar et al., 2009).

1.1.1. Glutaraldehyde crosslinking

Possible uses of glutaraldehyde for protein immobilization with the use of a pre-activated supplement or the treatment of proteins adsorbed on the support via amino groups with glurataldehyde molecules are among the possible studies that have been tried (Hwang et al., 2004). In fact, both protocols appear to have similar mechanisms following each other: immobilizing proteins using ionic interactions first, then using covalent modification. In the immobilization strategy part, only the groups containing immobilization are chemically modified, while in the other strategy the entire surface of the proteins is chemically modified (Zhou et al., 2001).

In immobilization on the pre-activated support, the aldehyde groups formed by modification of the amino groups of the support react with the basic amino acid groups of the enzymes. In such studies, the pH of the reaction medium should be in the range of 7.0-8.5. If the pH is high, the support activated with glutaraldehyde shows less stabilization (L´opez-Gallego et al., 2005). At such pH values, the reactivity of Lysine groups (ε -amino) is not exactly as expected and multiple covalent bonding is thought to reduce the density of the molecule. On the other hand, if the enzyme is first attached to the solid support and then treated with glutaraldehyde molecules under suitable conditions, it can allow an effective crosslinking under certain reaction conditions (Fern´andez et al., 1995).

1.2. Polymers

Synthetic water soluble polymers are reagents that play a very important role in biotechnology. These materials provide effective support to the stabilization science in being a stabilization agent and immobilization host. Recently, the role of these polymers in protein purification and enzyme stability is an important approach that has come to the fore. The general approach in enzyme stabilization is that there are suppressions to prevent protein openings. Protein engineers have tried many methods by adding materials that can provide stabilization. These additional materials added to the environment; Substrate, product, inhibitor, cofactor, allosteric effector and other ligand molecules, as well as metal ions, ionic species, organic solvents, proteins, polymers and lipids. Among these, synthetic and natural polymers are among those that have been tried frequently. It is possible to interpret the effect of polymers on stabilization under the following headings (Bryjak, 1995);

1) To have a preferable hydration or minimal surface energy in order to maintain the protein forces and to form the correct conformation,

2) Ability to increase solvent viscosity,

3) Encapsulation of enzymes thanks to the polymer chain network,

4) To protect the enzymes that are partially separated in the solvent from adverse effects,

5) Being able to protect enzymes from proteolytic enzymes,

6) Some of them can show antibacterial and antifungal activity.

For this purpose, many researchers have tried water-soluble polymers (Feng et al., 1993). Some of these polymers: Poly (vinylpyrrolidone), poly (vinylacetate), poly (vinylalcohol), poly (methylacrylic acid), poly (benzethylene sulfonate), poly (N, N-dimethyl aminopropylene bromide), poly (2-hydroxyl 1, 3 -dimethylamino propylene chloride), poly (ethylene glycol), poly (ethyleneimine) (Zhang et al., 1999).

1.2.1. The use of silicon in stabilization

Mikroelektronik malzemeler arasında silikon yüksek olgunluk ve düşük değerde bir teknolojiye sahiptir. Ayrıca, bazı araştırmacı grupları, silikonun biyosensör uygulamalarında inovatif yaklaşımlarda da bulunmuşlardır. Porlu silikonlar kimyasal ve biyolojik sensör, medikal diagnostik, optik bant geçiş filtreleri, mikrokimyasal reaktör, mikro yakıt hücreleri uygulamalarında yoğun bir şekilde araştırılmaktadır (Koh et al., 2007). Silikon tabanlı matrikslerin enzim immobilizasyonunda, stabilizasyonunda ve biyokimyasal aktivasyonların korunmasında çok kullanışlı bir destek malzemesi olduğu da gösterilmiştir (Libertino et al., 2008). Özgün yarı iletken özelliğinden ve gelecekteki entegratif kompleks sistemlerde mikro ölçekte yapılan işlem proseslerin gelişmesine izin verebilecek potansiyele sahip olduğundan dolayı ilgi çekmekte ve kabul görmektedir (Sahare 2014), (Lars et al., 2009), (Jennıfer et al., 1999).

Use of Dextran and Polyethyleneimine in stabilization

Although enzyme immobilization is required in many industrial processes, conditions such as large volume substrate, insoluble substrate, cofactor requirements and use in membrane reactors have emerged that researchers need to contain enzymes in dissolved form. In such cases, they reported the use of aldehyde-dextran and polyethyleneimine, with very good results in chemical, physical crosslinking and confinement of subunits of multimeric enzymes (Garcia et al., 2013). The polymer allows coating of large protein surface ratios that will allow long distance enzyme-polymer bonds and fully crosslinking of the subunits (Fuentes et al., 2004).

It has been reported that especially dextran and polyethyleneimine are effective not only in the stabilization of multimeric but also monomeric enzymes in dissolved form. It has also been mentioned that these polymers can protect the biological molecule from oxidation, aggregation and surface ethics. Crosslinking of proteins in multimeric protein solutions with aldehyde-dextran can be easily achieved (Garcia et al., 2013).

Polyethyleneimine polymer has primary, secondary and tertiary amino group density with high ionization capacity. In fact, this polymer has been shown to be highly capable of adsorbing protein through ionic changes occurring at neutral pH values. (Fig 2).

Increasing the incubation time of the enzyme and polyethyleneimine decreases the desired enzyme stabilization and enzyme modification under correct conditions is also very important (Bolivar et al., 2009). Otherwise, separation may occur and stabilization of enzymes may not occur. The stabilization of the polyethyleneimine-enzyme complex even

under harsh conditions is through the advanced covalent cross-linking of both the enzyme and the polymer with glutaraldehyde (Mazzaferro et al., 2010)

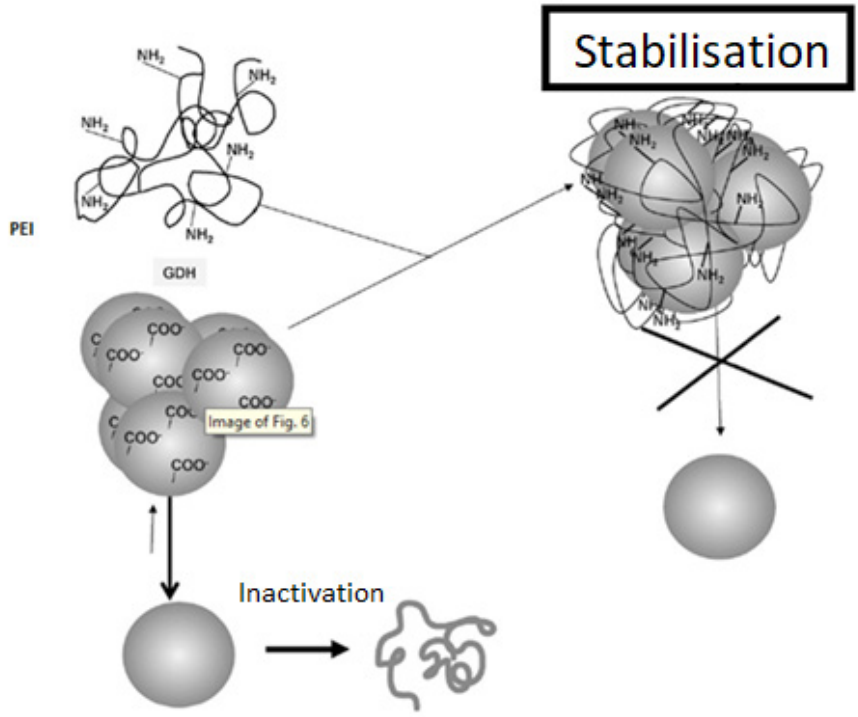

Figure 2. *Schematic representation of the proposed mechanism in the stabilization of glutamate dehydrogenase with polyethyleneimine (Garcia et al., 2013).*

2. The use of enzymes in biosensor applications

Biosensors; It is widely used in clinical diagnostics, medical applications, process inspection, bioreactors, food quality control, agriculture and veterinary diagnosis and quality control, preparation of bacterial and viral diagnostic kits, pharmaceutical production, industrial wastewater inspection, mining, military defense industry (Kökbaş et al., 2013). Today, the area where biosensors are used most frequently is medical diagnosis and diagnosis.

Biosensors are analytical systems formed by combining physicochemical analysis systems and biological materials. In biosensors, the high specificity of the biological system is combined with the detection sensitivity of the physical analysis system. Initial biosensor studies L.C. It is an enzyme-based glucose biosensor prepared by Clark in 1962 by immobilizing glucose oxidase enzyme on the amperometric electrode

surface (Bartlett, 2008). Enzymes have been an indispensable element of the bioactive layer in the biosensor history due to their substrate specificity and effective immobilization. Regardless of what basic sensor transmission system is in question today, the advantage of enzyme electrodes stands out in practical and commercial applications. The biggest factor in this result is the presence of a large number of enzymes that can be used in the direct or indirect analysis of almost any substance related to living systems. Besides the known enzymes, the potential existence of unknowns, the availability of hundreds of commercial enzyme preparations in the market and the increase in this number day by day are indications that the indisputable superiority of enzyme sensors will continue. In recent years, the findings on organic phase enzymology have revealed the feasibility of quite different analyzes in the organic phase with enzymes. These findings will contribute to both the increase in the number of enzyme sensors that can be designed and the widespread use of application areas (Beilen et al., 2002).

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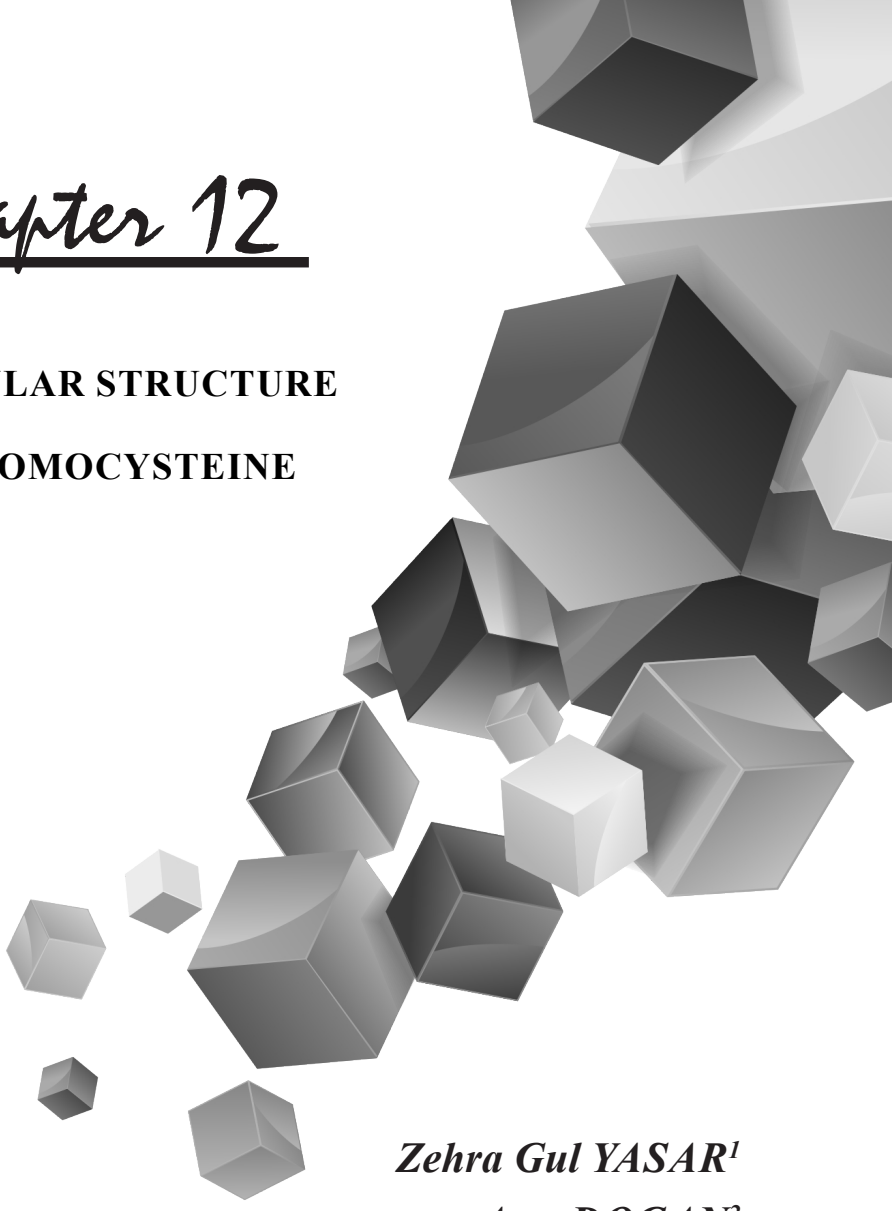
Chapter 12

VASCULAR STRUCTURE AND HOMOCYSTEINE



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Introduction

Cardiovascular diseases are among the leading causes of death in developed countries and are spreading rapidly around the world. Despite current treatments, these diseases are common(Spyridopoulos & Andres, 1998). Many vascular diseases such as atherosclerosis, post-angioplasty restenosis, stent restenosis, and post-transplant coronary arteriopathy, which are among cardiovascular diseases, are characterized by intimal hyperplasia. The smooth muscle cells of the blood vessels normally produce vascular tone(Owens, Kumar, & Wamhoff, 2004). In response to overexposure to various active substances such as cytokines and growth factors, vascular smooth muscle cells (VSMC) in the elastic lamina can differentiate, secrete extracellular matrix components that make up neointimal tissue and form a layer(Ginnan, Guikema, Halligan, Singer, & Jourd'heuil, 2008; Schwartz, 1997). The development of intimal hyperplasia results in vasoconstriction and is clinically manifested by myocardial infarction. However, it has not been fully clarified how various factors affect proliferation in VSMC depending on the injury. In other words, the etiology of intimal hyperplasia is not fully known in general(Ginnan et al., 2008).

Also, it has been reported that there is a positive relationship between cardiovascular diseases and the increase in blood homocysteine (Hcy) level, and it may play a role in the development of post-angioplasty restenosis and atherosclerosis in the general population(Masaki et al., 2007). Although cardiovascular diseases are observed in hyperhomocysteinemic patients with a blood Hcy level above 15 μmol / L, the mechanisms underlying this process are still uncertain today. Possible causes have been reported as vascular endothelial damage and dysfunction, coagulation and fibrinolytic system disruption, abnormal lipid metabolism, increased proliferation of VSMC, and monocyte activation(Buccianti et al., 2004; Heinz, Kropf, Luley, & Dierkes, 2009; Jakovljevic, Gasic, Kovacevic, Rajkovaca, & Kovacevic, 2015). The most emphasized among these mechanisms is the proliferation of VSMC. Some studies suggest that Hcy can induce cellular proliferation(Dalton, Gadson, Wrenn, & Rosenquist, 1997; Liu et al., 2008) by enabling the activation of the protein kinase C pathway(Dalton et al., 1997) and the expression of c-fos, c-myb of the MAPK pathway(Woo, Dudrick, & Sumpio, 2000). However, in these studies so far, the mechanism underlying Hcy-induced proliferation in VSMC is largely unknown.

A better understanding of the mechanisms that control the proliferation and differentiation of smooth muscle cells, which form an important part of the vascular structure, and the effect of hcy on the vascular structure

will make a significant contribution to the prevention and treatment of vascular diseases.

Vascular Wall

The vascular wall in the adult organism consists of a three-layered structure that includes the tunica intima, media, and adventitia (Figure 1). The innermost layer is the tunica intima layer formed by a single row of endothelial cells on the basement membrane that covers the inner surface of the vessel. The subendothelial layer formed by loose connective tissue that may contain sparse smooth muscle cells and the internal elastic lamina that provides flexibility and strength to the endothelial cells is located under the basal membrane. This lamina contains a spaced structure that feeds the cells located deep within the vessel wall and allows diffusion of various substances. The middle layer (tunica media) consists mainly of the concentric layer of smooth muscle cells arranged in the form of a helix and elastic fibers that contribute significantly to homeostatic vascular tone. The outermost layer (tunica adventitia or tunica externa) is a fibro-elastic connective tissue that surrounds vessels consisting of longitudinally arranged collagen and elastic fibers. This layer, which was thought to be only a simple connective tissue for a long time, is embedded in the tissue surrounding the vessel and contains vascular vasorum and nerve fibers. Their thickness varies greatly depending on the size of both the media and the adventitia(Shalaby et al., 1995; Worsdorfer et al., 2017; Zengin et al., 2006).

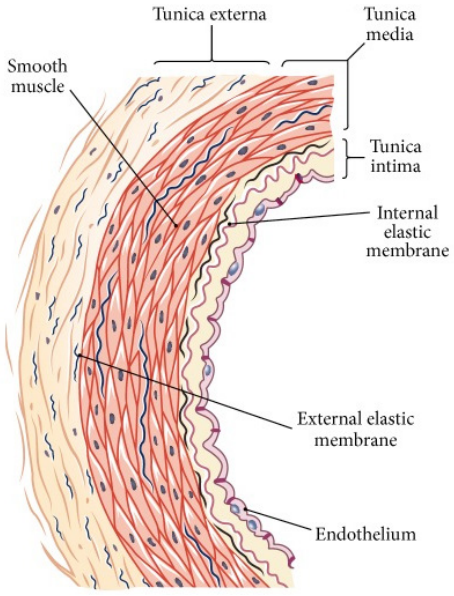


Figure 1. Schematic view of the arterial wall structure. *(Hammes, 2015)*

Smooth Muscle Cells

Smooth muscle cells in the veins, airways, urogenital system, and digestive tract have a contractile feature that can change the shape-size of the surrounding organ or allow it to move. The smooth muscle cells located in the vascular wall change the diameter of the lumen by directly contracting the vascular wall(Batu, 2016; Fitridge & Thompson, 2011). Each smooth muscle cell is surrounded by a basal lamina and reticular fibrillar network. The basal lamina and reticular fibrillar network contribute to muscle tone by playing a role in transforming the force generated by each smooth muscle fiber into collective action. These cells are spindle-shaped when they are not contracted. The nucleus of the cell is located in the center of the cell; Organelles such as mitochondria, granulated endoplasmic reticulum cisternae, free ribosomes, glycogen granules, and the Golgi apparatus are densely located at both ends of the nucleus(Batu, 2016; Fitridge & Thompson, 2011).

The thin ends of smooth muscle cells are tightly connected with the bulging middle part of neighboring cells, and these gap junctions provide communication between cells. Small molecules and ions pass through these connections and cause contraction of smooth muscle tissue simultaneously. smooth muscle cells have a cytoskeleton made up of thin filaments such as actin and tropomyosin, thick filaments containing myosin, and intermediate filaments such as desmin and vimentin(Batu, 2016; Brozovich et al., 2016; Fitridge & Thompson, 2011).

The contractile activity of the smooth muscle is controlled by hormones, autonomic nerves, and various factors. Like the cardiac muscle and skeletal muscle, smooth muscle contraction is dependent on calcium and Increased intracellular calcium concentration causes contraction of smooth muscle. Smooth muscle sarcolemma contains voltage-gated Ca^{2+} channels. Besides, smooth muscle contains the endoplasmic reticulum (sarcoplasmic reticulum, SR), which acts as a reservoir for Ca^{2+}. When stimulating hormones, neurotransmitters or drugs bind to their receptors in the cell membrane, they cause calcium release from SR to myoplasma.

Smooth muscle cells have a feature that proliferates regularly and in case of damage, they respond to this damage by mitosis(Batu, 2016; Boswell, Joris, & Majno, 1992; Brozovich et al., 2016; Fitridge & Thompson, 2011; GÜNEŞ; Mazurek et al., 2017).

Vascular Smooth Muscle Cells

VSMC is one of the three main cell types that make up the blood vessel wall, in addition to endothelial cells and fibroblasts. According to histological classification, VSMC forms the tunica media in the vascular structure and is the thickest layer. Smooth muscle cells are separated from each other by elastic lamellae. They are linked to each other by gap

junctions(Boswell et al., 1992; Brozovich et al., 2016). VSMC are fusiform, approximately 200 μm in length, 5 μm in diameter, and contain a large nucleus in the center(Fitridge & Thompson, 2011). VSMC does not contain the T-tubule / SR system commonly found in striated muscle. However, instead of these, there are structures called caveols. These structures increase the cell surface area. VSMCs can migrate to the tunica intima layer by multiplying under the influence of growth factors such as fibroblast growth factor (FGF) and thrombocyte-derived growth factor (PDGF) synthesized by neighboring endothelial cells. While this event contributes to the repair of the vascular wall under normal physiological conditions, it may cause thickening of the wall and consequently atherosclerosis in pathological conditions(Batu, 2016; Boswell et al., 1992; Brozovich et al., 2016; Fitridge & Thompson, 2011; Xie, Ritchie, Huang, Zhang, & Chen, 2011).

The extracellular matrix is composed of large proteins and polysaccharides that provide a scaffold for vascular tissue cells and act as binding agents to hold the cells together. The main source of extracellular matrix proteins is VSMCs. The extracellular matrix plays an important role in normal function and phenotypic changes of VSMCs, in many diseases such as atherosclerosis, restenosis, hypertension, and aneurysm.

Normally, smooth muscle cells in blood vessels proliferate at a very low rate and contain contractile proteins, ion channels, and signal molecules necessary for the contractile function of the cell. Unlike skeletal and cardiac muscle, smooth muscle cells have exceptional plasticity and can cause effective and reversible changes in response to changes in local environmental factors(Owens, 1995). In response to vascular damage, cell proliferation, migration, and synthetic capacity of smooth muscle cells increase and play a critical role in vascular repair(Owens et al., 2004).

When the smooth muscle cells in the vascular wall are examined in terms of structure, there are two different phenotypes, contractile and synthetic. In healthy adult blood vessels, smooth muscle cells often have the contractile phenotype characterized by a slow proliferation rate. With immunohistochemical staining of proteins such as calphonin, smooth muscle alpha-actin (α-SMA), and smooth muscle myosin heavy chain (SM-MHC), it can be shown whether smooth muscle cells are in the contractile form(Mantella, Quan, & Verma, 2015).

The contractile type of VSMCs provides the regulation of myogenic tone in the blood vessel, blood pressure, and blood flow. As a result of damage to the vascular wall, contractile type VSMCs turn into a synthetic phenotype. Synthetic VSMCs, in addition to their proliferation and migration properties, cause an increase in substances that form the

extracellular matrix such as collagen, elastin, and proteoglycans(Alexander & Owens, 2012; Mantella et al., 2015; Rzucidlo, 2009).

Vascular Smooth Muscle Cell Proliferation

Uncovering this proliferative mechanism is critical, as the abnormal proliferation of VSMCs plays an important role in the pathogenesis of vascular diseases such as atherosclerosis, by-pass graft stenosis, and hypertension(Ishii et al., 2001). Proliferation signal transduction pathways in VSMC are quite complex. In the literature, it is reported that 3 types of receptors on the cell membrane activate signaling pathways that cause proliferation. These are receptor protein tyrosine kinases (RTK), G protein-coupled receptors (GPCR), and integrins(Köksoy, 2002). Signal transduction in proliferation takes place as a communication pattern between the pathways of these receptors. In this way, the initial stimulation is increased several times, turning into a signal cascade where many receptors and kinases work together. In VSMC, signal transduction pathways associated with RTK, integrin, and GPCR form a complex system that interacts with each other and common signal path proteins are activated. Activation of the system causes the activation of mitogen-activated protein kinase (MAPK) family members, phospholipase C (PLC), and phosphoinositide-3-kinase (PI3K) by activating many mechanisms in the intracellular environment(Köksoy, 2002). Activation of these kinases causes them to cross into the cell nucleus and activate transcription factors through phosphorylation(Lenormand et al., 1993). Thus, when the receptors located on the membrane are activated, they provide genetic regulation in the nucleus. MAPK is one of the most important steps in VSMC proliferation. Therefore while it plays an active role in almost all proliferative signaling pathways, inhibition causes disruptions in apoptosis and DNA synthesis. Regulation of MAPK activation and consequently Proliferation is carried out by MAPK phosphatases(Köksoy, 2002).

It has been shown that these receptor pathways lead to cell proliferation in the formation mechanism of diseases such as atherosclerosis and bypass stenosis. However, it is not known what the key molecules or mechanisms that turn the proliferation mechanism, which is normally under tight control, into an uncontrolled state(Buemi et al., 2001).

Homocysteine (Hcy),

An amino acid containing sulfhydryl is an intermediate product that occurs in the normal biosynthesis of methionine and cysteine amino acids(Faeh, Chiolero, & Paccaud, 2006). In the human body, 1% of the Hcy in the blood is free, 70% is bound to proteins, and the rest is in reduced or oxidized form(Faraci, 2003).

It is important to clarify the methionine metabolic pathway because of its association with various pathological conditions (Figure 5). Methionine is converted to S-adenosylmethionine (SAM) by the methionine adenosyltransferase enzyme(Huang, Yuan, Zhang, Zou, & Li, 2008; Kaul, Zadeh, & Shah, 2006). This pathway is essential in providing methyl groups to activate biomolecules such as DNA, creatine, and phospholipids. SAM is demethylated to S-adenosylhomocysteine (SAH) as a product of these methyl-transferase reactions; SAH is also hydrolyzed to Hcy (Moat et al., 2004).

After Hcy is formed, it is metabolized by two metabolic pathways: remethylation and transsulfuration. Remethylation is the vitamin-dependent pathway that converts Hcy to methionine via 5-methyltetrahydrofolate reductase (MTHFR) and methionine synthase(Dinleyici et al., 2006). The activity of MTHFR is largely dependent on the presence of folate (vitamin B9) and cobalamin (vitamin B12). Remethylation appears to be the primary regulator of fasting and high plasma Hcy concentrations(Moat et al., 2004). Transsulfuration requires vitamin B12 to convert Hcy to cysteine through a two-step process involving the vitamin B6-dependent enzyme cystathionine β-synthase and cystathionase. Ultimately cysteine is converted into sulfate and excreted in the urine (Huang et al., 2008).

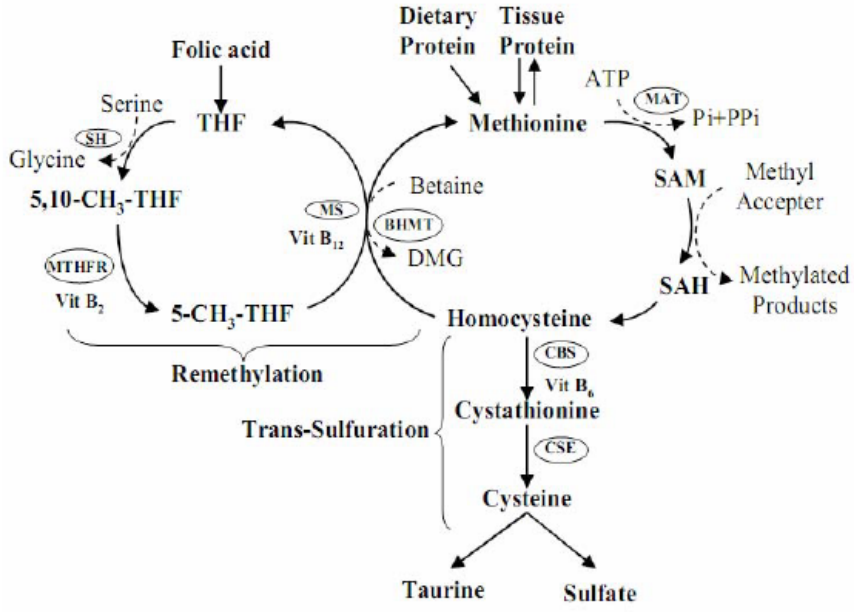


Figure 5. Metabolism of homocysteine*(Huang et al., 2008).*

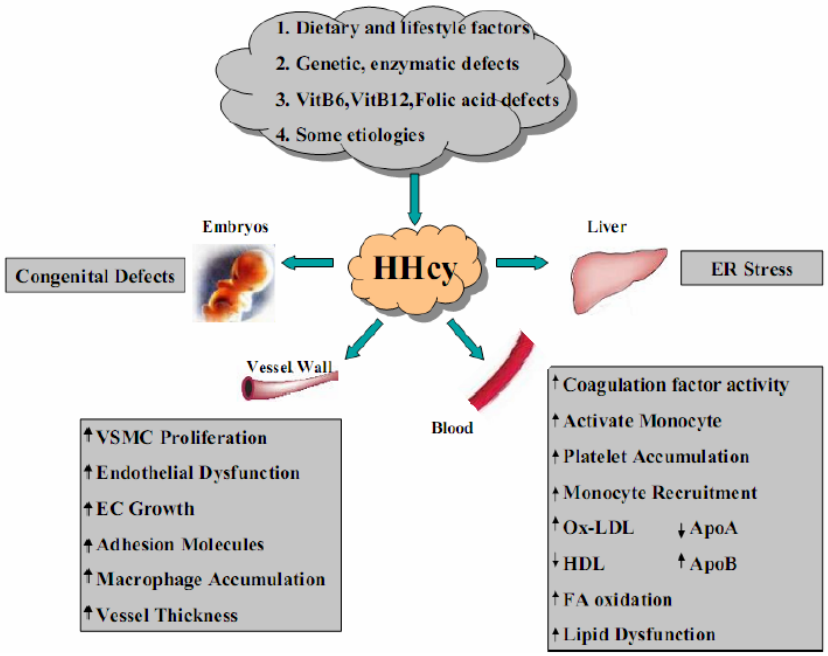
MS: Methionine synthase; CBS: Cystathionine β-synthase; CSE: Cystathionine γ-lyase; MTHFR: Methylenetetrahydrofolate reductase;

BHMT: Betaine-homocysteine methyltransferase; MG: Dimethylglycine; 5,10-CH3-THF: 5,10-Methylene-Tetrahydrofolate; 5-CH3-THF: 5-methyl Tetrahydrofolate; THF: Tetrahydrofolate; SH: Serine hydroxymethyltransferase; Pi: Orthophosphate; PPi: Pyrophosphate.

In healthy subjects, the total plasma hcy concentration in the fasting state are among the lowest is 5.0 to 15.0 μM (Baszczuk & Kopczynski, 2014). An increase in Hcy level over 15 μmol / L in the blood is called hyperhomocysteinemia (HHcy)(Guo, Chi, Xing, & Wang, 2009). When this level in blood is between 16-30 μmol / L, it is classified as mild, between 31-100 μmol / L, moderate, and above 100 μmol / L, as severe HHcy(Hankey & Eikelboom, 1999). Increasing protein dietary intake, genetic defects, and vitamin deficiencies in Hcy metabolism cause rapid increase in Hcy level in plasma(Hamelet, Demuth, Paul, Delabar, & Janel, 2007; Rosenberg, 1996; Selhub, 1999; Selhub, Jacques, Wilson, Rush, & Rosenberg, 1993; Verhoef & de Groot, 2005). HHcy can be caused by a nutritional deficiency of folate, vitamin B6, and vitamin B12(Curro et al., 2014). Folate, vitamin B12, and, to a lesser extent, blood vitamin B6 levels are inversely related to total Hcy(Curro et al., 2014; Hankey & Eikelboom, 1999).

It is believed to be associated with moderately elevated Hcy concentrations of various medications, alcohol, tobacco, coffee, old age, and menopause, as well as certain diseases such as kidney and thyroid dysfunction, cancer, psoriasis, and diabetes(Faeh et al., 2006). The main way of clearing Hcy from plasma is the kidneys, and its elevation may be due to kidney failure. Total Hcy levels were found to be quite high in patients with chronic kidney disease(van Guldener, 2006).

The prevalence of HHcy can vary significantly within the population and is likely due to age, diet, and genetic background(Faeh et al., 2006). Older age, male gender, smoking, coffee consumption, high blood pressure, unwanted lipid profile, high creatine, and malnutrition are some of the factors associated with increased Hcy levels. On the other hand, lower Hcy levels have been shown with increased physical activity, intake of folate and vitamin B12, and moderate alcohol consumption. In vegetarians, the risk of HHcy increases due to the decrease in plasma vitamin B12 levels(Shenoy, Mehendale, Prabhu, Shetty, & Rao, 2014).



Şekil Figure 6. The alleged pathogenesis of cardiovascular diseases seen in HHcy*(Huang et al., 2008).*

HHcy: hyperhomocysteinemia; EC: endothelial cell; VSMC: vascular smooth muscle cell; ER: endoplasmatic reticulum; Ox-LDL: oxidized low-density lipoprotein; ApoA: Apolipoprotein A; ApoB: Apolipoprotein B; HDL: high-density lipoprotein.

Increased plasma Hcy level appears to be an important and independent risk factor for the pathogenesis of many cardiovascular diseases (Figure 6). It has been reported in many studies that Hcy is a risk factor for atherosclerosis in coronary, cerebral and peripheral vessels, and its proliferative effect on VSMC is seen as one of the most important mechanisms by which it induces vascular diseases(Hansrani, Gillespie, & Stansby, 2002). For the first time in 1969, Dr. McCully suggested that the high Hcy level seen in infancy caused widespread vascular lesions(McCully, 1969). The increase in plasma Hcy causes VSMC proliferation, and it has also been shown to cause ventricular hypertrophy and significantly increase systolic blood pressure. It has also been reported that Hcy has the potential to affect vascular reactivity through its role in VSMC proliferation and extracellular matrix accumulation(Mujumdar, Hayden, & Tyagi, 2000). Today, the effect of high Hcy levels on cardiovascular diseases is explained by some possible mechanisms. According to the autoxidation mechanism, Hcy rapidly oxidizes to disulfide Hcy or Hcy thiolactone in plasma. During this reaction, reactive oxygen radicals such as superoxide radicals and hydrogen

peroxide are formed. While these cause damage to the vascular endothelium, they initiate lipid peroxidation by affecting LDL particles. According to another mechanism, nitric oxide released from endothelial cells binds to this molecule to counteract the toxic effects of Hcy. However, as a result of long-term exposure to HHcy, lipid peroxidation, and endothelial nitric oxide synthase production and release are reduced. As a result, the endothelium is exposed to oxidative damage, and its integrity is broken. It is also reported that hcy acts directly on vascular smooth muscle cells and stimulates cell proliferation. Although it is not known which mediator Hcy uses in the membrane to produce this effect on VSMC, it is suggested that it stimulates cell proliferation through a possible N-methyl-D-aspartate (NMDA) like receptor(Chen et al., 2005) (Figure 7) (Hansrani et al., 2002).

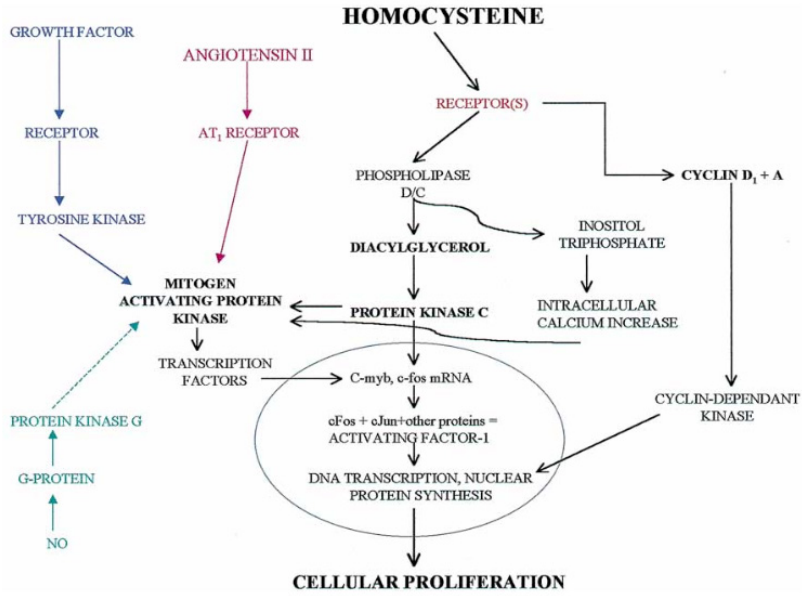


Figure 7. Mechanisms by which cellular proliferation in VDKH can be induced by Hcy*(Hansrani et al., 2002).*

It has been reported that Hcy allows cells by stimulating cyclin A(Tsai et al., 1996) and D1(Neganova & Lako, 2008), which provide the transition of cell division from G1 to S phase and have an important role in S phase, to re-enter the cell cycle via cyclin-dependent kinase(Hansrani et al., 2002).

It is suggested that Hcy also activates phospholipases such as C and D. Activation of phospholipases leads to an increase in intracellular calcium ions by causing DAG, IP3 synthesis, PKC activation, and it may also cause activation of DNA transcription factors and MAPK that stimulates cellular proliferation(Hansrani et al., 2002). Also, it has been reported that Hcy indirectly causes VSMC proliferation due to its effect on extracellular

matrix proteins. (Tyagi, 1998).

Although the effect of Hcy on proliferation has been tried to be explained with various signaling pathways as mentioned above, the mechanism underlying its effect on cardiovascular diseases has not been fully elucidated. Therefore, further studies are needed to evaluate the Hcy's role. (Hansrani et al., 2002).

Although the effect of VDKH proliferation induced by HHcy in the pathogenesis of cardiovascular diseases is complex and not well understood it is thought that activation of the renin-angiotensin system (RAS) may also play an important role here. In animal studies investigating the relationship between HHcy and RAS, it has been reported that Ang II increases collagen accumulation in mouse arteries with HHcy(Neves et al., 2004). In addition to these, it has been reported that ventricular hypertrophy and increased systolic blood pressure, which develops due to increased Hcy in the plasma due to methionine intake with diet, were treated with the administration of AGTR1 antagonist valsartan(Kassab et al., 2006). It has also been shown in studies that vascular responses to Ang II, such as intracellular Ca^{2+} release(Mujumdar et al., 2000) and contraction response in carotid vascular tissue(Bonaventura et al., 2004), increase in the presence of high Hcy in isolated aortic VSMCs. When these studies are examined, it is thought that there is an interaction between HHcy and RAS in cardiovascular diseases, but the mechanism of this interaction is unknown.

Conclusions

Although cardiovascular diseases are observed in hyperhomocysteinemic patients, the mechanisms underlying this process are still uncertain today. Possible causes are reported to be various pathologies such as vascular endothelial damage and dysfunction, impairment in coagulation and fibrinolytic system, abnormal lipid metabolism, increased proliferation of VSMCs, and monocyte activation(Buccianti et al., 2004; Heinz et al., 2009; Jakovljevic et al., 2015). The most emphasized among these mechanisms is the proliferation of VSMCs. Some studies suggest that Hcy can induce cellular proliferation by enabling activation of the protein kinase C pathway(Dalton et al., 1997) and the expression of c-fos, c-myb of the MAPK pathway(Dalton et al., 1997; Liu et al., 2008; Woo et al., 2000). However, in these studies so far, the mechanism underlying Hcy-induced proliferation in VSMCs is largely unknown.

However, the relationship between HHcy and various pathologies can be elucidated by investigating the molecular mechanisms of Hcy-induced metabolism and other non-cardiovascular aspects of its deleterious effects. Therefore, new studies and researches are needed.

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Chapter 13

AN ARTISTIC TOUCH TO PEOPLE WITH SCHIZOPHRENIA: *DANCE AND MOVEMENT THERAPY*

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1.Schizophrenia

1.1. Definition of Schizophrenia

Schizophrenia is one of the common severe psychological disorders, and it is an important public health problem which may cause severe losses in the individual's life (Sadock et al., 2016). It is a psychosis which begins generally at early ages and where the individual alienates to habitual perception and interpretation styles and experience significant disturbances in his/her own senses, thoughts and behaviors (Dulgerler, 2014, Ozturk and Ulusahin, 2011).

1.2. Epidemiology of Schizophrenia

The prevalence of schizophrenia in the general population is approximately 1%. It is equally common among men and women. However, onset age and course of the disorder differ among both sexes. It begins among men at earlier ages compared to the women; and prognosis of disease is better in women. The onset of schizophrenia is very rare before 10 years and after 50 years old (Sadock et al., 2016; Dulgerler, 2014).

1.3.Etiology of Schizophrenia

Although the exact cause of schizophrenia is unknown, several theories have been proposed. Some of them are genetic predisposition, stressful life events, physical and sexual abuse experienced during childhood, trauma and infections experienced by the mother before, during and after childbirth, conditions leading to hypoxia inside the mother's womb, changes in biochemical factors (dopamine, serotonin, glutamate, gamma aminobutyric acid (GABA), estrogen) and changes in the volume of grey and white matter in the brain (Sadock et al., 2016; Ozturk and Ulusahin, 2011).

1.4. Symptoms of Schizophrenia

Although schizophrenia symptoms are generally classified as positive and negative, it has been proposed to be gathered under three categories also including cognitive disorders in recent years. Positive symptoms include delusion, hallucination, disorganized thoughts and behaviors. On the opposite, absence of typical functions such as blunting in affect, social withdrawal, unwillingness or inability to take initiative and difficulty in starting an activity represent negative symptoms (Stahl and Schwartz, 2016; Arango and Carpenter, 2011). Cognitive symptoms of schizophrenia are assessed as impaired attention and verbal fluency disorder, problems regarding information processing and sequential learning, sustaining

attention, focusing, concentration, prioritization and problems in regulating behaviors based on social cues (Stahl and Schwartz, 2016).

1.5. Treatment of Schizophrenia

Particularly antipsychotic-containing pharmacological treatments, electro-convulsive therapy, cognitive-behavioral therapies, psychosocial skill education, family therapy and psychosocial therapies are used in the treatment of patients with schizophrenia (Dulgerler, 2014).

1.5.1. Psychosocial Approaches in Schizophrenia Treatment

It is known that antipsychotics alleviate most symptoms of schizophrenia and reduce exacerbation rates in the long term. However, performed studies have shown that antipsychotic drugs are not much effective on the negative symptoms since negative symptoms tend to persist for a longer time and their treatment is more difficult compared to the positive symptoms (Dauwan et al., 2016; Kalisz and Cechnicki, 2016; Remington et al., 2016). Moreover, long term use of antipsychotic drugs generally has severe side effects. These effects, that are called as extrapyramidal side effects, may affect life quality of the individuals in a negative way. This circumstance limits the use of drugs by the patients (Hanevik et al., 2013). Therefore, the National Institute for Health and Clinical Excellence (NICE) emphasized the need for presenting psychosocial approaches besides the medications for the improvement in the symptoms of patients with schizophrenia regarding psychological, social and functional difficulties (Thornicroft et al., 2004; Kirkbride et al., 2012).

Therapy programs, which combine pharmacological and psychosocial treatments and support patients with schizophrenia by integrating medication treatment with psychosocial approaches, have gained importance (Yildiz, 2000; Kirkbride et al., 2012). Psychosocial therapies are accepted as important approaches for optimizing psychosocial functionality by decreasing the severity of negative symptoms (Cechnicki et al., 2011). Psychosocial approaches are helpful in fixing problems such as quality of life, interpersonal relations, treatment incompliance and dysfunction. Psychosocial approaches used in the treatment of people with schizophrenia are family therapy, group therapy, cognitive-behavioral therapy, social skill development therapy, psychoeducation and art therapy (Heinssen et al., 2000).

Clinicians have turned to art therapy increasingly in order to meet the healthcare needs of the patients. Although the use of creative arts for the treatment of psychological disorders was first emerged more than half a century ago, there has been a growth of literature describing its potential

benefit over the past three decades (Attard and Larkin, 2016; Carr et al., 2013; Feirstein, 2016; Fenner et al., 2017)

2. Art Therapy

2.1. Definition of Art and Art Therapy

Art can simply be described as the expression of imagination and creativity. It has positive contributions to the individuals with its rehabilitating aspect and also a potential to heal brain damage, developmental disability and many mental diseases. In this context, "Art Therapy" helps people to recognize and express their own feelings and thoughts by using artistic creativity (Sayar, 2006).

Art, an ongoing phenomenon since the existence of humanity, and creation process is empowering, cathartic and healing by nature. For that reason, the use of art as a therapy method has been based on prehistoric times and a variety of cultures. Forms of expression created by art can be used for the expression of a series of feelings including the ones that are hard to describe by the patients or clients (Cakmak et al., 2020).

Art therapy is described as "a mental health occupation that uses creative process of art-making in order to enrich and improve physical, emotional and mental beings of the individuals of all ages" by American Art Therapy Association. In other words, art therapy is the use of various art materials during diagnosis and treatment in order to aid causing a positive improvement among the individuals, resolving conflicts, decreasing physical and mental problems, solving problems and coping with stress (Coskun, Yildiz and Yazici, 2010; Geue et al., 2010).

Art therapy is the process of expressing one's internal experiences and feelings, that she/he suppressed and could not express verbally, through artistic elements. The focus of therapist is not related to the aesthetic values of art-making or the resulting artistic material, it is on the therapeutic needs that an individual can express without having concerns about performance (Malchiodi, 2011).

What matters in the therapy session is the inclusion of the client in the creative process during the therapy, selection of art activities which are situation-specific and beneficial to the client, and facilitation of sharing visual, auditory or sensory imagery and experiences of the client with the therapist following the creative process. At this point, what makes art therapy stronger than the other methods is to address even the most painful issues without disturbing the person (Steele and Kuban, 2003).

Hidden feelings, wishes or fears of the client are seen as the products that appear in non-verbal and symbolic language. In this aspect,

psychological problems of various severity and diagnosis may be treated by this method. Considering that the creation process in art may bring the people down to their deepest layers and by protecting the defense mechanisms of the clients when necessary, it is highly important to choose artistic instrument and technique to be used in therapy session as allowing them to yield as far as they are ready (Malchiodi, 2011).

2.2. History of Art Therapy

The use of art therapy, which has begun to be used in 1940s, as a therapeutic intervention dates back to 1960s. The term 'art therapy' was first used by the artist Adrian Hill in 1942 in a study on patients with tuberculosis. Hill discovered that painting did not only let patients spend time, but also it enabled patients to express their concerns and traumas (Akhan, 2012; Case and Dalley, 2006; Malchiodi, 2005).

2.3. Benefits of Art Therapy

Since the 21st century, art therapy has been used on the children, adults, families and groups in many various environments in order to enhance self-understanding and to aid emotional repairs (Malchiodi, 2011).

Art therapy contributes to solve problems, reduce stress, improve interpersonal relations, manage the behaviors, raise self-consciousness, gain insight and increase self-esteem. It has been revealed that individuals can raise their self-consciousness, cope with traumatic events, increase the pleasure they get from life and improve their cognitive skills due to art-making and reflecting this to art processes and products (American Art Therapy Association, 2020).

2.4. The Use of Art Therapy in Psychiatry

Art therapy is often used by the mental health professionals with the idea that it can facilitate the emergence of emotinal conflicts suppressed by the individuals. While it strengthens the therapeutic relationship through communication in one hand, it also facilitates to establish a connection between conscious and unconscious processes of the individual on the other hand (Demir and Yildirim, 2017). Moreover, the patient has a passive nature during the treatment process using only medications, he/she switches to a client-centered active nature by finding the opportunity to participate in his/her own treatment (Killick and Schaveiren, 2003).

Art therapy is used in many diseases such as developmental delay, learning disorder, anxiety disorders, depression, neurological disorders and post-traumatic stress disorder. Medical art therapy can be used also in pain control and in reducing stress during pregnancy (Oz Celikbas, 2019).

2.4.1. The Use of Art Therapy in Schizophrenia Patients

Art therapy which can be used for the individuals of all ages is an effective method especially for the ones who have difficulty in expressing themselves verbally (Demir and Yildirim, 2017). At this point, therapeutic use of art has a very valuable function especially for the individuals who lack or have a limited verbal communication to understand or try to understand themselves (Patterson, 2007).

There are many studies in the literature regarding art therapy in people with schizophrenia (Gajic, 2013; Leurent et al., 2014; Crawford et al., 2012; Caddy et al., 2012). In the study by Montag et al. which was carried out with 58 schizophrenia patients in 2014, half of the patients were given only standard treatment and the other half was given standard treatment with art therapy two times a week for 6 weeks. Significant decreases were found in the positive and negative symptoms in the group which was applied art therapy.

Hung and Ku applied a semi-structured art therapy to two patients with schizophrenia for four times a month, and assessed positive and negative symptoms. At the end of the study, score of positive symptoms scale was dropped from 90 to 65 and negative symptoms scale was dropped from 69 to 45 in the first patient. In the second one, these were from 114 to 92 for positive symptoms and from 94 to 69 for negative symptoms. In another study performed with paetients with schizophrenia and their relatives, it was reported that art therapy reduced anxiety and depression levels of the patient relatives and positively affected social skills in individuals with schizophrenia (Sarandol et al., 2013).

2.5. Types of Art Therapy

The types of art therapy are visual art therapy, music therapy, dance and movement art therapy, drama/theatre therapy and creative writing therapy. As these therapy types can be applied individually, more than one therapy can be used in the same session (Chiang et al., 2019). Besides, various books, movies and documentaries, museums, games, art of marbling and traditional arts can be used in art therapy (Oz Celikbas, 2019).

All types of art therapy use artistic acts; and each of them has their own role and characteristic in therapeutic work. For instance, music therapy is aimed at releasing emotions and socialization with playing musical instruments or singing songs in groups whereas visual art therapy is more personal and individualized. Dance/movement therapy offers interactive options and can shape relationships (Cathy and Malchiodi, 2003).

3. Dance and Movement Therapy

3.1. Description of Dance and Movement Therapy

Dance and movement therapy is the way of discovering emotions of the individual nonverbally; i.e. without language and words (Ren and Xia, 2013). American Dance Therapy Association (ADTA) describes dance and movement therapy as "psychotherapeutic use of movement as a process that promotes an individual's emotional, social, cognitive and physical integration" (ADTA, 2011). Dance therapy differs from the other art therapy forms in terms of its contribution to both physical and psychological improvement by establishing mind-body connections (Chen et al., 2016; Levine and Land, 2016).

In the dance and movement technique, neither problem analysis as in therapy nor aesthetic concerns as in dance studies are in question. The aim of dance therapy is not to teach dancing, it is to provide well-being of the individual by increasing the range of movement. It is to understand and express the messages given by the body and to understand and tell the unthinkable (Nyström and Lauritzen, 2005). Basic principle of dance and movement therapy is based on in-depth observation over the movements of the individual and their association with the individual's internal Dynamics because movements of the individual reflect his/her interpersonal, spiritual and cultural patterns. Therefore, there is a direct relationship between movement and meaning. Since individual's movement is aimed at meeting a requirement and coping with the environment at the same time, it reflects his/her relationship with the surrounding and problem solving style; and it also helps to adapt different circumstances. Dance therapist observes the movements of the individual, and enables him/her to perceive and recognize his/her movements and to widen his/her movement repertoire. As a result, individual's consciousness increases and he/she may develop new mechanisms in verbal communication.

Features of dance and movement therapy such as integrating mind, body, emotion, creativity and morale, including relaxation, breathing and imagination techniques in the therapeutic process, providing to improve the use of touching, reflecting (mirroring), simultaneity and body empathy, providing to improve positive physical and emotional coping methods and ensuring emotional healing make it possible to be applied among various patient groups (Altan Sarikaya et al., 2017).

3.2. History of Dance and Movement Therapy

As the healing effect of dance has been known since ancient ages, it has been used as a therapeutic method in cases such as childbirth, death and disease. However, the emergence of dance therapy as an integrated

therapy method corresponds to the second half of the twentieth century. The advances both in modern dance and psychiatry have played a significant role during the development of dance therapy (Hanna, 2006).

The method was first developed by the dancer Marian Chace in 1940. She used dance and body movements on the patient with severe mental problems; and provided a deep understanding of the aspect of body movements that freely expresses emotions (Nyström and Lauritzen, 2005).

Modern dance movement was founded by dance therapy pioneers Mary White House and Turdy Schoop in the mid 20th century; and it was influenced by psychodynamic theory in the 1940s with the transformation of observation, interpretation and dance elements into practice. In 1960s, there were also some studies which were carried out regarding the interaction between nonverbal expression style and cognitive functions. The importance of dance and movement therapy has increased with the establishment of American Dance Therapy Association in 1966 and the development of education and certification programs in this field (Bostancioglu and Kahraman, 2017). Dance and movement therapy is influenced by an eclectic group of conceptual frameworks including psychodynamic theory, Gestalt theory and humanistic theory today (Bostancioglu and Kahraman, 2017).

3.3. Benefits of Dance and Movement Therapy

Dance and movement therapy allows individuals

- to socialize within a large group again,

- to use nonverbal creative expressions during expressing their emotions,

- to increase muscle coordination and movement capability,

- to enhance self-esteem,

- to recognize self and body,

- to contribute physical and psychological development by relaxing the body (Sheets-Johnstone, 2010).

3.4. Practice of Dance and Movement Therapy

Dance and movement therapy, that improves body and soul integration and social communication, is practiced by the therapists who have been trained in this field on healthy and patient groups at several areas varing from health to education as one-to-one session or group therapy. The therapist helps individual to make a connection between his/her dance/

movement and emotions and to interpret this connection (Levy Fran, 2005).

3.5. The Use of Dance and Movement Therapy in Psychiatry

Dance and movement therapy is frequently used in psychiatric field. Considering its positive effects on negative symptoms and anger control especially in patients with anxiety, depression and schizophrenia, it has been thought that dance and movement therapy may help to keep patients' symptoms under control (Altan Sarikaya et al., 2017). Dance and movement therapy may be used in mental health rehabilitation units, nursing homes and day care centers (Ren and Xia, 2013).

3.6. Dance and Movement Therapy in the Patients with Schizophrenia

While positive symptoms benefit more from the antipsychotic drugs among the people with schizophrenia, dance and movement therapy was found to be more effective on negative symptoms and physical findings (Röhricht and Priebe, 2006; Lee et al., 2015; Martin et al., 2016; Pohlmann et al.,2017).

There are some studies on dance and movement therapy among the individuals with schizophrenia in the literature. In a study which was carried out to examine the effect of dance and movement therapy on psychotic symptoms and affect among the people with schizophrenia, it was observed that there was a significant decrease in the anger and depression levels of the patients in experimental group compared to the control group; and it was found to be effective especially on negative symptoms (Lee et al., 2015). It has been indicated that non-cognitive negative symptoms such as emotional blunting and motor retardation could be also improved by the promotion of sensory awareness in dance and movement therapy (Röhricht and Priebe, 2006). Moreover, in another study including 45 individuals with schizophrenia, it was determined that a significant recovery was seen in the negative symptoms of patients who were applied standard care and dance therapy compared to the ones who were applied only standard care (Xia et al., 2009). In the other two studies where control group was applied visual art therapy (n=36 and n=31), significant improvements were recorded in the group which was applied dance therapy in terms of cognitive functionality, quality of life and physical findings (walking distance, balance and power) (Chen et al., 2016; Kaltsatou et al., 2015).

Lastly, a recent study which was performed in 2020 on 31 patients with schizophrenia by using a mixed method, patients reported that their self-confidence and anger control were enhanced, they felt better in self-

care, problem solving, recall and learning and they could communicate better with other people at the end of dance and movement therapy which was applied for twice a week for 10 weeks. In the same study, it was also determined that there was a decrease in the severity of negative symptoms, an increase in the feelings such as peace, joy and enjoying life and future planning and an improvement in interpersonal relationships.

4. Conclusion

Schizophrenia is one of the common severe mental disorders and an imporant public health problem that may lead to severe losses in individual's life (Sadock et al., 2016). Literature data have shown that standard care is not so effective on the negative symptoms of the patients with schizophrenia and dance and movement therapy may be beneficial at this point. It has been observed that dance and movement therapy is highly effective on negative symptoms, functionality, communication, problem solving, learning and muscle movement coordination among the people with schizophrenia.

The use of dance and movement therapy in the field of psychiatric health is based on the ability to express self and the thought of human integrity. It is estimated that patients' thoughts may change and thus, disease symptoms will be improved through physical interaction during the therapy. Dance and movement therapy is a power that unleashes patients' own resources in coping with their problems and it has become important to perform dance studies in psychiatry nursing since psychiatry nurses may help patients to use this power.

The features of dance and movement therapy show that dance and movement promote to communicate with other people and to explain personal ideas; and thus, improve mental health. Nursing intervention is required especially for the amelioration of the negative symptoms of people with schizophrenia who experience problems in communication and self-expression. At this point, dance and movement therapy during which patients can express themselves non-verbally is accepted as a nursing intervention (Ravelin et al., 2006).

In conclusion, it is considered that training of psychiatric nurses on dance and movement therapy in line with their interests will both improve their own well-being and increase the quality of care they provide. At the same time, it is estimated that performing mind-body focused practices such as dance movement therapy with patients hospitalized in service environment will contribute to the therapeutic communication with the patients (Altan Sarikaya et al., 2017).

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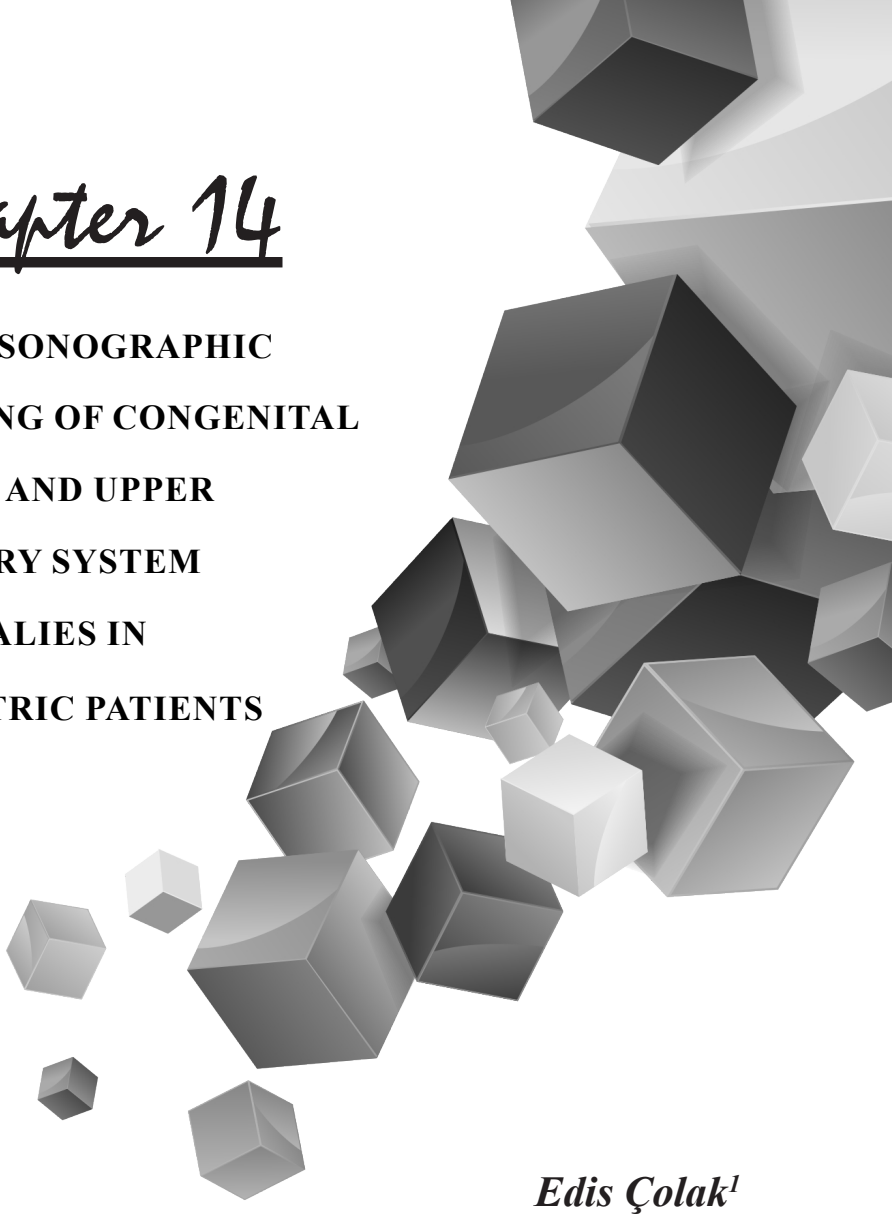
Chapter 14

ULTRASONOGRAPHIC IMAGING OF CONGENITAL RENAL AND UPPER URINARY SYSTEM ANOMALIES IN PEDIATRIC PATIENTS



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Introduction

Congenital renal and upper urinary system anomalies (CRUUSA) occur in 10% of the population with an overall incidence of 1 in 200 births. CRUUSA represent approximately 30% of all prenatally detected congenital abnormalities and about 40% of all renal diseases(Zhang, Wang, Sun, Jia, & Shen, 2011). Although CRUUSA are usually asymptomatic and are found incidentally on ultrasonographic (US) imaging, they can be associated with other renal conditions such as nephrolithiasis, infection, hypertension, and renal failure. As nearly two-thirds of children with end-stage renal disease had an underlying CRUUSA, ultrasonography during pregnancy and the child's first year of life is crucial for early diagnosis and management of complications(Seikaly, Ho, Emmett, Fine, & Tejani, 2003). US is widely accepted first-line imaging modality recommended for evaluation of the renal and urinary system in pediatric patients, due to its lack of radiation, non-invasiveness, repeatability, low cost, and easy availability. Radiologists play an important role in the diagnosis and characterization of CRUUSA. Good knowledge of the variable US presentations of CRUUSA helps in detecting complications at an early stage to prevent their progression.

This paper describes the US imaging features of renal and upper urinary system anomalies in infants and children.

Ultrasonography technique

Ultrasonographic (US) imaging of kidney and upper urinary system in pediatric patients is performed in a quiet environment with a high-resolution ultrasound machine equipped with a 5–9 MHz sector, convex, and linear probes(Paliwalla & Park, 2014). A 3.5 MHz convex probe is commonly used in older children. The patients are usually examined in the supine and lateral decubitus positions with arms extended above the head. Prone and oblique positions may be a reasonable choice for obese patients. An agitated child can be examined in the arms of a parent. Sedation is almost never necessary.

The kidneys and upper urinary system are scanned in both the transverse and coronal planes, ensuring that the whole kidney together with the proximal ureter is evaluated.

Classification of CRUUSA

Based on embryological development, CRUUSA can be classified into four categories: anomalies in the renal parenchyma, anomalies of renal position, anomalies of renal fusion, and anomalies of the upper urinary system.

Anomalies in the renal parenchyma

Anomalies in renal parenchyma include renal agenesis, renal hypoplasia, supernumerary kidney, and multicystic dysplastic kidney.

Persistent fetal lobulation, a hypertrophied column of Bertini, junctional parenchymal defect, and dromedary hump are considered as normal anatomical variations of the renal parenchyma.

Renal agenesis

Unilateral renal agenesis affects approximately 1 in 1000 newborns and is defined by a complete absence of one kidney(Paliwalla & Park, 2014). It is usually accompanied by the ipsilateral agenesis of the ureter and ureteric orifice in the bladder. The left kidney is commonly affected. Compensatory hypertrophy of the contralateral kidney usually maintains normal renal function. The prognosis of unilateral agenesis depends on the existence of contralateral hypoplasia, dysplasia, urethral dilatation, contralateral nephrolithiasis, and infection, or associated anomalies of the reproductive system(Ramanathan et al., 2016). Renal agenesis may also be associated with rarer genetic syndromes such as Fraser syndrome and VACTERL syndrome(Cascio, Paran, & Puri, 1999). Ultrasonographic images of the unilateral renal agenesis, as shown in Figure 1, confirm the absence of one kidney with compensatory growth of the contralateral kidney. A presence of a hypoechoic psoas muscle is a very important landmark for renal agenesis on US.

Bilateral renal agenesis occurs in only one out of 5000 births with a male: female ratio of 2: 1 to 3: 1. This anomaly is part of the oligohydramnios syndrome, pulmonary hypoplasia, limb, and face anomalies (classic Potter's syndrome). It is fatal within minutes or hours after birth(Ramanathan et al., 2016).

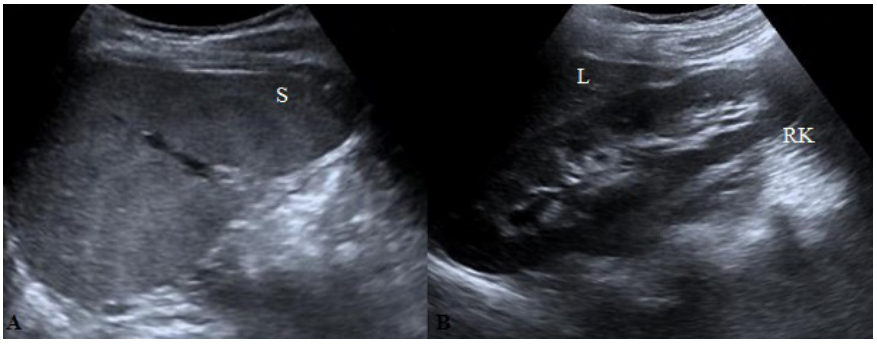

Figure 1. Renal agenesis. Longitudinal views of the renal ultrasonography show (A) absent left kidney and (B) compensatory hypertrophy of the right kidney. S - spleen. L – liver. RK – right kidney.

Renal hypoplasia

Renal hypoplasia is defined as an abnormally small kidney with a reduced number of pyramids, papillae, and calyces. The overall incidence is 1 in 400 births(Ramanathan et al., 2016). The kidney is histologically normal but has a lesser number of nephrons. Renal function may be normal or mildly reduced. While global hypoplasia has a good prognosis, especially when the contralateral kidney compensates the reduced function; segmental hypoplasia may cause hypertension that rarely requires surgical removal.

A representative ultrasound image of renal hypoplasia is shown in Figure 2.

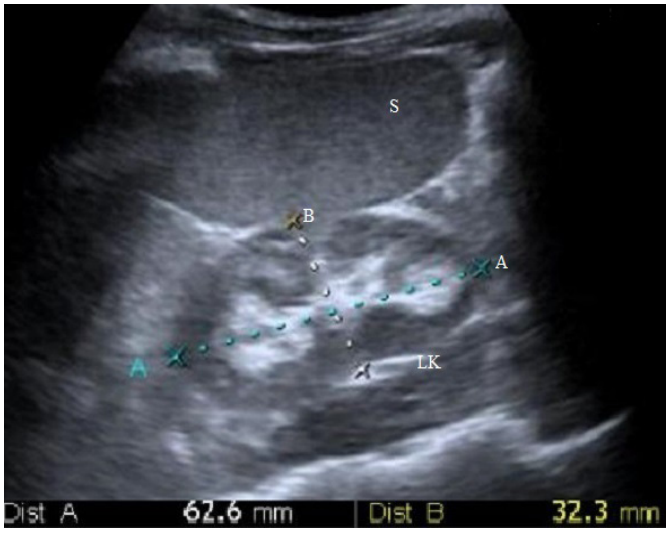

Figure 2. Renal hypoplasia. Longitudinal sonogram shows small dimensions of the left kidney with a normal parenchymal thickness. A- longitudinal dimension. B-transverse dimension. S - spleen. LK - left kidney.

Supernumerary kidney

The supernumerary kidney is a very rare anomaly with approximately less than 100 cases reported to date(Oto, Kerimoglu, Eskicorapci, Hazirolan, & Tekgul, 2002). The supernumerary kidney is defined as the presence of an accessory kidney in addition to two kidneys. It may be fused to the other kidneys and associated with other urogenital anomalies such as ureteral atresia, ureteral duplication, urethral atresia, and vaginal atresia[4].

This anomaly is usually asymptomatic and does not require treatment unless accompanied by infection, hydronephrosis, and nephrolithiasis.

Multicystic dysplastic kidney (MCDK)

MCDK affects approximately 1 in 4300 pregnancies and is characterized by the existence of multiple, non-communicating cysts of different sizes (1–9 cm). A thin, echogenic, and minimal to no functional renal parenchyma may be seen around the edges of the cysts(Schreuder, Westland, & van Wijk, 2009). MCDK is the most common cause of a cystic mass in the fetal abdomen. An association with hypertension and malignancy has been reported in subjects with MCDK. Prognosis of this anomaly depends on the function of the contralateral kidney, and coexistence of additional anomalies such as ureteropelvic junction obstruction or ureteric stenosis.

Figure 3 shows the ultrasonographic appearance of MCDK presented with multiple small-sized cysts accompanied by a thin echogenic renal cortex. Bilateral MCDK is incompatible with life (Ramanathan et al., 2016).

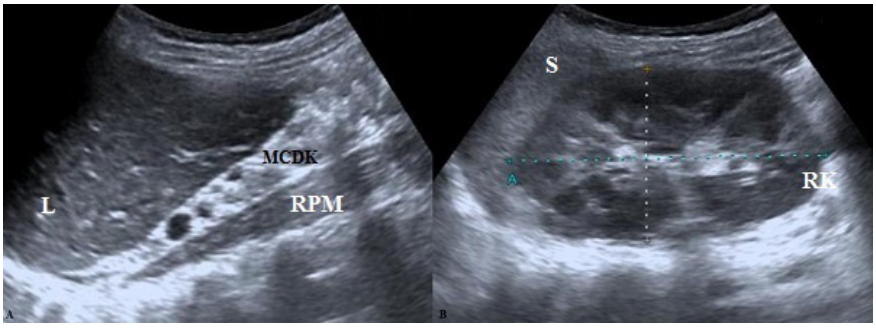

Figure 3. Multicystic dysplastic kidney. Longitudinal views of the renal ultrasonography show (A) small-sized cysts in the right renal fossa and (B) compensatory hypertrophy of the left kidney. MCDK- multicystic dysplastic kidney. L – liver. RPM- right psoas muscle. S - spleen. RK – right kidney.

Persistent fetal lobulation

Persistent fetal lobulation is a normal variation of the kidney detected in 4% of children and 10% of the adult population(Bhatt, MacLennan, & Dogra, 2007). It occurs due to the failure of the normal fusion of the renal lobules.

As shown in Figure 4 fetal lobulations may mimic focal cortical defects, however the indentations from fetal lobulation are more distinctive and occur between the normal medullae of the kidney.

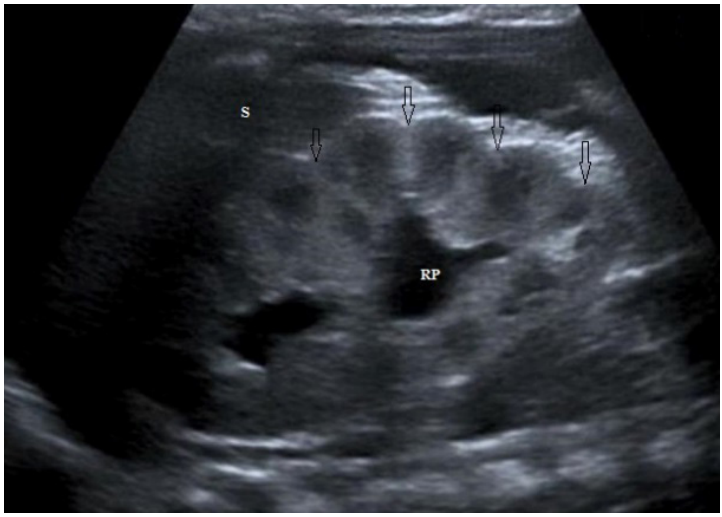

Figure 4. Persistent fetal lobulation. Longitudinal sonogram of the left kidney shows **distinctive** *indentations of the fetal lobulations (black arrows). The renal pelvis is dilated. RP- renal pelvis. S – spleen.*

Hypertrophied column of Bertini

The hypertrophied column of Bertini is considered as a normal variation, commonly seen as a rounded or triangle formation with sharp edges at the middle third of the kidney(Algin, Ozmen, & Gumus, 2014). A column of Bertini may extend from the medullary pyramids to the renal sinus and may divide the kidney into two parts. This variation may be mistaken for a renal mass[8].

However, US imaging can identify the hypertrophied column of Bertini, by the presence of the renal pyramids and vascular structures similar to the vascularity of renal parenchyma.

Junctional parenchymal defect

The junctional parenchymal defect is a common variant of the kidney that occurs as a result of an incomplete embryonic fusion of two parenchymatous parts called ranunculi(Ramanathan et al., 2016). It can be easily recognized with US as a hyperechoic triangular region due to the extension of sinus fat into the cortex. It can be differentiated from the focal cortical defect by its usual location in the upper pole and interpolar region of the kidney.

Dromedary hump

Dromedary hump is considered as a normal variation, seen as a bulge on the lateral surface of the middle third of the left renal cortex. It is

caused by the pressure of the spleen to the kidney during embryological development(Bhatt et al., 2007). The presence of the renal calyces and echogenicity identical to renal parenchyma can help to distinguish Dromedary hump from the renal tumor.

Anomalies of renal position

Renal ectopia

Renal ectopia, an abnormally located kidney, occurs when the kidney is found out of the normal anatomical position. About 60% of ectopic kidneys are located in the pelvis. Rarely it may be seen in the abdomen and thorax(Barakat & Drougas, 1991). An ectopic kidney can be "simple" when it is on the same side of the expected position, or "cross-ectopic" when it is contralaterally located. The overall incidence of the simple ectopic kidney is 1 per 3000 births(Barakat & Drougas, 1991). The cross-ectopic kidney is rare and occurs only in one out of 7000 newborns(Kakitsubata, Kakitsubata, Watanabe, Natsuda, & Miyanaga, 1993). Renal ectopia increases the incidence of ureteropelvic obstruction, vesicoureteral reflux, and nephrolithiasis (Guarino et al., 2004). Prognosis of this anomaly depends on the coexistence of additional genitourinary, cardiovascular, respiratory, and skeletal systems malformations (Guarino et al., 2004).

The ultrasound image of the simple ectopic kidney is shown in Figure 5 and is characterized by the presence of a pelvic malrotated kidney located on the same side of the expected position.

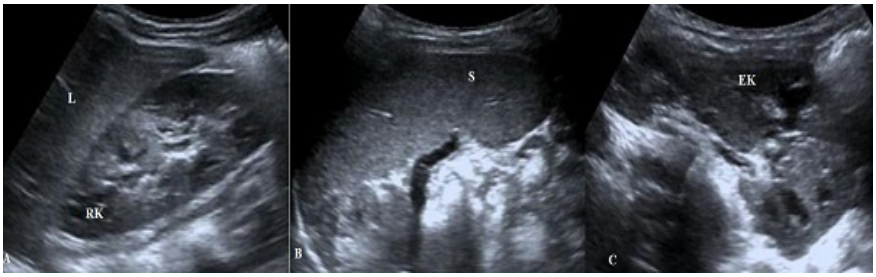

Figure 5. Simple ectopic kidney. (A) Longitudinal sonogram shows a normal appearance of the right kidney. (B) Longitudinal sonogram shows empty left renal fossa. (C) Transverse sonogram shows ectopic kidney in the left iliac fossa. L – liver. RK – right kidney. S – spleen. EK-ectopic kidney.

The ultrasound image of the cross-ectopic kidney is shown in Figure 6 and is characterized by the presence of a small, lobulated, and malrotated kidney. The pelvic kidney is located on the opposite side and is not fused with the orthotopic kidney.

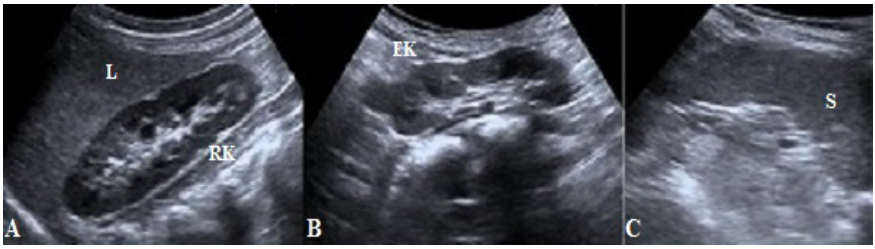

Figure 6. Cross-ectopic kidney. (A) Longitudinal sonogram shows a normal appearance of the right kidney. (B) Transverse sonogram shows small ectopic kidney in the right iliac fossa. (C) Longitudinal sonogram shows empty left renal fossa. L – liver. RK – right kidney. EK-ectopic kidney. S – spleen.

Anomalies of renal fusion

Anomalies of renal fusion include horseshoe kidney and crossed fused renal ectopia.

Horseshoe kidney

The horseshoe kidney (Ren arcuatus) is the most common form of renal fusion seen in approximately one in every 500 newborns. It is twice more frequent in males than in females. In 90% of cases, lower poles of the kidneys are fused by an isthmus of fibrous or normal renal tissue. In most of the cases, the isthmus lies at the level of the L4 vertebra, just below the origin of the inferior mesenteric artery, in 20% of cases, the isthmus is located in the pelvis; and in the remaining cases, it is placed at the level of the lower poles of the kidneys(Fazio, Razvi, & Chin, 2003). The fusion of the upper renal poles is rare. The ureters pass medially and anteriorly over this isthmus.

The horseshoe kidney increases the incidence of hydronephrosis, nephrolithiasis, infection, and renal malignancy. Horseshoe kidney is often associated with abnormalities of the central nervous, cardiovascular, gastrointestinal, and skeletal system(Dyer, Chen, & Zagoria, 2004).

The ultrasound image of the horseshoe kidney is shown in Figure 7 and is characterized by the fusion of the lower poles of the kidneys by an isthmus.

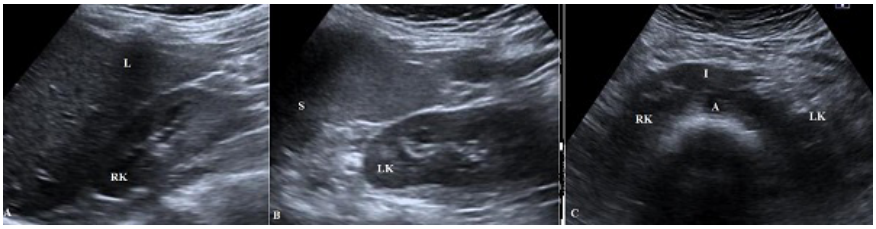

Figure 7. Horseshoe kidney. (A) Longitudinal sonogram shows a poor visualization of the inferior pole of the right kidney. (B) Longitudinal sonogram shows a poor visualization of the inferior pole of the left kidney. (C) Transverse sonogram shows fusion of the lower poles of the kidneys by isthmus. L – liver. RK – right kidney. S – spleen. LK – left kidney.I – isthmus. A– aorta.

Crossed fused renal ectopia

Crossed fused renal ectopia is the second most common fusion anomaly of the kidney and occurs when the ectopic kidney is displaced across the midline and fused inferiorly to the orthotopic kidney. Nearly 85% of the cross ectopic kidneys are fused(Goodman, Norton, Carr, & Yeh, 1986). Left-to-right ectopia is more commonly seen.

Six subtypes of crossed fused renal ectopia are defined according to Mc Donald and Mc Clellan(Goodman et al., 1986). In decreasing order of incidence, they are:

A- Inferior crossed fused kidney: the upper pole of the ectopic kidney is fused inferiorly to the orthotopic kidney (most common type).

B- S-shaped or sigmoid kidney: the superior pole of the ectopic kidney is fused to the inferior pole of the orthotopic kidney, and the renal sinus of the ectopic kidney is oriented laterally.

C- Unilateral lump kidney: fusion of the kidneys occurs over a wide margin and is located more inferiorly.

D- L-Shaped kidney: ectopic kidney lies inferiorly and horizontally fusing with the lower pole of the orthotopic kidney.

E- Unilateral disc kidney: the fusion occurs along the medial borders of the kidneys.

F- Superior crossed fused kidney: the lower pole of the ectopic kidney is fused with the upper pole of the orthotopic kidney.

The ureter of the ectopic kidney passes the midline and inserts into the bladder on the opposite side. The predominant urological problems include vesicoureteral reflux, ureteropelvic obstruction, infection, and nephrolithiasis. Genitourinary, cardiovascular, and skeletal system abnormalities may be associated(Boyan, Kubat, & Uzum, 2007).

Anomalies of the upper urinary system

Anomalies of the upper urinary system include ureteropelvic junction obstruction, duplex collecting system, hydrocalyx, congenital megacalyces, and calyceal diverticulum.

Ureteropelvic junction obstruction

Ureteropelvic junction (UPJ) obstruction is the most common cause of neonatal hydronephrosis. It is defined as a narrowing of the UPJ of the kidney, preventing the passage of urine to the urinary bladder(Liang et al., 2002). The UPJ obstruction may be caused by congenital stenosis, absence of peristalsis at the junction, abnormal insertion of the ureter, or compression by an inferior polar accessory artery. It is seen most often in boys (65%) and more frequently affects the left kidney (60%). It can be bilateral in 25 to 30% in newborns. Its prevalence is estimated at 1/500 births(Ismaili & Piepsz, 2013). Prognosis depends on the function of the contralateral kidney and coexistence of pyelonephritis or nephrolithiasis. Bilateral chronic UPJ obstruction leads to renal failure.

The typical ultrasound appearance of UPJ obstruction is shown in Figure 8 and is characterized by the abnormally dilated renal pelvis, mild dilatation of the calyces, and normal ureter.

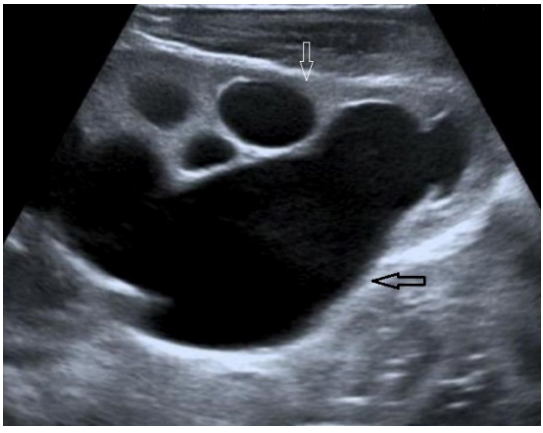

Figure 8. Ureteropelvic junction obstruction. Longitudinal sonogram shows dilated renal pelvis (black arrow) and cystic dilatation of the calyces (white arrow).

Duplex collecting system

A duplex collecting system is a very common anomaly with an incidence of 1 in 100 live births(Fernbach, Zawin, & Lebowitz, 1995). It is twice more frequent in females. The left and right sides are equally affected. This anomaly occurs due to the incomplete fusion of the superior and inferior renal moieties. The renal cortex of the superior moiety may appear echogenic with one or several small cysts.

According to the degree of fusion, a duplex collecting system may be classified as(Fernbach, Feinstein, Spencer, & Lindstrom, 1997):

bifid renal pelvis: Figure 9 demonstrates two separate renal sinus fat echoes. It is a normal variation.

incomplete Y-shaped ureteral duplication: the two ureters fuse anywhere along their course.

incomplete inverted Y-shaped ureteral duplication: the two ureteral orifices drain from a single kidney.

incomplete V-shaped ureteral duplication: the two ureters fuse near or in the bladder wall.

incomplete blind-ending ureteral duplication: one of the ureters is not connected to the bladder or the kidney.

complete ureteral duplication: the two ureters drain separately into the bladder.

According to the Weigert-Meyer rule, in the complete ureteral duplication (duplex kidney), the upper moiety ureter is usually obstructed due to the ectopic drainage or ureterocele. The lower renal moiety is commonly dilated as a result of vesicoureteral reflux(Inamoto, Tanaka, Takemura, & Ikoma, 1983).

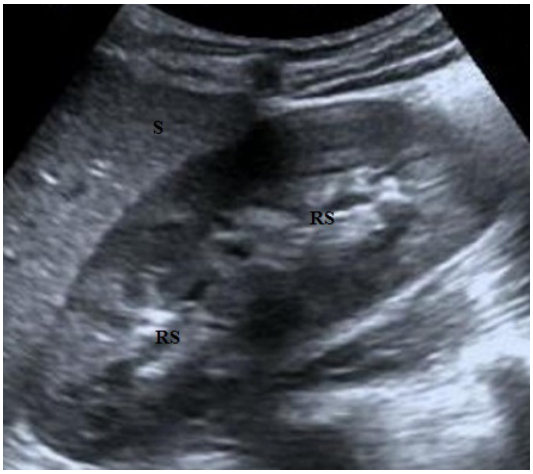

Figure 9. Bifid renal pelvis. Longitudinal sonogram of the left kidney shows two separate renal sinus echoes. *RP- renal pelvis. S – spleen. RS – renal sinus.*

Hydrocalyx

A hydrocalyx is a dilated calyx due to an obstructed calyceal infundibulum. The obstruction may be congenital or acquired. Fraley

syndrome is a condition where the infundibulum of the superior calyx is obstructed by the crossing renal artery branch(Sungur, Caliskan, & Lokman, 2018). It is usually asymptomatic and does not require treatment unless accompanied by infection or calculi.

Congenital megacalyces

Congenital megacalyces are defined as an abnormal dilatation of the renal calyces. It occurs due to the hypoplasia of the renal pyramids with resultant enlargement of the calyces. It may be mistaken for hydronephrosis(Kozakewich & Lebowitz, 1974). However, US imaging can identify the congenital megacalyces by the presence of the normal renal pelvis without evidence of obstruction. It increases the incidence of infection and nephrolithiasis. A congenital megaureter may be associated(Pieretti-Vanmarcke, Pieretti, & Pieretti, 2009).

Calyceal diverticulum

A calyceal diverticulum is a cystic dilatation of the calyx that occurs due to the eventration of the renal calyx into the renal cortex. It communicates with the collecting system which provides passage of urine in the calyceal diverticulum(Auge, Munver, Kourambas, Newman, & Preminger, 2002). Urine stasis increases the incidence of recurrent urinary tract infection and stone formation. Approximately 50% of patients with calyceal diverticulum have nephrolithiasis. It can be differentiated from the simple renal cyst by its contrast material filling on intravenous urography or computed tomography urogram.

Conclusion

Congenital renal and upper urinary system anomalies include a wide range of conditions varying from normal anatomical variations to conditions that seriously impair the renal function or are incompatible with life. Due to the widespread use of prenatal and postnatal ultrasonography, the incidences of incidentally found congenital renal anomalies have increased during the past years. Early prediction of complications and appropriate management are crucial for preventing progressive and irreversible renal failure. Therefore, the radiologists take the essential role in identifying the renal congenital anomalies on imaging, and the initial diagnosis is an integral part of a comprehensive multidisciplinary approach.

Keywords: Congenital anomalies, kidney, ultrasonography, upper urinary system

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Chapter 15

LINGUALIZED OCCLUSION MODALITY IN PROSTHETIC TREATMENT

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Definiton of Lingualized Occlusion

Lingualized occlusion which is a non-anatomic occlusion represents a technique to achieve esthetic and functionally successful articulation in mandibular maxillar jaw relations (Phoenix RD, & Engelmeier RL, 2010.). The occlusion concept is described in the glossary of prosthodontic terms as ‘ this form of denture occlusion articulates the maxillary lingual cusps with the mandibular occlusal surfaces in centric occlusion, working and nonworking mandibular positions.’ (Ferro, KJ et al,) The benefits of the lingualized occlusion concepts were recognized by a number of researchers (Parr, GR, Loft GH, 1982, Jacobson, T. E., & Krol, A. J.,1983, Engelmeier, R. L., & Phoenix, R. D. ,2019). These benefits can be drawn as following (Engelmeier, R. L., & Phoenix, R, 2019):

✓ The concept is in accordance with the basis of neutrocentric occlusion.

✓ Technique of adjusment teeth is simple

✓ Esthetic is good

✓ Bolus can be penetrated

✓ Lateral forces can be directed toward residual alveolar ridges

✓ Stability is good even in parafunction cases

✓ The concept can be prefered in Class II, Class III cases and cross-bite jaw relationships.

However, the lingualized occlusal concept have disadvantages should be considered in some clinic conditions (Williamson et al.,2004, Becker CM, Swoope CC & Guckes AD., 1977):

✓ Nonanatomic teeth used on maxillar denture can cause unaesthetic appearance and patient can be unpleaseant when talking and smiling.

✓ Porcelain-porcelain occluding teeth pairs can cause the clicking sound during functional movements and patient can feel uncomfortable.

In the early 20th century, the lingualized occlusion concept was defined by Alfred Gysi. He informed that normal resorption pattern of edentulous jaws caused posterior cross-bites for the 60% of edentulous patients (Gysi, A. 1927). Constructing a well balanced occlusion with anatomic teeth for such patients is difficult. So, he patented and manufactured "cross bite posterior teeth" (Alfred, G.,1928) (Figure 1). Each mandibular tooth has a shallow depression that is in a harmony with linear cusp of each maxillary tooth. These teeth were can easily be arranged to prevent dislodging mastication forces. Similarly, Alfred Lüthy, improved non-anatomic teeth design (Alfred L.,) (Figure 2). Felix

French modified posterior teeth to adjust occlusion with free to movement in all mandibular positions (French FA., 1954). These nonanatomic teeth have some advantages and disadvantages.

The main advantages of nonanatomic teeth design (Lang BR & Kelsey CC., 1972, Engelmeier, R. L. ,2019.) :

1. Nonanatomic teeth can be more easily arranged than anatomic teeth. Nonanatomic teeth allow mandible movements without any interference.

2. Nonanatomic teeth direct occlusal force vertically to the supporting tissues. Horizontal occlusal forces considered more destructive can be decreased with nonanatomic teeth without inclined occlusal morphologies.

3. Non-anatomical teeth requires less precision in recording the centric relationship. Because the teeth can be arranged to close in various position.

4. Nonanatomic teeth are convenient to adapt maxilla and mandibular relationship in Class II and Class III cases.

5. Non-anatomical teeth can adapt to changes in vertical and horizontal jaw relations that occur as a result of reduction and remodelling process in residual bone tissue. It is possible to easily adapt to such changes with occlusal adjustments.

6. Rebasing and relining can be more easliy achieved with nonantomic teeth.

7. Nonanatomic teeth provide centralization of occlusal plane in accordance with the residual bone tissue. So, denture stability can be increased.

The main disadvantages of nonanatomic teeth design (Lang, BR., & Kelsey, CC.,1972, Engelmeier, R L., 2019.):

1. Chewing ability of nonanatomical teeth is less efficient than the chewing ability of anatomic teeth.

2. Bolus can cover the occlusal surfaces of the nonanatomical teeth because of inadequate escape cannot be achieved during chewing. Therefore, the decrease in chewing efficiency may cause an increase in chewing pressure.

3. Nonanatomical teeth are not as aesthetic as anatomical teeth.

4. Nonanatomic teeth can cause horizontal forces during lateral or diagonal chewing movements.

5. Patients can be negatively effected in terms of food penetrability of nonanatomic occlusal designs.

6. When nonanatomical teeth used on a curved ridges, the balance of denture can be jeopardized.

7. If nonanatomical teeth are not in accordance with jaw movements during chewing, denture bases can not stay stable and excessive friction can occur.

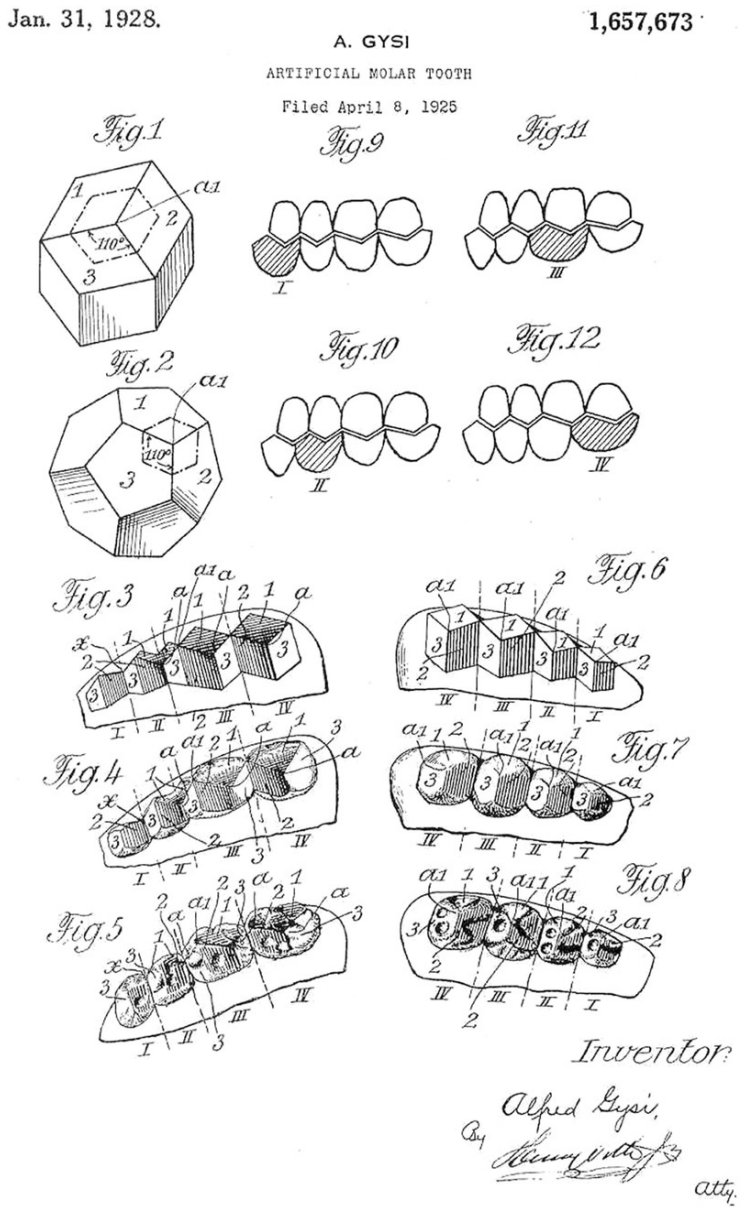

Figure 1. Cross-bite Posterior Teeth was designed by Gysi and manufactured by the Dentist's Supply Company of New York.

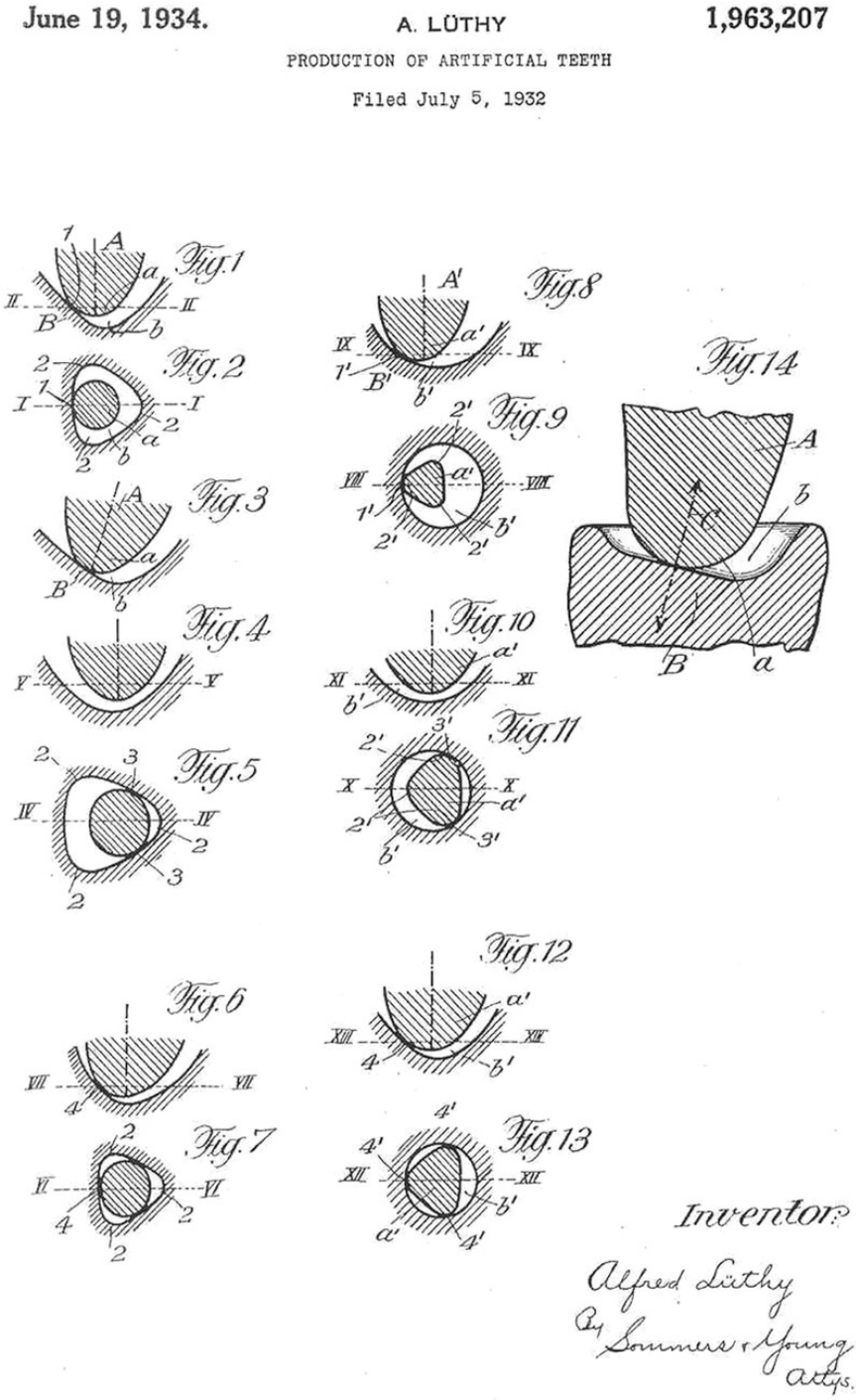

Figure 2. Teeth with mortar and pestle occlusal were designed and patented by Alfred Lüthy received in 1934.

Although marketing of these special designed teeth for the lingualized occlusion in 1930s, the biomechanical feature of the occlusion was widely described by S. Howard Payne, prosthodontist, U.S.A. in 1941 (Payne SH., 1941). As Payne described technique and reported his researches, the lingualized occlusion concept became popular (Engelmeier, R. L., & Phoenix, R, 2019). Considering Payne's article, modifying of 30-degree teeth was used to adjust a mortar-and-pestle regulation. During eccentric mandibular movements, the maxillary posterior palatinal cusps and the mandibular posterior teeth remain in contact. Conversely, there are no any contact between the buccal cusps of maxillary posterior teeth and the opposing mandibular teeth. Payne stated that these arrangements could maintain balance of denture via reducing the effect of lateral chewing forces. Additionally, the concept could support remained hard and soft tissues by directing chewing forces to center of the mandibular residual bone (Payne SH., 1941., Payne SH., 1955).

Within the past years, a variety of anatomical and non-anatomical teeth designs were offered for the lingualized occlusion (Lang, B. R., & Razzoog, M. E., 1983a, Lang, B. R., & Razzoog, M. E., 1983b). Mostly, maxillary posterior teeth which have cusp angles of ≥30 degrees and cusp angles of mandibular posterior teeth which are of ≤20 degrees are used together. Other popular combination is the usage of maxillary posterior teeth with 30- to 33-degree cusp and mandibular posterior teeth with 0-degree cusp (Phoenix RD, & Engelmeier RL, 2010.).

The lingualized occlusal concept is compatible with the technical basis of complete denture planning (Engelmeier, R. L., & Phoenix, R. D. ,2019). The lingual surfaces of mandibular molar teeth are placed between slightly divergent two lines. These two lines begin from the mesial surface of the mandibular canines, one of the lines goes to the lingual side of the retromoler ridge and the other to the buccal side. So, the occlusal surfaces of the mandibular posterior teeth are placed between these two lines. Thus, the placement of the mandibular teeth simulates basic teeth position. Because, mandibular molar teeth place in a passive area where the pressures of the tongue and cheek neutralize each other (Parr, GR &Loft GH., 1982). Also, the arrangement of mandibular posterior teeth supports control over on the position of maxillary posterior teeth. The maxillary posterior teeth are placed towards the buccal side of the alveolar ridge which have tough bone structure. These teeth arrangement have a positive effect the stability of mandibular dentures (Ohguri, T., Kawano, F., Ichikawa, T., & Matsumoto, N., 1999). The lingualized occlusion concept can be applied in following endications (Kimoto, S. et al., 2006, Curtis TA., et al., 1988.) :

- ✓ For patients with high esthetic demands,

- ✓ For patients who have severe resorbed alveolar ridges,
- ✓ For Class II and Class III maxillo-mandibular relations,
- ✓ For higher intermaxillary relations,
- ✓ For decreasing horizontal forces owing to chewing and parafunctional movements.

For improving the stabilization of denture in cases which 80 ° or less angle the between occlusal plane and ridges,

- ✓ For improving the retantion of dentrues in cases which conventional complete maxilla denture occluding with implant retained removable mandibular denture.

There are some important technical points to arrange lingualized occlusion. Firstly, the articulator is set. Horizontal condylar guidance is arranged according to maxilla and mandibula relation records. The guidance settings should not exceed 5 degrees. Considering Hanau's formula (L = H/8 + 12), lateral condylar guidance is adjusted (Javid NS & Porter MR., 1975., Phoenix, R. D., & Engelmeier, R. L. 2010). Incisal guidance is established as less 20 degrees from the horizontal guidance records. For each side, the lateral components of incisal guidance is arranged at 5 degrees. Secondly, maxillary posterior teeth with 30-33 degree cusp and mandibular posterior teeth which have 0-degree cusp or swallow occlusal morphology are selected (Kelly, E. 1977., Murrell, G.A., 1974.) . In cases with severe residual ridge resorption, the occlusal plate should be narrowed (Levin, B., 1977. Murrell, G.A., 1974.). Secondly, selective grinding is performed on the mandibular posterior teeth. Thus, the edge ridges are lowered and the occlusal plane with a slight concavity can be prepared (Kelly, E., 1977., Becker, C.M., Svvoope, C.C., Guckes, A.D., 1977 ., Murrell, G.A., 1974.). Thirdly, the buccal cusps of mandibular posterior teeth should not be in contact with the maxillary posterior teeth in centric occlusion. The palatinal cusps of maxillary posterior teeth should be in contact with the mandibular posterior teeth in centric occlusion (Murrell, G.A., 1974.). The palatinal cusps of maxillary posterior teeth should articulate with the occlusal surfaces of posterior mandibular teeth in working, and balancing sides. Maxilla posterior teeth can be slightly rotated in the buccal direction to avoid clashing of buccal tubercles on the working side (Gronas, D.G., Stout, C.J., 1974.). Thus, the mandibular denture can be prevented from lateral dislodging. Finally, antero-posterior interferences can be reduced in order to provide better balance in protrusive jaw movement.

In the present days, the lingualized occlusion concept has been well understood. The concept can be prefered for complete dentures and

implant retained dentures. Various researches presented clinical results of the lingualized occlusion concept.

The Lingualized Occlusion Concept and Complete Dentures

With the loss of natural teeth, oral functions are physiologically impaired. Edentulous patients can not properly perform oral functions such as chewing, smiling and speaking. This unpleasent situation can endanger the individual's self-confidence and disrupt social life (Schierz, O., & Reissmann, D., 2016.). Therefore, prosthetic treatment is quite important in edentulous cases.

In recent times, implant-retained treatments are widely prefered. Although the successful results of implant treatments, conventional complete dentures continue to be a popular treatment option. Because, complete dentures can be applied as a convenient treatment for patients who have severe systemic diseases and patients can not received implant treatment owing to economical reasons (Boven GC, Raghoebar GM, Vissink A, Meijer HJ.,2015.).

The movements of complete dentures are different natural teeth under occlusal forces. Because, chewing forces can be transmitted to the whole denture from occlusal surface of an artificial single tooth (Bhambhani, R., Joshi, S., Roy, S. S., & Shinghvi, A., 2020.). The stability and retention of complete dentures are supported by remained tissues. Therefore, stabilization and retention can be considered as main factors for succesful treatments.

The occlusion arranged for complete dentures has a impact on the masticatory functions and stability of complete dentures (Raghavan, R., Shajahan, P. A., & Purushothaman, P.,2020.). Three different occlusion concepts which are canine guided occlusion, bilateral balanced occlusion and lingualizde occlusion can be constructed in complete dentures (Maddula, RT., Ariga, P., & Jain, AR., 2018.) . The lingualized occlusion concept is primarily suggested to improve stabilization of complete dentures. There are various clinical studies evaluate the impact of occlusion type on chewing efficiency of complete dentures.

In a previous clinical research, the effect of lingualized occlusion and bilateral balanced occlusion comparatively was evaluted (Kimoto, S., et al., 2006.). There were no difference between the chewing efficiencies of two occlusion concepts. However, the retention and patient satisfaction results of complete denture with lingualized occlusion were found as better than the results of complete denture with bilateral balanced occlusion. Clough et al. compared the lingualized occlusion and monoplane occlusion in their clinical research. They stated that 67% of the patients preferred lingualized

occlusion due to satisfied chewing efficiency of the concept (Clough, HE., et al., 1983.). Patients expectations with their complete denture is crucial to enhance oral health-related quality of life. Former clinical studies evaluted the impact of various factors on satisfaction of patient who use complete denture (Yamaga, E., Sato, Y., & Minakuchi, S., 2013., Deniz DA & Kulak Ozkan Y.,2013., Bajoria AA, Saldanha S, Shenoy VK., 2012., Gaspar MG. et al., 2013.). Chewing efficiency, speech,phonetic and esthetic are main factors related with patients satisfaction with their complete denture. So, the clinically stable and well functional mandibular denture is stated as an outstanding determinant factor for the patient's satisfaction (Alfadda, S. A. ,2014). Similarly, studies evaluating patients' satisfaction with their complete dentures stated that 85.9% of patients' dissatisfaction was related to the deficiency of denture retention (Bilhan, H. et al., 2012, Bilhan, H. et al., 2013.). Especially, the stability of mandibular denture and occlusal arrangement for determining denture retention are key factors to improve patient' satisfaction (Peroz I., et al., 2003., Zhao K., et al., 2013.) So, higher satisfaction results with lingualized occlusion than those for bilaterally balanced occlusion can be attributed to the improved chewing muscle activity (Singh, S., Mishra, S. K., & Chowdhary, R., 2019). Because, high voluntary contraction can obtained in anterior temporalis and masseter muscles via lingualized occlusion concept. Improved muscle activity can decreased chewing time in complete dentures with lingualised occlusion (Deniz DA & Kulak Ozkan Y.,2013). The form of lingualized occlusion is more able to cut and chew food compared to bilateral balanced occlusion design (Kimoto S et al., 2006). A number of investigations have proposed different clinical results of lingualized occlusal scheme constructed in complete dentures. The absence of dislodging chewing forces between nonanatomic mandibular posterior teeth and occluding maxillary posterior teeth can be advantage of lingualized occlusion for complete dentures (Dawson PE., 2006). This occlusal arrangement between occluding posterior teeth increase chewing ability, stability and comfort of complete denture. In a former study, chewing efficiency and ability of cutting food was defined in higher for lingualized occlusion compared with bilateral balanced occlusion (Heydecke G. et al., 2008). On the other hand, these results are not in accordance with another research. According the findings of the research, there were no difference between chewing ability of the lingualized occlusion and bilateral balanced occlusion (Wiens JP& Priebe JW., 2014). The masticatory efficiency of various occlusal arrangement for complete dentures searched in various studies (Zarb GA, Bolender CL, & Carlsson GE., 1997., Shirani M, Mosharraf R & Shirany M., 2014.) , and it conlcuded that chewing efficiency is effected mainly remained ridge (Paleari AG.,2012, Shirani M, Mosharraf R & Shirany M., 2014.). Also, the occlusal arrangement of complete denture for severe resorbed

ridges is important. Because, lever balance and controlling of chewing forces can be managed by occlusal scheme (Heydecke G. et al., 2007). Lingualized occlusion can be adapted to different ridge types preventing lateral interferences and cuspal interferences (Clough HE. et al., 1983., Kawai Y. et al., 2017) .

The chewing ability of lingualized occlusion and bilateral balanced occlusion have been comparatively assessed. The general results of the researches in a novel review claimed that the chewing efficiency of lingualized occlusion was superior than bilateral balanced occlusion and canine guided occlusion (Maddula, R. T., Ariga, P., & Jain, A. R., 2018). In a randomized clinical trial, chewing performances of two different occlusal designs: lingualized occlusion and balanced occlusion were comparatively evaluated. Chewing performance and mandibular movements were examined at 3 months and 6 months. According to the results of the study, the participants with balanced occlusion indicated impairment in the chewing performance. Also, it was observed that a significant decrease in stability of the dentures with balanced occlusion. The researchers stated that lingualized occlusion indicated better chewing performance for participants with severe resorbed alveolar ridge than balanced occlusion (Matsumaru Y., 2010.).

It can be concluded that the lingualized occlusion concept is beneficial modality for complete dentures. Especially, lingualized occlusion can be a convenient solution for cases which are difficult to ensure stability and chewing performance of complete denture.

The Lingualized Occlusion Concept and Implant Retained Overdentures

Implant retained overdenture has become a popular modelity for edentulous patients. The retention and stability of overdentures can be strengthened by means with implants. Therefore, implant-supported overdenture can be meet to patient's expectations and oral functional rehabilitation rather than complete dentures (Walter M, Marre´ B & Eckelt U., 2000.).

Occlusion of overdenture is regarded as a important factor for clinical success. Because, excessive occlusal forces was considered as a contributing factor for overdenture failure and decreasing of marginal bone surrounding implant (Abd El-Dayem, et al., 2016.). Also, mechanical complications on overdentures such as fracture of denture, screw loosening or fracture and implant fracture/or failure can be results of occlusal overload (Schwarz MS, 2000). In a previous study, it was emphasized the chewing efficiency was directly related with the occlusal anatomy of teeth (Khamis MM.,

1988). Three differenct occlusal designs (lingualized occlusion, 30-degree teeth, and zero-degree teeth) were found effective on the chewing cycles and masticatory efficiency of implant supported mandibular overdentures. Occlusal arrangment of implant supported overdenture carefully examined to avoid destructive forces.

Lingualized occlusion concept were introduced for using in complete dentures. But, it can direct masticatory forces vertically to the alveolar crest (Reitz, J. V.,1994). So, the concept can also be applied in implant supported mandibular overdentures (Aarts, J. M., Payne, A. G., & Thomson, W. M., 2008). In a study, the lingualized occlusion and bilateral balanced occlusion were comparatively evaluated for implant supported overdentures. After six moths follow-up, it was indicated that masticatory efficiency in lingualized occlusion is higher than bilateral balanced occlusion (Fayad, M., et al., 2016).

Wismeijer et al suggested that lingualized occlusion should be considered when planning conventional maxillary dentures opposed to implant supported mandibular overdenture (Wismeijer D, et al., 1995). The suggestion is based that the implant to be protected from horizontal forces and chewing forces should be directed to vertically implants.

Conclusion

The lingualized concept is not a new introduced technique for prosthetic treatments. But, it is still prefered for both conventional complete denture and implant supported dentures thanks to its biomechanical and esthetic advantages. The lingualized occlusal concept can provide for freedom in centric relationship and, also during mandibular movements. Additionally, the chewing forces can be diverted vertically to residual alveolar bone. As a result, the lingualized concept will be continued to applied and searched in clinical researches.

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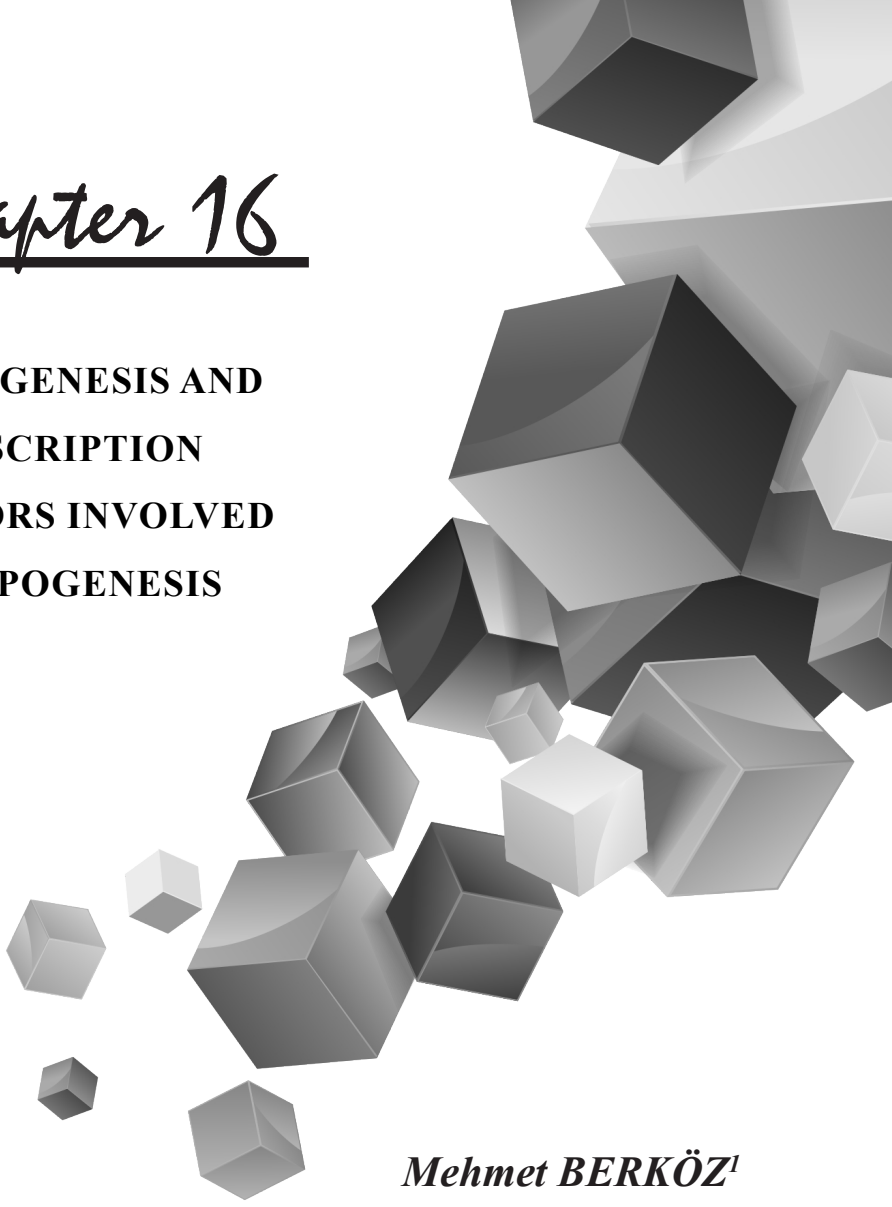
Chapter 16

ADIPOGENESIS AND TRANSCRIPTION FACTORS INVOLVED IN ADIPOGENESIS



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Overview

Obesity is an independent risk factor for non-insulin-dependent diabetes mellitus (NIDDM), hypertension and coronary artery disease, and is therefore a major contributor to morbidity and mortality. It results from a breakdown in energy balance, leading to increased lipogenesis. Adipose tissue serves as a major site in the body for energy metabolism and homeostasis, where triglycerides are stored during periods of excess nutritional intake and mobilized in the form of free fatty acids during periods of nutritional deprivation. Adipocytes are the fat storing cells found in adipose tissue which respond to external stimuli that control the balance between lipolysis and lipogenesis. In order to combat obesity, it is important to understand the steps that initiate and control preadipocyte growth and differentiation. Adipogenesis is regulated by the expression of transcription factors which initiate and maintain the adipogenic gene program by transactivating differentiation-specific genes. Although the regulation of preadipocyte differentiation has been extensively studied, many of the mechanisms that control the adipogenic program and the conversion of mesenchymal stem cells into committed preadipocytes are yet unknown. There are many known proteins expressed by adipocytes which are differentiation specific and thereby can be used as markers of the mature fat cell phenotype, i.e. glucose transporter-4 (GLUT4), glycerol phosphate dehydrogenase, fatty acid binding protein (aP2), adipsin, acetyl CoA carboxylase. There are also several transcription factors that have been identified which play a role during various phases of the differentiation process. The two major families of transcription factors are the CCAAT enhancer binding proteins (C/EBPs) and the peroxisome proliferator-activated receptors (PPARs). In addition to transcriptional control of the adipogenic gene program, differentiation of preadipocytes is accompanied by dramatic morphological changes, where well spread fibroblastic cells round up into large adipocytes laden with fat droplets. This active alteration in morphology is not simply due to an accumulation of lipid droplets, but appears to be a part of the differentiation program.

Differentiation is achieved by the responsiveness of committed preadipocytes to external stimuli, i.e. growth factors, cytokines and the extracellular matrix (ECM), whose signals are transduced by membrane receptors to intracellular signaling pathways that converge on specific genes involved in growth, differentiation and cytoskeletal remodeling. This dynamic process results in a terminally differentiated fat cell whose intracellular and extracellular environments have been dramatically altered.

Obesity and Non-Insulin-Dependent Diabetes Mellitus (NIDDM)

Until recently, obesity was considered a common disease of western society, but now it has become a concern among many cultures as a result of modem lifestyles worldwide fast food, sedentary lifestyle, lack of exercise. In addition, there is a genetic component of this disease. Obesity is a leading risk factor for the development of NIDDM, or Type 2 diabetes, which affects approximately sixteen million people in the United States. Reports from the Diabetes Research Working Group describe NIDDM as approaching epidemic proportions. Although NIDDM was formerly referred to as adult onset diabetes, affecting adults over the age of 40, it is now increasingly common in children, especially among minorities. Type 2 diabetes encompasses defects in both insulin action and secretion, where the body fails to respond to circulating glucose and the insulin-producing pancreatic beta cells try to overcompensate by overproducing insulin. As the disease progresses, however, the beta cells eventually fail to respond to glucose and are unable to produce sufficient insulin. Hyperglycemia is a major contributor to the microvascular complications associated with diabetes that result in retinopathy, nephropathy and neuropathy, which can lead to eventual blindness, kidney failure and amputation, respectively. Obesity contributes to insulin resistance; thus it exacerbates this disease.

As fat tissue develops, it normally becomes increasingly responsive to insulin. The chronic obese state, however, is often accompanied by a loss of this responsiveness, which has been classically attributed to the down-regulation of insulin receptors. Basic research has revealed the possibility that intracellular responses to insulin are also likely candidates in rendering a state of insulin resistance. For instance, down-regulation and abnormal phosphorylation of intracellular molecules like insulin receptor substrate-1 (IRS-1) may be critical. To this end, recent studies have demonstrated that a constitutively active subunit of phosphatidylinositol 3-kinase (PI3-kinase), the p110 catalytic subunit, results in serine/threonine phosphorylation of IRS-1 leading to decreased tyrosyl phosphorylation of this molecule in response to insulin (Egawa *et al*., 1999). In fact, lesions can occur at several different levels, including receptors (i.e. insulin and leptin), increased or decreased secretion of proteins from fat cells (i.e. TNFα and leptin), down-regulation of key transcription factors (i.e. C/EBPα and PPARγ), or altered states of phosphorylation of intermediate signaling molecules and transcription factors. The severity of NIDDM is attributed to its effect on virtually every tissue in the body with long-term complications. This leads to personal debilitation of the individual and enormous health care costs. The treatment of NIDDM is primarily centered around treatment of the complications resulting from the disease and changes in personal lifestyle. While NIDDM cannot be abolished among genetically susceptible

individuals, its onset can be delayed and its severity can be reduced by proper management of body weight. It is estimated that over 97 million Americans are overweight of whom 22% are obese. Obesity itself is a complicated disease with a genetic predisposition. Besides being a risk factor for NIDDM, obesity is a significant health problem worldwide. Accordingly, there is a quest to understand the normal pathways and factors leading to the production of fat cells and the storage of triglycerides in order to identify molecules which might become targets of therapeutic agents to combat obesity.

Differentiation of Preadipocytes in Culture

Most of the knowledge regarding the mechanisms that regulate growth and differentiation during adipogenesis has been acquired by employing cells in culture. This has been greatly aided by the availability of established cell lines that mimic adipogenesis *in vitro*. As is the case with nearly all *in vitro* model systems, however, cells differentiated *in vitro* never acquire the mature phenotype compared to in vivo models. In part this is due to the lack of three dimensional structure, matrix components, mitogens and cytokines in the external environment, and the absence of other cell types that compose tissues in the body. Similarly, adipocytes *in vitro* display lowered insulin-responsive glucose uptake as compared to in vivo models. Nevertheless, two particular cell lines that have been extensively used are the 3T3-L1 and 3T3-F442A mouse preadipocytes which were originally selected from disaggregated 17- to 19-day Swiss mouse embryos for their ability to accumulate cytoplasmic triglycerides (Fujimoto *et al*., 2005; Jayarathne *et al*., 2019). The most compelling evidence indicating that these cells do in fact represent good models for *in vitro* adipogenesis was demonstrated by the subcutaneous injection of 3T3-F442A cells into nude mice at an anatomical site lacking adipose tissue. Within five weeks, mature fat pads developed at the site of implantation. These were histologically indistinguishable from normal adipose tissue and were not malignant (Bi and Kuang, 2015).

Although the 3T3 cell lines have undergone commitment to the adipocyte lineage, exposure to external inducers is required to initiate differentiation. The variable dependence of each cell line on exogenous inducers is attributed to the fact that they are arrested at different stages of development. In the case of 3T3-L1 preadipocytes, differentiation is induced by exposure of a post-confluent monolayer of cells to dexamethasone (DEX), isobutylmethylxanthine (MIX), insulin and 10% fetal bovine serum (FBS). Differentiation of F442A preadipocytes, however, is achieved by exposure to only insulin and FBS, or growth hormone under serum-free conditions. Due to their minimal requirement for external inducers, the 3T3-F442A cells are proposed to be more developmentally advanced;

thus, it is presumed that they were isolated at a later stage during the initial clonal selection. Nevertheless, both cell lines have been shown to faithfully mimic adipocytes in vivo by displaying similar characteristics with regard to their gene expression and metabolic properties. Biochemical analysis of these adipocytes has demonstrated that the accumulation of cytoplasmic triglycerides is closely related to the coordinate expression of enzymes required for fatty acid and triglycerol synthesis. In addition, electron micrograph studies have revealed that mature 3T3 adipocytes possess the ultrastructural features of adipocytes *in situ* (Kuri-Harcuch *et al*., 2019).

In addition to the 3T3-L1 and 3T3-F442A cell lines, other cells have been shown to undergo adipogenesis as a result of exposure to chemical manipulations. Embryonic stem cells derived from mouse totipotent blastocysts differentiate into adipocytes upon treatment with retinoic acid (Xu *et al*., 2018). Treatment of multipotent mouse embryo 10T1/2 cells with the demethylating agent 5'-azacytidine also gives rise to adipocytes (Shin and Ajuwon, 2018). From these cells, the TA1 cell line was established, which differentiates into fat cells in response to 10% FBS, insulin and DEX (Chapman, 1984). The OB17 cell line and its derivatives were isolated from adipose precursors in epididymal fat pads of genetically obese adult ob/ob mice (mice lacking leptin). These cells undergo adipogenesis in response to 8% FBS, insulin and T3 (triiodothyronine) (Koenig, 2005). All of the established preadipocyte cell lines serve as an important tool to address fundamental questions regarding specific stages of differentiation.

Recently, serum-free culture conditions have been developed using the 3T3-L1 and 3T3-F442A cells, other preadipocyte cell lines (i.e. OB1771 and 1276 cells) and stromal preadipocytes in order to assess the requirements for external inducers which are permissive for differentiation using chemically defined medium (Boccellino and D'Angelo, 2020). While the effect of certain mitogens varies in serum-free versus serum-containing medium, under both conditions the external inducers insulin or IGF-1, glucocorticoids and cyclic AMP (cAMP) are considered necessary for the induction of differentiation. In addition, recent investigations suggest that long chain fatty acids and peroxisome proliferators also function as inducers of differentiation for certain preadipocyte cell lines (Chun *et al*., 2019). Both long chain fatty acids and peroxisome proliferators are activators/ligands of members of the PPAR transcription factor family. One member of this family, PPARγ, is implicated as a key regulator of adipogenesis. Although these PPAR activators and ligands cannot stimulate full differentiation alone, they appear to act synergistically with other external inducers to initiate differentiation. It follows that there are at least four second messenger signaling pathways that are known to play a role during adipogenesis. These include the IGF-1-activated tyrosine

kinase pathway, which leads to the activation of PI3-kinase/Akt and the Ras-Raf-MEK-Erk pathways the glucocorticoid pathway; the cAMP-dependent protein kinase pathway, which activates cAMP-dependent protein kinase A (PKA), among other proteins; and the fatty acid-activated receptor pathway (Petersen *et al*., 2008).

Stages of Preadipocyte Differentiation

Differentiation of 3T3-L1 preadipocytes can be divided into three stages: early, middle and terminal. Cells entering the early stage are already committed to the adipogenic lineage from a mesenchymal stem cell. Experiments using demethylating agents have demonstrated that treatment of the 10T1/2 mesenchymal cell line with 5'-azacytidine leads to the development of preadipocytes, myoblasts and chondroblasts, suggesting that hypomethylation of genomic DNA may activate regulatory genes that trigger the commitment of these precursor stem cells into several lineages (Shin and Ajuwon, 2018). To date, however, identification of these regulatory genes has been unsuccessful, although two recently identified basic helix-loop-helix transcription factors are potential candidates: twist and scleraxis. Twist is expressed in early somites. During somite compartmentalization, its expression is restricted to the sclerotome and excluded from the myotome. A role for this transcription factor in mesodermal cell fate has been suggested. In fact, ectopic expression of M-twist (the mouse homolog of twist) seems to antagonize muscle formation by interfering with MyoD DNA binding and by inhibiting transactivation by myocyte enhancer factor-2 (Izumi *et al*., 2018). In addition, a mesodermal tripotential cell line derived from teratocarcinoma cells (C1 clone) that expresses M-twist can be induced to become adipoblasts in the presence of insulin and dexamethasone (Jie *et al*., 2009; Shin and Ajuwon, 2018).

Once committed to the adipocyte lineage, a prerequisite of differentiation is growth arrest. The conventional means of arresting cell growth is by contact inhibition of cells grown to high density. The 3T3-F442A preadipocytes have also been shown to differentiate following growth arrest by suspension in a viscous methylcellulose-containing medium. Two to four days following cessation of growth, the cells are presumed to be in G_0, and are induced to differentiate. As mentioned previously, 3T3-L1 cells are induced to differentiate by exposure to the "adipogenic cocktail" consisting of DEX, MIX and insulin (DMI). Immediately following exposure to the adipogenic inducers, this growth arrested, quiescent population of cells re-enters the cell cycle and undergoes at least one round of cell division. This event has been coined "clonal expansion". It is generally accepted that serum activation of confluent cells (Balb/c 3T3 fibroblasts) can induce DNA synthesis but not cell division. It is probable that clonal expansion is unique to signaling

events induced by insulin since it results in a doubling of the population. It is not known, however, whether any potent mitogen can elicit the same response. This notion that mitosis can occur in a quiescent monolayer of cells is intriguing. Although it is not clear why clonal expansion occurs, it appears to be a prerequisite for differentiation. In fact, under conditions where clonal expansion does not occur, or occurs minimally, i.e. in the presence of DEX and MIX, the cells do not differentiate to the same extent. It is possible that DNA replication in these cells alters the accessibility of promoter elements to transactivating factors that control differentiation by functioning as positive or negative regulators. Following clonal expansion, preadipocytes enter a quiescent phase referred to as the G_D state of growth arrest. This second state of quiescence is permissive for differentiation (Pekala and Moss, 1983).

Differentiation of preadipocytes seems to require the activation of specific signaling pathways which in turn activate or inhibit regulatory proteins by altering their state of phosphorylation. These intermediate molecules may modulate early differentiation. To this end, two pathways which may play an important role during differentiation are the PI3-kinase and mitogen-activated protein (MAP) kinase pathways (Dupont *et al*., 2003).

During the early stage of adipogenesis that coincides with clonal expansion (0 - 24 hours), differentiating preadipocytes express markers including lipoprotein lipase, adipocyte determination and differentiation factor 1 (ADD1), and the mouse equivalent of the human cc2 chain of Type VI collagen. In addition, C/EBPβ and C/EBPδ are expressed in response to MIX and DEX, respectively, and retinoid X receptor a and y (RXRα and RXRγ) are induced (Baillie *et al*., 1998).

As clonal expansion ceases, two important transcription factors are expressed, PPARγ and C/EBPα, followed by the coordinate activation of adipose-specific genes including aP2, glycerol phosphate dehydrogenase, adipsin, GLUT4 and stearoyl-CoA desaturase 1. In addition, the cells lose their fibroblastic morphology due to a downregulation of cytoskeletal proteins, actin, tubulin and vimentin, and they begin to accumulate small cytoplasmic lipid droplets. This is the mid-stage of differentiation. During this phase, accumulation of adipose-specific genes and proteins continues. Furthermore, mRNA levels of some genes are progressively suppressed (i.e. C/EBPδ) (Hyun *et al*., 2010). Although the distinction between mid-stage and terminal differentiation seems subtle, it has been reported that during the early and middle stages of differentiation, adipocytes can dedifferentiate, re-enter the cell cycle and undergo mitosis. This is achieved by disruptinalphag cell-cell contact or exposing the cells to inhibitors of differentiation like tumor necrosis factor alpha (TNFα) or retinoic acid

(Nagahara *et al*., 2016). During terminal differentiation, however, mature adipocytes lose the ability to dedifferentiate. Studies performed in Balb/c 3T3 cells demonstrate that once differentiation is past a particular point, the cells are incapable of dedifferentiating and become committed to a state of terminal differentiation. During this late stage of differentiation, the cells express abundant amounts of insulin-responsive GLUT4 protein, adipsin and aP2. In addition, the cytoplasmic triglyceride droplets coalesce and become unilocular, delineating a typical signet ring appearance (Wu *et al*., 1998; Baillie *et al*., 1998; .

Despite the fact that up to a particular stage, adipocytes can dedifferentiate and become fibroblastic, there seems to be a difference between undifferentiated and dedifferentiated fibroblasts. Recently Xing *et al*. (1997) showed that a preadipocyte marker which is down-regulated during differentiation, Pref-1, is not re-acquired by dedifferentiated preadipocytes. Pref-1, preadipocyte factor-1, is a transmembrane protein consisting of EGF repeats. The expression of Pref-1 is restricted to preadipocytes and is dramatically down-regulated to undetectable levels during their differentiation. In fact, cells that do not undergo differentiation in response to the adipogenic inducers express high levels of Pref-1. This implicates Pref-1 as a critical regulatory protein in determining the permissive state of cells to differentiate. Furthermore, ectopic expression of this protein in 3T3-L1 preadipocytes inhibits their differentiation. In fact, it is the ectodomain of this protein that is considered to be anti-adipogenic. Pref-1 is cleaved at two sites in the extracellular domain, yielding a dominant large 50 kDa and a smaller 31 kDa set of soluble proteins. The 50 kDa protein contains an EGF repeat and is phosphorylated by A-kinase. The observation that Pref-l cannot be re-acquired by dedifferentiated cells indicates that dedifferentiated preadipocytes and undifferentiated preadipocytes possess unique properties (Sul *et al*., 1998; Wang *et al*., 2006; Hudak and Sul, 2013).

Adipogenic Transcription Factors

CCAAT Enhancer Binding Proteins (C/EBPs)

The C/EBP family of transcription factors consists of three major members, C/EBPα, C/EBPβ and C/EBPδ, which exhibit similar DNA-binding affinities and readily form homo- and heterodimers through a conserved leucine zipper domain. A flanking basic region facilitates binding of the dimers to regulatory elements in the promoters and/or enhancers of many genes expressed by adipose tissue, as well as in other metabolically active tissue such as liver. Despite sharing sequence homology and having related DNA-binding properties, the various C/EBPs control distinctive sets of target genes. This has been demonstrated in

different tissues where C/EBPα and C/EBPβ are shown to be reciprocally expressed. Under normal circumstances, tissues such as liver, fat and gut express C/EBPα which presumably regulates the expression of certain tissue-specific genes. During acute phase response, upon exposure to cytokines, however, C/EBPα is down-regulated and C/EBPβ is highly expressed resulting in the down-regulation of C/EBPα-responsive genes and activation of acute-phase genes. Similarly, in fat, treatment of mature adipocytes with TNFα results in a down-regulation of C/EBPα, with a concomitant down-regulation of fat-specific genes and the induction of C/EBPβ (Guo *et al*., 2015; Moseti *et al*., 2016; Mota de Sá *et al*., 2017).

It follows that the expression of each C/EBP transcription factor during differentiation occurs at a discrete time which is consistent with a specific role for each protein in controlling the sequential activation of the adipogenic gene program. Both C/EBPβ and C/EBPδ are induced within the first few hours following exposure of post-confluent 3T3-L1 preadipocytes to the adipogenic cocktail. Specifically, exposure of cells to MIX, which is an inhibitor of phosphodiesterase, results in increased cAMP levels and activates protein kinase A. PKA in turn activates cAMP-response element binding protein (CREB), which interacts with CRE binding sites in the C/EBPβ promoter, resulting in the induction of this early adipogenic transcription factor. DEX induces C/EBPδ expression via the glucocorticoid receptor. Studies have shown that these two early transcription factors cooperate with DEX to induce the expression of PPARγ. C/EBPα, on the other hand, is expressed between days 2 and 3 of differentiation (Moseti *et al*., 2016; Mota de Sá *et al*., 2017).

It has been shown that C/EBPα gene expression precedes the synthesis of several enzymes and proteins that are characteristic of mature adipocytes. In fact, the genes for many adipogenic proteins contain C/EBP regulatory elements within their promoters and enhancers. Definitive proof that C/EBPα is important during differentiation stems from studies performed *in vitro* and *in vivo* to "knockout" the expression of this transcription factor. The *in vitro* studies involved constitutive expression of anti-sense C/EBPα RNA in 3T3-L1 preadipocytes which blocks the expression of several adipogenic proteins, including aP2, stearoyl-CoA desaturase 1, GLUT4 and C/EBPα itself. The result is a lack of triglyceride accumulation in the cytoplasm. Other investigators have shown that homozygous knockout mice for the C/EBPα gene die within eight hours of birth due to hypoglycemia that may result from impaired expression of the genes encoding enzymes that control glucose metabolism in the liver (i.e. phosphoenolpyruvate carboxy kinase). In fact, administration of glucose to these mice enabled their survival for 40 hours; however, there was limited maturation of both white and brown adipose tissue in the knockout

mice compared to their normal litter mates. Further evidence supporting the importance of C/EBPα during differentiation is demonstrated by Lin *et al*. (1993) who show that conditional expression of C/EBPα in 3T3-L1 preadipocytes results in differentiation of these cells independent of exogenous inducers. Furthermore, Freytag *et al*. (1994) show that ectopic overexpression of this transcription factor in several fibroblastic cell lines induces adipogenesis. Accordingly, a great deal of evidence exists which implicates a critical role for C/EBPα during adipocyte differentiation (Moseti *et al*., 2016).

Three additional isoforms belonging to the C/EBP family have been identified; C/EBPγ, C/EBPε and CHOP10/GADD153/C/EBPτ. C/EBPγ, also known as Ig/EBP-1, binds a cis-regulatory element of the immunoglobulin gene enhancer. C/EBPε, or CRP-1, together with CEBPδ was identified by low-stringency probing using C/EBPα gene sequences. Not much is known about the biological function of the γ and ε isoforms and whether they are expressed in adipose tissue (Carlson *et al*., 1993; Batchvarova *et al*., 1995; Carrière *et al*., 2004).

CHOP10 (GADD153) (C/EBPτ) is a small nuclear protein that was cloned from a 3T3-L1 adipocyte library based on its ability to dimerize with C/EBPβ. Its shared cDNA homology with the growth arrest and DNA damage inducible gene 153 (GADD153) cloned from a Chinese ovary cell library showed that these are indeed the same proteins. In fact, CHOP10 is activated upon growth arrest and DNA damage. While CHOP10 is considered a member of the C/EBP family of transcription factors and shares their sequence homology, the basic region of this transcription factor has diverged significantly from the three classic C/EBP members in that proline and glycine amino acids in this region have replaced conserved residues that interact with conventional C/EBP regulatory elements. This difference in the basic region of CHOP10 is presumably responsible for its ability to inhibit the transactivation activity of the C/EBPs on promoters containing C/EBP sites. Therefore, CHOP10 can act as a dominant negative inhibitor of the C/EBPs. Further support of this notion is that forced expression of CHOP10 in 3T3-L1 cells inhibits adipogenesis possibly by interfering with the expression of C/EBPα. While CHOP10 expression is high in proliferating 3T3-L1 cells, its expression declines dramatically upon exposure of these cells to the adipogenic cocktail. Upon removal of the external inducers from the culture medium, however, it is transiently re-expressed, thereby indicating that it may play more than one role. In fact, a recent study demonstrates that CHOP10-C/EBPβ heterodimers can interact with a unique DNA binding site, thus supporting the notion that CHOP10 may also function as a positive regulator of transcription. There is no evidence, however, suggesting a positive role for CHOP10 during

adipogenesis. Instead, all the evidence during preadipocyte differentiation suggests that this transcription factor blocks differentiation by dimerizing with C/EBPα when it is overexpressed, thereby preventing C/EBPα from interacting with its target genes. It also appears to inhibit the induction of PPARγ by C/EBPβ and C/EBPδ heterodimers (Carlson *et al*., 1993; Batchvarova *et al*., 1995).

Peroxisome Proliferator-Activated Receptors (PPARs)

PPARs are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors. Three members of this family have been identified thus far, PPARα, PPARδ and PPARγ, which exhibit specific tissue distribution in adult animals and during development. These transcription factors bind DNA by forming heterodimeric complexes with retinoid X receptors. The resultant PPAR:RXR complex is unable to transactivate gene expression, however, in the absence of an appropriate ligand for each nuclear factor. In the case of RXR, its natural ligand is 9-cis-retinoic acid. The natural ligand for PPARs is yet unknown although several structurally diverse natural and synthetic compounds have been shown to activate these receptors either directly or indirectly by unknown mechanisms. Some of these ligands/activators are peroxisome proliferators, thiazoladinediones (TZDs), fatty acids, prostaglandin and leukotriene derivatives, and the endogenous adrenal steroid dehydroepiandrosterone (Lefterova *et al*., 2014; Thangavel *et al*., 2017).

PPARγ, which is abundant in white fat, was cloned and identified as a component of the adipogenic transcription factor ARF6 that binds the enhancer of the *aP2* gene. It is considered a key regulator of adipocyte differentiation, since its expression is one of the earliest events during adipogenesis *in vitro*. Evidence that this transcription factor may function as a master switch during adipogenesis stems from studies demonstrating that its ectopic expression in fibroblasts promotes adipogenic conversion. In fact, ligand-activated PPARγ regulates the expression of several adipocyte-specific genes, including phosphoenolpyruvate carboxy kinase, aP2 and leptin. Some of the ligands/activators of PPARγ are long chain fatty acids, prostaglandin J2 and TZDs. TZDs are a group of synthetic compounds that are able to induce adipogenesis in culture by functioning as ligands for PPARγ. These drugs are also insulin-sensitizers that can lower blood glucose levels and suppress leptin gene expression when administered to diabetic animals. In 1997, one TZD, troglitazone, was introduced into clinical trials in Japan, the UK and USA. The studies were suspended, however, following adverse effects on liver function. Recent investigations by Wiesenberg *et al*. (1998) demonstrate that a novel TZD, BRL49653, is able to transiently reduce blood glucose levels enhanced by pharmacological doses of dexamethasone. This results in a progressive

decline in body weight, consequently leading to overall improvement of insulin resistance by enhancing insulin action. These findings suggest a pivotal role for PPARγ and its ligands in controlling adipocyte development and glucose homeostasis (Tontonoz *et al*., 1995; Wiesenberg *et al*., 1998).

Adipocyte Determination and Differentiation Factor-1 (ADD1)/ Sterol Regulatory Element Binding Factor-1 (SREBP1)

ADD1 is a member of the basic helix-loop-helix-leucine zipper family of transcription factors that binds to two distinct DNA sequences. It has been implicated in both adipocyte differentiation as well as cholesterol metabolism. ADD1 is expressed at high levels in white fat, brown fat, and liver. Its mRNA level is elevated in committed preadipocytes compared to other fibroblasts and it is induced during differentiation of 3T3-L1 and 3T3-F442A cells. The human homologue of ADD1 was previously identified and cloned as sterol regulatory element binding factor-1 (SREBP1). Unlike other basic helix-loop-helix proteins, ADD1/ SREBP1 has dual DNA- binding specificity in that it interacts with high affinity to both an E-box motif (ABS: atCACGTGta) and a non-E-box sequence (ATCACCCCAC). The non-E-box sequence was previously identified as the sterol regulatory element-1 while the E-box element has been shown to correspond to a carbohydrate-responsive element. This unique dual DNA binding specificity of the ADD1 homodimer is controlled by a single tyrosine residue in the basic domain. Several genes including those involved in lipogenesis like fatty acid synthase and spot-14-synthase have been determined to contain a consensus ADD1-binding sequence in their promoters which can be transactivated in *in vitro* assays by ADD1. The promoter of the C/EBPα gene also contains an E-box element (CACGTG) that is similar to the ADD1 binding site. It is therefore possible that ADD1 is involved in C/EBPα transcription. Recent studies have strongly implicated ADD1 as a determination factor in adipocyte differentiation. In fact, expression of a dominant negative form of ADD1, which inhibits DNA binding of the wild type protein, interferes with the normal process of differentiation in 3T3-L1 cells. Furthermore, under conditions that are permissive for full differentiation, ectopic expression of ADD1 in NIH 3T3 fibroblasts has been shown to promote a low level of adipocyte differentiation and augment PPARγ transcriptional activity of an aP2 promoter/reporter construct. Therefore, one possible role for ADD1 is that it induces the expression of genes like fatty acid synthase, which in turn lead to the production of a natural ligand for PPARγ. Alternatively, it is possible that ADD1 induces the activation of a co-activator that is necessary for PPARγ transcription (Kim *et al*., 1995; Amemiya-Kudo *et al*., 2002; Hsu *et al*., 2007; Seo *et al*., 2009).

Effectors That Promote Adipocyte Differentiation and Inhibit Differentiation

Growth factors play an important role in cell proliferation and differentiation. Their signals are transduced intracellularly resulting in specific cues indicating whether cells should grow, differentiate, or apoptose. More recently, it has been shown that some of these intracellular signaling events are redundant in their function (Pritchard *et al*., 1995). For instance, Parrizas *et al.* (1995) demonstrate that while IGF-1 signaling via the PI3-kinase pathway has been regarded as a survival mechanism for cells, in fact signaling via p42/p44 MAP kinase by IGF-1 also seems to protect cells from apoptosis. Similarly, it has been tempting to characterize growth factors as mitogenic or differentiation factors, but we now know that this is far too simplistic a classification for any individual growth factor. It is already known that growth factors can have distinct functions in different tissues. The classic example is basic fibroblast growth factor (bFGF) which is thought of as being predominantly a mitogen; however, it induces differentiation of nerve cells (Moscatelli and Quarto, 1989).

The classification of growth factors in metabolically active tissue like fat is even more difficult. There has been ample debate in recent years with regard to signaling events that may promote or inhibit differentiation. Much of these deliberations are attributed to the fact that most studies regarding pathways downstream of insulin and IGF-1 have been performed in mature adipocytes. Undifferentiated preadipocytes and mature adipocytes are quite different and may have distinct responses to external stimuli. It follows that comparisons between an undifferentiated and terminally differentiated population cannot be made. Insulin and IGF-1 switch from being mitogenic to adipogenic, while bFGF is initially mitogenic and then becomes anti-adipogenic. The studies presented are an attempt to understand the signaling events that bring about the opposing effects of these growth factors and their resulting effect on adipogenic transcription factors (Moscatelli and Quarto, 1989; Dupont *et al*., 2003).

IGF-1 and pharmacological doses of insulin are potent inducers of differentiation. IGF-1 enhances the differentiation of 3T3-L1 preadipocytes in a dose-dependent manner under both serum-containing and serum-free conditions, and it stimulates differentiation of primary preadipocytes. Unlike insulin/IGF-1, other mitogens are generally considered inhibitors of adipogenesis, perhaps due to the downstream signaling events in response to their mitogenic effects. Basic FGF has been shown to have anti- adipogenic effects in several preadipocyte cell lines in the presence of serum, while it has no inhibitory effect under serum-free conditions. Insulin/IGF-1 and bFGF activate at least two signaling pathways: PI3-kinase and p42/p44 MAP kinase. An understanding of how these signaling

pathways regulate adipogenesis will be relevant to the goals of combating obesity because particular signaling molecules may serve as potential targets in the quest for discovering anti-obesity drugs (Li *et al*., 2000).

Insulin and Insulin-Like Growth Factor-1 (IGF-1)

The interaction of insulin with its cell surface receptor triggers both metabolic and mitogenic responses. Both insulin and IGF-1 have been extensively studied for their ability to promote differentiation of preadipocytes. In fact, at high concentrations, it is believed that insulin signals via the IGF-1 receptor, but at low doses when insulin signaling occurs only through the insulin receptor, adipogenic differentiation is inefficient. Despite the requirement of high doses of insulin to differentiate preadipocytes, it is unclear how the signaling pathways transduced by this effector regulate early adipogenic transcription factors (Le Marchand-Brustel, 1999; MacDougald and Lane 1995).

IGF-1 belongs to a family of growth factors named insulin-like growth factors due to their structural homology to insulin, their insulin-like metabolic effects and their mitogenic ability. Another member of this family is IGF-2, which has been shown to play a role during development, growth and cell survival. Elevated levels of IGF-2 have been reported in many human tumors in association with elevated levels of the type-I IGF receptor (IGFR). Normally, IGF-2 is cleared from the circulation by interacting with the IGF-II/mannose-6-phosphate receptor (Le Marchand-Brustel, 1999; MacDougald and Lane 1995).

IGF-1 is a 70 amino acid polypeptide that is synthesized by a variety of cells and acts in a paracrine and autocrine manner. It is also produced by the liver in response to growth hormone followed by release into the circulation. The circulating form of IGF-1 comprises the largest pool of IGFs in the body and functions as an endocrine hormone. Like insulin, IGF-1 is translated as a precursor polypeptide consisting of five domains: BCADE. The A and B domains resemble the corresponding A and B domains of insulin. The C domain resembles that of the proinsulin molecule. The D domain has no homology to insulin. The E domain found at the C terminal end consists of either 35 residues in the case of IGF-1a or 77 residues in the case of IGF-1b; nevertheless, this domain is removed from the final product, resulting in the same product formed from both IGF-1a and IGF-1b precursor polypeptides. Like insulin, IGF-1 is composed of one intrachain and two interchain disulfide bonds and it is postulated to fold into a similar configuration as insulin (Laron, 2001).

The insulin and type-I IGF receptors are both heterotetrameric proteins composed of two α and two β subunits linked by disulfide bonds. The α subunits contain the extracellular ligand binding domain, while the

β subunits contain the transmembrane and intracellular domains. It is the intracellular domains that possess intrinsic tyrosine kinase activity. Both the insulin receptor and the type I IGFR are similar in size, structure and amino acid sequence, with the greatest homology located in the tyrosine autophosphorylation domain. In addition, like most membrane-bound proteins, both receptors are glycosylated (MacDougald and Lane 1995; Laron, 2001).

IGF-1 can also act as a ligand for the type II IGFR, which is structurally different from the type-I receptor. This is a mannose-6-phosphate receptor composed of a 250 kDa monomer which does not have tyrosine kinase activity although it can be a substrate for membrane-associated tyrosine kinase phosphorylation. The type-II IGFR/mannose 6-phosphate receptor is found on both the cell surface and the luminal face of the trans golgi reticulum membrane. It plays an important role in directing lysosomal enzymes to lysosomes, as well as clearing IGF-2 from the circulation. In addition, it is postulated to be linked to guanine nucleotide binding proteins, inositol phosphate turnover and calcium influx. The dominant receptor in preadipocytes is the type-I IGFR, which declines during differentiation, while expression of the insulin receptor increases (Aguirre *et al*., 2016).

In addition to their role in metabolism and mitogenesis, both insulin and IGF-1 induce differentiation in certain cell types and inhibit apoptosis. In fact, the role of insulin/IGF-1 as a cell survival factor has been proposed to involve both the PI3-kinase and the Ras-Raf MAP kinase pathways, as well as perhaps the $p70^{s6kinase}$ pathway. The involvement of each of these pathways has been demonstrated by the exploitation of specific inhibitors of each. It is evident, therefore, that there is considerable overlap and reinforcement among the various signaling pathways (Bertrand *et al*., 2006; Timmer *et al*., 2018).

Basic Fibroblast Growth Factor (bFGF)

Basic FGF, like IGF-1, signals via a receptor tyrosine kinase, and like IGF-1 it has pleiotropic effects in different tissues. For instance, although bFGF is generally believed to be a potent mitogen, it also promotes differentiation of nerve cells. In addition, bFGF is involved in wound healing, tissue repair and hematopoiesis (Vaseenon *et al*., 2020).

bFGF, also known as FGF-2, is one of eighteen distinct members of the FGF family. Exogenous bFGF undergoes nuclear localization in a receptor-dependent manner, while endogenous bFGF translated from the conventional start site remains cytosolic. Members of the FGF family bind with varying potential to four FGF receptors (FGFR1-4) which are single transmembrane tyrosine kinase receptors. While FGFs bind with

high affinity to their respective receptors, some investigators argue that interaction of FGFs to low affinity heparan sulfate proteoglycans (HSPGs) is initially essential in aiding the interaction of FGF to its tyrosine kinase receptor and the ensuing biological response. Upon interacting with its receptor, bFGF stimulates receptor kinase autophosphorylation at several sites, followed by receptor association with putative substrates. The two most important phosphorylation sites on FGFRl with regard to biological activity are Tyr-653 and Tyr-654. Phosphorylation at these sites confers kinase activity resulting in subsequent phosphorylation of Shc, pp90, a Grb-2 associated phosphoprotein, *c-src*, phospholipase C-γ (PLC-γ), Raf-1 and the Erk kinases 1 and 2. It is binding of the Grb-2/SOS complex with phosphorylated Shc and pp90 that links FGFRs to the Ras signaling pathway, while *c-src* phosphorylates cortactin, a protein that binds filamentous actin, resulting in morphological changes of the cell (Mohammadi *et al*., 1996).

Basic FGF is initially made as a 155 amino acid precursor polypeptide and is proteolytically cleaved to produce a 146 amino acid protein consisting of two heparan binding sites, one near the amino terminus (residues 18-22) and one near the carboxy terminus (residues 107-110). Although bFGF can be secreted, the specific signal sequence and mechanism that directs its secretion are yet unknown. Nevertheless, it can be produced by a wide variety of cell types and, therefore, is ubiquitous throughout the body, being found in different tissues such as brain, retina, adrenal gland, kidney, bone and muscle. The wide distribution of bFGF throughout the organism might be attributed to its ability to bind heparan sulfate proteoglycans, which themselves are ubiquitous in the extracellular matrix surrounding many tissues. It follows that bFGF can be sequestered within the extracellular milieu of tissues whose cells might not produce or secrete it (Florkiewicz and Sommer, 1989).

The HSPGs consist of heparan sulfate side chains attached to a core protein. Several types of HSPGs exist on the cell surface, including syndecans 1-4 and glypican, a phosphatidylinositol-linked proteoglycan. The HSPGs are constitutively internalized and undergo varying intracellular fates. Glypicans are internalized and rapidly degraded with an approximate half-life of 30 minutes, resulting in a concomitant rapid degradation of bFGF; syndecans, on the other hand, are internalized and the heparan sulfate fragment of HSPG is cleaved, resulting in intact FGFR-bFGF-heparan sulfate complexes. This latter process clearly allows for a much longer half-life for the HSPG moiety, possibly resulting in protection of bound bFGF from proteolysis and, thereby, a prolonged effect of bFGF on the cell (Saksela and Rifkin, 1990).

Another mode of regulation of the biological activity of FGFs is by their interaction with their native receptors. FGFRs have different rates of internalization, and perhaps even different intracellular fates. It is postulated that the intracellular sorting of bFGF, based on the receptor to which it is bound, is important in the determination of the FGF-mediated cellular response (Vaseenon *et al*., 2020).

When bFGF binds to one of its tyrosine kinase receptors, several intracellular events are triggered. First, tyrosine kinase activity of the receptor is activated which leads to receptor autophosphorylation. Binding of the ligand also induces homodimerization and heterodimerization between the various receptor family members. This dimerization is necessary for receptor kinase activity in order for the various substrates of the FGFRs to be phosphorylated on tyrosine residues. In addition, receptor activation results in increased intracellular Ca^{2+} levels and phosphatidylinositol (PI) turnover. It is of interest that mutated FGFRs that are incapable of inducing an increase in PI turnover, elevating intracellular calcium levels, and phosphorylating PLC-γ, still retain the ability to phosphorylate other intracellular substrates resulting in cellular proliferation, indicating that FGF-induced mitogenesis is independent of PLC-γ activation and PI turnover (Akl *et al*., 2016).

The proliferative effect of bFGF is mediated through the Raf-1/MAP kinase pathway. Briefly, activated FGFRs serve as docking sites for two intracellular proteins, Grb2 and SOS. As mentioned previously, the Grb2/SOS complex interacts with Shc and pp90 and subsequently activates Ras protein by converting Ras-GDP to active Ras-GTP. Activated Ras in turn binds to and activates Raf-1, which in turn phosphorylates MAP kinase kinase (MEK). Activated MEK phosphorylates p42 and p44 (Erk 2 and 1, respectively) which are able to phosphorylate proteins on serine and threonine amino acid residues. Erk1 and Erk2 can also translocate to the nucleus where they phosphorylate c-Jun and c-Myc proto-oncogenes, resulting in increased transcriptional activity and a subsequent proliferative response of the cell (Buscà *et al*., 2016).

Signal Transduction Pathways That Influence Adipogenesis

Several signal transduction pathways can be activated by the effectors that regulate adipogenesis. The PI3-kinase pathway is implicated in insulin/IGF-1 signaling, yet it is unclear to what extent this pathway is necessary for the formation of adipose tissue. Many studies demonstrate that activation of PI3-kinase is essential for translocation of GLUT4-containing vesicles to the plasma membrane in fat and muscle tissue, enabling the sequestration of excess glucose from the extracellular milieu; however, its role in the expression and/or activation of adipogenic transcription

factors is thus far unknown. Equally unclear and controversial is the role of the MAP kinase pathway in regulating adipogenesis. While the MAP kinase pathway is generally associated with growth, in some cases it is important for differentiation. It is quite likely that adipogenesis in the body involves a continual process of activation and inhibition of differentiation and apoptosis to meet the ever changing needs of the organism. Thus extracellular factors that inhibit adipogenesis and/or induce apoptosis may also play a critical role in the homeostasis of adipose tissue. In this regard, it has been shown that several mitogens can inhibit differentiation of preadipocytes in culture. While the signaling pathways that are triggered by many of these effectors are known, their role in regulating the expression and/or activity of transcription factors during adipogenesis is poorly defined (Mora *et al*., 1995).

Phosphatidylinositol 3-Kinase (PI3-Kinase)

Following stimulation of the insulin and/or IGF-1 receptors by insulin or IGF-1, a key molecule recruited to the receptor is IRS-1. This is a 160 kDa protein consisting of 12 tyrosine residues. Six of these residues begin with the sequence motif YMXM and two begin with the motif YXXM. The p85 isoform of PI3-kinase becomes tightly associated with the phosphorylated YXXM motifs on IRS-1 via its SH2 domains and subsequently activates the p110 catalytic subunit. Activated p110 in turn phosphorylates phosphoinositides at the D-3 position of the inositol ring to generate phosphatidylinositol 3-monophosphate from phosphatidylinositol, phosphatidylinositol 3,4-bisphosphate from phosphatidylinositol-4- monophosphate, and phosphatidylinositol 3,4,5-triphosphate from phosphatidylinositol-4,5-bisphosphate. In addition, PI3-kinase phosphorylates proteins on serine/threonine residues. The specific biological role of each of these phosphatidylinositides is yet to be determined; nevertheless, PI3-kinase appears to be involved in many cellular processes including cell motility, the *ras* pathway, vesicle trafficking and secretion, apoptosis and growth. The availability of selective inhibitors of PI3-kinase provides a powerful tool to study the function of this enzyme in intact cells. The two most widely used inhibitors are Wortmannin, a fungal metabolite, and LY294002, a synthetic inhibitor similar in structure to Wortmannin. LY294002 functions as a competitive inhibitor of ATP binding on the p110 catalytic subunit of PI3-kinase; therefore, its binding is reversible, making it possible to wash out the inhibitor (Maeda *et al*., 2003; Liu *et al*. 2003; Gharbi *et al*., 2007).

Recent investigations suggest that PI3-kinase participates in mechanisms that control the differentiation and function of adipocytic cells. Inhibition of PI3-kinase by either Wortmannin or LY294002, or by expression of a dominant negative mutant of p85 prevents insulin-stimulated

glucose uptake in rat primary adipocytes and 3T3-L1 adipocytes and inhibits glycogen synthase and DNA synthesis. In fact, studies using SH2-containing 5' inositol phosphatase (SHIP) implicate phosphatidylinositol 3,4,5-triphosphate in regulating insulin-induced GLUT4 translocation, growth factor-induced cytoskeleton rearrangement and DNA synthesis. Furthermore, exposure of 3T3-L1 preadipocytes to Wortmannin prevents their differentiation into adipocytes (Gharbi *et al*., 2007).

Three downstream targets of the PI3-kinase pathway and potential mediators of insulin/IGF-1 action during preadipocyte differentiation are protein kinase B (PKB), $p70^{s6kinase}$ and glycogen synthase kinase-3 (GSK-3). PKB, also known as Akt and Rac, is a serine/threonine kinase with a pleckstrin homology domain. Akt is activated by PI3-kinase following the exposure of adipocytes to insulin/IGF-1 and is dependent on PI3-kinase enzymatic function for its activation (Egawa *et al*., 1999). In fact, activation of this kinase requires a dual mechanism involving binding of phosphatidylinositol 3,4-bisphosphate to its pleckstrin homology domain, as well as serine/threonine phosphorylation by one or more Akt kinases, which are presumably themselves stimulated by lipid products of PI3-kinase. There is increasing evidence that Akt functions downstream of PI3-kinase and is a likely mediator of many biological responses of effectors that activate PI3-kinase. Constitutively active forms of Akt produced by ectopically targeting the kinase to membranes has been shown to result in spontaneous differentiation of 3T3-L1 preadipocytes in an insulin- independent manner, and to stimulate glucose uptake by inducing translocation of GLUT4 to the plasma membrane. Akt has also been implicated in regulating leptin production in adipocytes. In spite of these important observations, however, little is known about the mechanisms by which Akt regulates adipogenesis. For instance, it is yet to be demonstrated whether this kinase translocates to the nucleus to enhance transcription of adipogenic genes by regulating the expression and/or activity of adipogenic transcription factors (Beg *et al*., 2017).

Akt may also enhance translation of particular proteins since it has recently been demonstrated to activate $p70^{s6kinase}$, an insulin-responsive enzyme that regulates the translation of mRNAs encoding proteins involved in the cell cycle by phosphorylating ribosomal protein S6. Constitutively active forms of Akt and membrane-targeted p110 subunit of PI3-kinase, which renders it constitutively active, both activate $P70^{S6kinase}$. Furthermore, this activation is sensitive to inhibitors of PI3-kinase (Klippel *et al*., 1996).

Other targets of PI3-kinase include 6-phosphofructo-2-kinase, GSK-3 and mammalian target of rapamycin (mTOR). GSK-3 was first discovered as a result of its ability to phosphorylate and inactivate glycogen synthase, the regulatory enzyme involved in glycogen synthesis in mammals. The

activity of GSK-3 is itself regulated by various effectors. In the case of insulin induction of glycogen synthesis, activation of PI3-kinase leads to the Akt-dependent phosphorylation of GSK-3, which inhibits its activity concomitant with the activation of protein phosphatase-1 (PP-1). Both these events are necessary for glycogen synthase activity. Although it is believed that GSK-3 is phosphorylated by Akt, the exact mechanism regulating its activity is unclear since constitutively active Akt and membrane targeted p110 inhibit the insulin effects on this enzyme. Nevertheless, it is quite apparent that GSK-3 modulates many other cellular processes that involve gene regulation, based on the diverse array of its other substrates which include transcription factors (c-Jun), the cAMP-response element binding protein CREB, and a translation factor (eIF2B). Recent studies have also shown that insulin causes the dephosphorylation of eIF2B at serine 540, the site that is phosphorylated by GSK-3 (Klippel *et al*., 1996; Hermida *et al*., 2017).

Mitogen Activated Protein Kinase (MAP Kinase)

MAP kinase signaling cascade is activated by tyrosine kinase receptors upon binding of their respective ligands. Stimulation of this signaling pathway involves recruitment of SH2-domain-containing proteins to phosphorylated tyrosine residues on the activated receptor, followed by protein interactions involving GTP-bound Ras with Raf which leads to the activation of the dual specificity MAP kinase kinase 1 (MEK1) and finally to the p42/p44 MAP kinases, Erk2 and 1, respectively. Activated MAP kinases translocate to the nucleus where they phosphorylate target transcription factors and thereby exert their effects on cell growth and differentiation. More recently, the notion of resident nuclear MAP kinases in some cell types has been intimated since phosphorylation of myelin basic protein used as a substrate was detected in nuclear extracts within 1 minute of insulin stimulation without MAP kinase translocation, suggesting the possible translocation of MEK itself (Pritchard *et al*., 1995).

The role of the p42/p44 MAP kinases during differentiation is unclear and seems to depend on their level and duration of activation, as well as the particular system in which they are studied. For instance, exposure of myoblasts to bFGF results in a low and prolonged activation of Erk1 and Erk2 that inhibits myogenesis; exposure to a low dose of IGF-1, on the other hand, results in acute activation of these kinases and promotes differentiation. The opposite is true in PC-12 pheochromocytoma cells in which long-term activation of MAP kinase by nerve growth factor induces differentiation, whereas short-term activation by epidermal growth factor (EGF) stimulates growth. The precise role of these MAP kinases during adipogenesis is yet unknown. It has been suggested that activation of MAP kinase at a particular stage of adipogenesis can prevent fat cell formation

by inhibiting PPARγ; however, studies by us and others demonstrate that the role of MAP kinases is complex: early activation in response to the adipogenic inducers stimulates adipogenesis, while its inhibitory action appears to occur later (Pritchard *et al*., 1995).

Morphological Remodeling

Tissue remodeling is a dynamic process that occurs during development, wound healing, and bone remodeling. It involves alterations in the extracellular matrix laid down by cells including its degradation by several families of extracellular proteases. These families of proteases include: serine proteinases (i.e. plasminogen-urokinase plasminogen activator system, leukocyte elastases), cystein proteinases (i.e. cathepsin D and L) and the zinc-dependent matrix metalloproteinases. The extensive alteration in cell morphology that occurs during adipogenesis involves a dramatic change in the expression of the adhesion apparatus. Most notably, there is a down-regulation of actin, tubulin, vimentin, vinculin and β integrin expression. In addition, there is extensive remodeling of the extracellular environment, whereby the production of certain extracellular matrix molecules (ECM) is down-regulated (i.e. fibronectin, collagen Type I), while others are enhanced (i.e. collagen Type VI, laminin) (Pranjol *et al*., 2015).

Matrix Metalloproteinases (MMPs)

Matrix metalloproteinases (MMPs) are a class of structurally similar zinc-dependent endopeptidases, of which there are more than 19 members. They function in the turnover of extracellular matrix molecules that constitute the pericellular environment, therefore, the precise regulation of their expression is critical to many normal and pathological processes. While all members of this family can degrade ECM components, recent evidence suggests that gelatinases may also degrade non-ECM molecules, such as myelin basic protein, interleukin-1β, and TNFα precursor. MMPs are secreted from cells as pro-enzymes into the extracellular milieu immediately following their synthesis. The pro-enzyme consists of a propeptide, a catalytic domain and a C-terminal domain. Latency of the proenzymes is attributed to the association of free cysteine residues of endogenous propeptides with the zinc molecule in the active site of the proenzyme (Klein and Bischoff, 2011). Disruption of this interaction by cleavage of the propeptide or disruption of the cysteine- zinc bond, activates the proenzymes. Activation of the proenzymes can also be achieved by non-proteolytic compounds, such as SH reactive agents [i.e. iodoacetate, 4- aminophenylmercuric acetate (APMA)], oxidized glutathione, HOCl, and denaturing agents (i.e. SDS, urea, NaSCN) and by heat treatment (Okada *et al*., 1992; Klein and Bischoff, 2011).

The expression of many MMPs is transcriptionally regulated by inflammatory cytokines, cellular transformation, hormones and growth factors. MMPs are further controlled by their activation from the pro-enzyme form, their interaction with ECM components, and their inhibition by endogenous inhibitors (i.e. TIMPs). These proteases play an important role during embryogenesis, morphogenesis, angiogenesis, and tissue involution, as well as diseases associated with unbalanced degradation of the ECM, such as arthritis, glomerulonephritis, atherosclerosis, tissue ulceration, periodontal disease, fibrotic lung disease and cancer cell invasion and metastasis (Meissburger *et al*., 2011).

Most investigations regarding MMPs is centered on pathological disorders. It was of interest to us to determine whether these matrix degrading enzymes play a role during normal biological processes. The differentiation of preadipocytes is particularly intriguing since unlike metastatic or cells involved in wound healing that degrade matrix to invade tissues, preadipocytes require remodeling of the surround ECM in order to alter their morphology and accumulate cytoplasmic lipids. An initial screen for collagenases, stromelysins and gelatinases was performed using proliferating preadipocytes, quiescent post-confluent cells and adipocytes at various stages of differentiation. The assays for collagenases (i.e. MMP-1, MMP-8, MMP-13 and MMP-18) and stromelysins (i.e. MMP-3, MMP-10, and MMP-11) showed no change among the various samples. It was only the zymogram for gelatinases, which include MMP-2 and MMP-9, that indicated alterations in the expression of these proteases among proliferating, quiescent and differentiated cells (Christiaens and Lijnen, 2006).

MMP-2, also known as gelatinase A (72 kDa), is unlike other members of this family in that it has been shown to bind the $\alpha_v\beta_3$ vitronectin receptor on the surface of invasive cells. This has been proposed as a mechanism by which MMP-2 acts as a growth and differentiation factor. Recent studies have also shown that MMP-2 activation is mediated by membrane type 1 MMP (MT-MMP), which functions either as an activator or a receptor of this protease. MT-MMPs contain a membrane spanning domain and play a unique role compared to the secreted members of the MMP family in that their location in the cell membrane results in proteolysis at discrete sites of the membrane-matrix junction, such as the leading edge of a migrating cell. The specific membrane-type MMP that activates MMP-2 is MT1-MMP, also known as MMP-14. Thus, MMP-2 regulation is quite complex. Not only is it regulated by external cytokines and hormones, but its regulation has also been demonstrated at the level of MMP-14 and TIMP-2 (Beliën *et al*., 1999; Creydt *et al*., 2010).

The role of MMPs may be important during adipogenesis since remodeling of the extracellular matrix is known to occur during differentiation. Furthermore, during the process of matrix remodeling, it is conceivable that growth factors associated with the matrix are released. For instance, bFGF associates with HSPGs, which themselves are tethered to the extracellular matrix. Degradation of collagen by the MMPs could release matrix bound bFGF which can act upon the cells and influence their growth and/or differentiation (Lilla *et al*., 2002).

In conclusion, understanding the molecular events governing the irreversible loss of proliferative potential during the adipocyte differentiation will contribute to an understanding of the balance between proliferation and differentiation. Uncoupling these two normally interdependent processes is an obligatory step in the generation of the transformed phenotype and cancer. Therefore, it is imperative to study the molecular coordination of differentiation and proliferation. The investigation of growth arrest in adipogenesis via transcriptional factors should provide more information on the mechanism of growth inhibition.

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Chapter 17

RESIN INFILTRATION TECHNIQUE IN MOLAR-INCISOR HYPOMINERALISATION - AN OVERVIEW

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MOLAR-INCISOR HYPOMINERALISATION

1. Introduction

Systemic-derived clinic hypomineralisation observed at the maturation phase of amelogenesis, of which etiology is not known well and by which one or more permanent first molar and permanent incisors can be affected, is defined as molar incisor hypomineralisation (MIH) (K. L. Weerheijm, Groen, Beentjes, & Poorterman, 2001). Tissue anomalies occur as a result of factors encountered in the histogenesis phase of mineralisation and organic matrix formation during the development of teeth (Güner & Salcıoğlu, 2016). The tooth development stages are affected by both genetic factors and environmental factors. Ameloblasts are very vulnerable in transition and maturation stages. If they are affected by long-term environmental or systemic factors during these periods, enamel hypoplasia or enamel hypomineralisation occur (Kühnisch et al., 2014).

2. Prevalence

MIH was suggested in the late 1970s by Swedish dentists working at community-based dental health services; however, the studies suggest that MIH's presence may have extended to earlier periods (Ogden, Pinhasi, & White, 2007). According to the prevalence studies before making MIH definition; it is seen that the prevalence has varied between 2.8-25% in the studies based on the present definition, whereas it has varied over a large range between 2.8-44% in the studies after the MIH has been defined (Willmott, Bryan, & Duggal, 2008). The difference of MIH incidence is considered to be caused by index, criteria, method used and variance of age groups, and variations among the countries' health systems and societies' socio-economic conditions (Durmus, Abbasoglu, & Kargul, 2013; Jälevik & Norén, 2000).

3. Etiology

Its etiology is not known well; however, it is considered that MIH may be caused by systemic, environmental, and medical factors encountered in prenatal and postnatal periods. A study determined that 78% of 151 children diagnosed with MIH have experienced a medical problem before, during, and after delivery. In another study, it was suggested that childhood diseases observed in the first three years of life are related to MIH. These childhood diseases include upper respiratory infections, bronchitis, pneumonia, asthma, varicella, otitis media, tonsillitis, measles, and rubella (Jälevik & Norén, 2000; Mittal, Goyal, Gauba, & Kapur, 2014; Wuollet, Laisi, Salmela, Ess, & Alaluusua, 2014). These disorders are regarded as etiologic factors of MIH because a sufficient amount of oxygen could not be supplied to ameloblasts due to systemic diseases in early childhood (Jälevik, Norén, Klingberg, & Barregård, 2001; Mittal et al., 2014).

It is considered that the use of antibiotics may also affect the etiology of MIH. However, whether antibiotics or the diseases caused by antibiotics causes to MIH is not precisely known (Jälevik et al., 2001; Wuollet et al., 2014).

In the studies, it was determined that the incidence of MIH is higher in children with systemic diseases. Some systemic diseases identified so far include nutritional deficiency, brain damage, neurological defect, cystic fibrosis, ophthalmic disorders, coeliac disease, gastrointestinal disorders, epilepsy, nephrotic syndrome, epidermolysis bullosa, lead poisoning, radiotherapy, treated cleft lip and palate, diabetes, thyroid and parathyroid disorders (Hall, 1989; Martínez et al., 2002; K. L. Weerheijm et al., 2003).

Furthermore, it is considered that the formation of genetic variations due to environmental factors during the phases of amelogenesis, especially in the last trimester of pregnancy, causes MIH (Jeremias et al., 2013; Kırzıoğlu & Çiftçi, 2009).

4. Histological Features of the Teeth with MIH

The defects of hypomineralized teeth are frequently observed on the tubercle crests and occlusal surfaces. While the level of amelogenin is average in hypomineralized teeth, the enamel protein ratio is high. These features distinguish teeth with MIH from amelogenesis imperfecta and fluorosis. Because the amelogenin level is higher in teeth with amelogenesis imperfecta and fluorosis (dos Santos & Maia, 2012), the dentin's Ca/P ratio under the hypomineralized enamel is compatible with the normal dentin. However, since C's level in the dentin under the hypomineralized enamel is high, its Ca/C ratio is lower than normal dentin (dos Santos & Maia, 2012; Güner & Salcıoğlu, 2016).

5. Characteristic Features of the Teeth with MIH

5.1. Pulpal status

Porous enamel creates a pathway for the passage of bacteria and other irritants. Inflammation leads to morphological and chemical changes in the pulp structure. In these patients, local anesthesia cannot be achieved with the desired efficiency, caused by recurrent sensitivity and decreased average activation threshold (dos Santos & Maia, 2012; Güner & Salcıoğlu, 2016).

5.2. Bacterial invasion into dentinal tubules

The bacteria penetrate through the defective enamel surface into the dentinal tubules. In severe cases, dentinal tubules constitute the main passageway. Since the dentin canals are wide during the eruption in permanent first molars, the bacterial invasion occurs rapidly (dos Santos & Maia, 2012; Güner & Salcıoğlu, 2016).

5.3. Enamel structure with defects

The quality of the remaining enamel tissue affects the success of the restoration. The most common problem during and after the restoration of the teeth with hypomineralisation is that the teeth can be broken easily or impair marginal harmony between the restoration and the tooth. The prism structure in the transition zone between the affected and unaffected enamel layer close to the affected side has changed. Therefore, problems may occur in retaining the restoration (dos Santos & Maia, 2012; Güner & Salcıoğlu, 2016).

5.4. Protein structure of enamel

The ratio of organic matter in the enamel layer of the hypomineralized teeth has increased compared to normal enamel and contains 8-21 times more protein, which is more common in hypomineralized teeth with brown enamel feature. The transmission between acid and hydroxyapatite crystals is decreased due to the increased protein content. Furthermore, increases were also observed in serum albumin, alpha-1-antitrypsin, and type I collagen levels in these teeth. Antithrombin III protein was also observed only in teeth with brown and yellow defects (dos Santos & Maia, 2012; Güner & Salcıoğlu, 2016).

6. Diagnosis of MIH

Permanent first molars and incisors should be carefully evaluated together with a detailed and comprehensive anamnesis to detect MIH's presence and make a diagnosis [15]. Developmental enamel defects and enamel hypoplasia can be confused with MIH. In enamel hypoplasia, the qualitative defects are characterized by locally reduced enamel thickness. Nevertheless, there are pits in one or more areas, and breakdown is observed in the partial or total enamel tissue reaching the dentin. It is difficult to distinguish between MIH and enamel hypoplasia because of post-eruptive fractures in molar teeth. Furthermore, MIH can be masked in children at high risk of caries. For all these reasons, it is easier and healthier to diagnose MIH when the permanent first molars erupt (Güner & Salcıoğlu, 2016; Wright, 2015).

During the diagnosis of MIH, rapid progression of caries increased sensitivity, and the difficulty of anesthesia are other factors that should be considered (Bhaskar & Hegde, 2014; Güner & Salcıoğlu, 2016).

7. Clinical Findings

The clinical criteria for the diagnosis of MIH developed by Weerheijm et al. (K. Weerheijm, Jälevik, & Alaluusua, 2001).

7.1. Presence of demarcated opacity

MIH is demarcated and typically seen as porous defects involving altered enamel translucency. Color changes ranging from white-cream to yellow-brown can be observed. The defective enamel is of normal thickness with a smooth surface, but the enamel structure is defective. The opacities are usually limited to the tubercle or incisal one-third of the crown (K. Weerheijm et al., 2001).

7.2. Post-eruptive enamel breakdown

Post-eruptive enamel breakdown (PEB) occurs by losing the initial form of enamel due to erosion and fraction following tooth eruption. The pre-existing demarcated opacities trigger this breakdown (K. Weerheijm et al., 2001).

7.3. Atypical restoration

Atypical restorations occurred in hipomineralize teeth extend to the buccal or the palatal smooth surfaces. At the borders of the restoration, opacity is usually noticed. In incisors, aesthetic restorations which are not associated with traumatic injuries and located on buccal surface can be observed (K. Weerheijm et al., 2001).

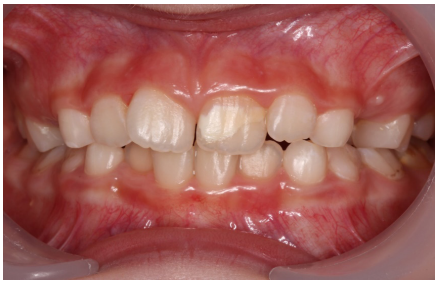

Figure 1. *Opacity in anterior central incisor with MIH*

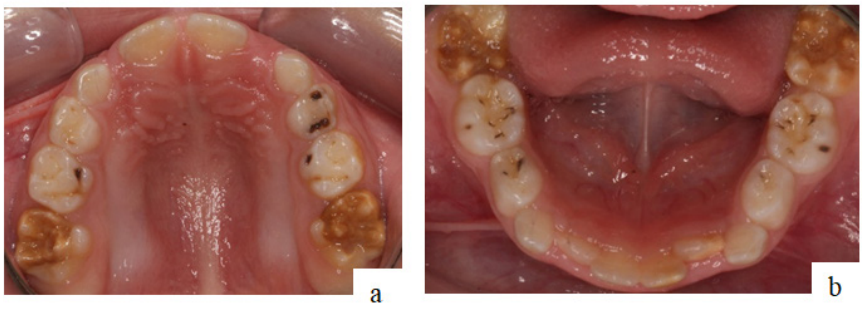

Figure 2. *Clinical appearance of maxilla (a) and mandible (b) of a boy with MIH. Notice the large affected areas with enamel loss and rapid cavity formation in the permanent molar teeth.*

7.4. Extractions due to MIH

Opacities or atypical restorations are not noticed on other permanent first molars inside the mouths considered to have molars lost due to MIH. Also, MIH can be suspected in cases where the absence of permanent first molars and well-demarcated incisors' opacities are seen. The extraction of incisors due to MIH is rare (K. Weerheijm et al., 2001).

8. Classification of MIH

8.1. Classification according to localization

MIH 1

One or more permanent first molars, along with one or more permanent incisors, are affected.

MIH 2

One or more permanent first molars are affected, and permanent incisors are not affected.

MIH 3

One or more permanent first molars are affected, and not all permanent incisors have erupted (Chawla, Messer, & Silva, 2008).

8.2. Classification according to the severity

It is determined according to the lesion's size and hypomineralisation degree (Mittal et al., 2014; Wright, 2015).

Mild MIH

- Limited and isolated opacities are observed in stress-free areas of permanent first molars.
- Discolorations range from white and cream to yellow-brown in the crown's upper part and on the masticatory surface.
- There are no enamel losses due to fractures in opaque areas.
- There is no tooth sensitivity.
- There are no caries in the affected enamel.
- Incisors are usually mildly affected.

Moderate MIH

- There are hypomineralized yellow-brown discolorations with more or less all crown crests affected.
- Limited opacities are observed on the incisal and occlusal triad of the teeth. No post-eruptive breakdown is observed.

- Post-eruptive breakdown or caries' presence is limited to 1 or 2 surfaces of the tooth and does not contain tubercles.
- There is no tooth sensitivity.
- The patient or family often has aesthetic concerns.

Severe MIH

- Post-eruptive breakdown exists and occurs, especially when the teeth are erupting.
- There are a defect and yellow-brown discoloration in crown morphology, which results in the extensive loss of enamel.
- There is tooth sensitivity.
- There are prevalent caries due to the frequently affected enamel.
- The breakdown of the crown may easily progress to the pulp.
- There are atypical restorations with defects.
- The patient or family has aesthetic concerns.

The effective etiological factors, the features of hypomineralized enamel, and diagnostic criteria are of great importance in revealing the measures and treatment methods to be followed (Mittal et al., 2014; Wright, 2015).

9. Treatment Approach for MIH

9.1. Risk identification and early diagnosis

Children with a putative history of etiological factors up to three in their medical history should be considered at risk and should be followed up routinely (William, Messer, & Burrow, 2006). Thus, it is necessary to plan protective applications and remineralization with the early diagnosis of defective surfaces that are susceptible to caries and erosion, an attempt to prevent loss of substance and caries that may occur in these mineralized areas should be made (Willmott et al., 2008; Wright, 2015).

9.2. Protective applications and remineralization methods

Oral hygiene education and diet control

Children and parents should be informed that these teeth are at a higher risk of decay than healthy teeth. Regular brushing habits should be gained, and doctor check should be made every three months (Willmott et al., 2008; Wright, 2015).

Children's nutrition should be evaluated regarding cariogenic risk factors and erosion potential, and necessary nutritional modifications

should be made. It should be explained to children and their families that snacks should be abandoned at snacks and cariogenic foods should be avoided (William et al., 2006)

The consumption of probiotic foods can be recommended since they contain calcium and decrease the levels of streptococcus mutants, one of the microorganisms that cause caries in saliva (Çaglar et al., 2005; Güner & Salcıoğlu, 2016).

Pits and fissure sealant application

Fissure sealant can be applied to prevent plaque accumulation and caries formation in mildly hypomineralized permanent first molars with preserved enamel integrity. Glass ionomer-based fissure sealants may be preferred in hypomineralized permanent first molars that are partially erupted and are erupting and in which humidity control is almost impossible. Resin-based fissure sealants can be used in hypomineralized teeth with the complete eruption and humidity control (Fragelli et al., 2017; Simonsen, 2002).

Remineralization

Remineralization treatment should be initiated in children with MIH in order to provide remineralization and desensitization. Casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) products, topical fluoride varnish, or gels can be used for remineralization and desensitization. Moreover, the teeth should be brushed with a fluoride-containing toothpaste and a soft toothbrush after each main meal (Lygidakis, Dimou, & Briseniou, 2008; Willmott et al., 2008).

9.3. Restorations

Restoration of hypomineralized permanent first molars

The treatment of permanent hypomineralized first molars constitutes many of these patients (Jälevik & Norén, 2000). The restoration of the affected permanent first molars becomes complicated due to the difficulty in administering anesthesia, increased anxiety in patients, and the problems concerning how much of the affected enamel will be removed and the selection of suitable restorative materials (Bodrumlu & Avşar, 2015; K. Weerheijm et al., 2001).

Many factors such as the degree of hypomineralisation, width, loss of tooth material, sensitivity, patient cooperation, age, development of malocclusion, and developmental missing teeth should be taken into consideration in the treatment decision of these teeth (Bodrumlu & Avşar, 2015; K. Weerheijm et al., 2001).

The selection of material should be based on the severity of the defect, the child's cooperation, and age (Bodrumlu & Avşar, 2015; Fayle, 2003). The adhesives should be selected because of atypical cavities formed after the removal of hypomineralized enamel. Glass ionomer cements (CIS) and resin-modified glass ionomer cements (RMCIS) are not recommended in areas under stress such as the occlusal surface of hypomineralized teeth. However, they can be applied until the permanent restoration is performed (Mahoney, 2001). Since resin composites are aesthetic materials with superior physical properties compared to CIS and RMCIS, they are frequently used to treat these teeth. In affected permanent first molars, resin composites' bond strength to hypomineralized enamel is less than the bond strength to normal enamel in both total and self-etch systems. In affected teeth, while the enamel's mineral content decreases, the protein content increases, which affects the acidification and bonding. Pre-treatment of enamel with 5% sodium hypochlorite is recommended to remove proteins (deproteinization) (Venezie, Vadiakas, Christensen, & Wright, 1994).

There are two approaches for determining the cavity margins during the restoration of the teeth affected by MIH (Fayle, 2003). The first approach is to remove all defective enamel after the decay is removed to increase the resin's retention and end the healthy enamel's cavity borders. This approach may significantly increase the cavity's size and shorten the restoration (Venezie et al., 1994). Another approach is to remove only a very porous enamel for a protective approach. There is a risk of poor bonding of the resin and marginal fracture in this approach's restoration since the hypomineralized enamel contains more protein (Fayle, 2003). In their two-year follow-up clinical study, Sönmez and Sarı (2017) reported that deproteinization and the method in which the entire defect was removed provided similar success. Therefore, deproteinization and the adoption of the protective approach principle are essential for protecting dental tissues.

Restoration of hypomineralized permanent incisors

Hypomineralized areas observed in patients with MIH incisors may lead to aesthetic concerns in the child and family. While microabrasion is an effective treatment method in shallow defects, bleaching with carbamide peroxide is effective in deeper yellow-brown defects (William et al., 2006)

The restorative treatment of incisions is avoided at first. However, if it affects the patient's quality of life, the patient may request the treatment. Aesthetics can be improved with indirect or direct composite veneers at the request of the patient. New generation composites can mask the defect without considering preparation, or large composite can be stacked on the tooth by preparation. However, it should not be forgotten that these teeth are young, immature teeth. Porcelain veneers should be delayed until late

adolescence, during which the tooth completely erupts, and the gingival structure becomes stabilized (William et al., 2006; Wright, 2002).

9.4. Resin Infiltration Technique

A new minimal interventional way to treat MIH in anterior teeth is the resin infiltration method. This method is based on the fact that low-viscosity light-polymerized resin can rapidly penetrate the porous enamel. The resin fills in the tooth's pores, replacing the lost tooth structure and stopping caries progression (Balmer, Toumba, Godson, & Duggal, 2012).

9.5. Extraction

The fact that the permanent first molars are severely affected can make restoration impossible, and extraction may be considered. When the extraction of the molars is considered, the vitality and restorability of the tooth, dental age, crowding in the buccal segment, occlusal relation, and the condition of other erupted, or unerupted teeth should be evaluated (Mahoney, 2001). If the tooth restoration is problematic or unsuccessful, the optimal time for extraction should be determined by following the tooth eruption and occlusion development. If the long-term prognosis of permanent first molars is low, the extraction of these teeth at the age of 8.5-9 is ideal. This age, which is the best time for the proper alignment of the second molars and the closure of cavities, corresponds to the time of bifurcation calcification of the mandibular permanent second molars. If the mandibular permanent first molars are extracted, the corresponding maxillary permanent first molars' compensation extraction should also be evaluated (Sönmez & Bezgin, 2018). Jalevik&Moller (2007) reported that the extraction of seriously affected permanent first molars was a good option against restoration.

RESIN INFILTRATION TECHNIQUE

The resin infiltration method is the current micro-invasive approach in treating initial caries without cavitation and lesions in the enamel. This technique closes the therapeutic gap between conservative applications and invasive treatment options and provides an alternative to invasive restorations used for white spot lesions in the anterior region.

1. Principles

Infiltrants are light-curing resin materials that can rapidly penetrate the capillary structure of the lesion body. These materials have low viscosity, low contact angles to the enamel, and high surface tension (Horuztepe, Ergin, & Gürgan, 2015; Sebastian Paris, Meyer-Lueckel, Cölfen, & Kielbassa, 2007).

The resin infiltration technique's main principle is based on preventing lesion progression by occluding the micro-porosities that provide diffusion pathways for acids and dissolved minerals (Horuztepe et al., 2015; Meyer-Lueckel & Paris, 2008). Although it is suggested that the bacteria trapped underneath may trigger the caries process except for occluding the top layer, it is also suggested that the bacteria in properly occluded cavities are not harmful (Horuztepe et al., 2015).

The hyper-mineralized superficial layer with a pore volume of 1% has an average thickness of 40 microns and prevents agents such as topical fluoride from progressing to sublayers of caries. Thus, it acts as a barrier that prevents resins from progressing to other layers of enamel caries. Therefore, this layer should be removed (Kielbassa, Mueller, & Gernhardt, 2009). For this purpose, hydrochloric acid (15% HCI) and orthophosphoric acid were used, and it was demonstrated that the application of HCI for 120 s is superior to the application of 37% orthophosphoric acid in the removal of the superficial layer of enamel lesions (Horuztepe et al., 2015; Meyer-Lueckel, Paris, & Kielbassa, 2007). Contrary to microabrasion application in the enamel, only 30-40 micrometer etching is made by this technique. With this technique, the intact and demineralized enamel is etched to the same extent because no pressure is applied (Kielbassa et al., 2009; Meyer-Lueckel et al., 2007).

2. Features that a resin infiltrant should have

- It should be hydrophilic and bacteriostatic.
- It should have high surface activity and low viscosity.
- It should not be toxic to mouth tissues.
- It should be able to polymerize into a solid-state.
- It should be resistant to mechanical and chemical irritants in the oral cavity.
- It should be aesthetically acceptable.
- It should have a high penetration capacity (Kim, Kim, Jeong, & Kim, 2011).

3. Content of the resin infiltrant

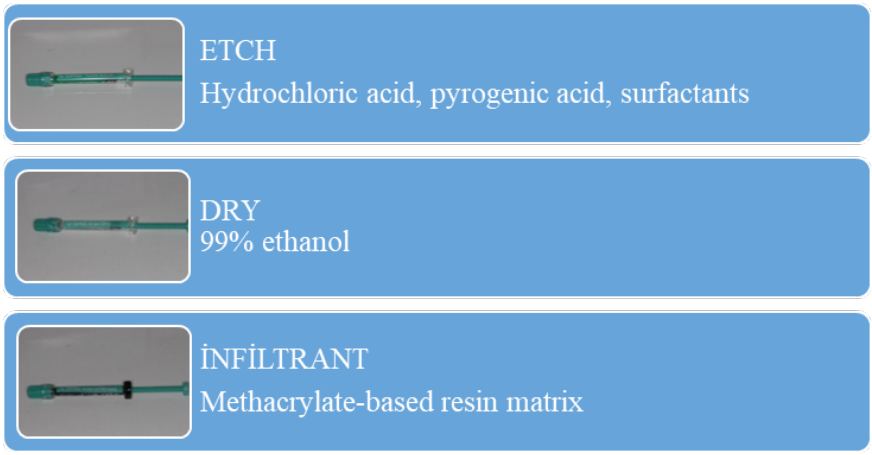

Figure 3. *Content of the resin infiltrant set (Icon®)*

When resin materials containing triethylene glycol dimethacrylate (TEGDMA) were compared with those containing bisphenol glycidyl methacrylate (Bis-GMA), they were found to be more successful in stopping the progression of lesions, which is associated with the higher penetration capacity of the resins containing TEGDMA (S Paris & Meyer-Lueckel, 2010). However, ethanol's addition to a TEGDMA-based infiltrant causes a slight reduction in penetration depth than the pure resin (Meyer-Lueckel & Paris, 2010).

4. Indications of resin infiltration treatment

- Anterior teeth affected by molar incisor hypomineralisation,
- White spot lesions observed on the smooth surfaces of the teeth after the fixed orthodontic treatment,
- Expansive opaque lesions on tooth surfaces,
- Hypoplasia caused by trauma,
- Fluorosis discolorations,
- Initial caries on the approximal surfaces (Kielbassa et al., 2009; S Paris & Meyer-Lueckel, 2010).

5. Advantages

- It supports the demineralized enamel mechanically.
- It protects straight, problematic areas.
- It permanently occludes superficial micro porosities and pores.

- It occludes pores in deep demineralized areas.
- It prevents the progression of the lesion.
- It reduces the risk of secondary caries.
- It delays the need for interventional treatment.
- No pulpal inflammation and associated post-operative sensitivity risk occur after the application.
- It does not cause gingivitis and periodontitis.
- It masks opaque white lesion appears as a result of using on demineralized labial surfaces.
- It is a method that is easily acceptable by patients (Kielbassa et al., 2009; S Paris & Meyer-Lueckel, 2010).

6. **Application (Icon®)**

Before starting the application, professional prophylaxis is applied to the affected and adjacent tooth, and any residues are removed. Then, the non-thermoplastic rubber dam is applied (Figure 4a). To ensure the application tip is designed for the approximal region to reach here, the teeth are separated by about 50 microns by the moderate force applied by plastic wedges in the kit.

The approximal tip is screwed onto the Icon etch syringe in the kit, and the clear strip in the application tip is placed in the interdentium. It should be paid attention to align the green side of this strip with the enamel area to be treated because the acid is applied through this area. Another tip is used for the application of HCI to vestibular regions. The syringa shaft is turned 1.5-2 turns, and a sufficient amount of HCI is applied to the lesion and left for 2 minutes (Figure 4b). Because the enamel surface appears chalky, it must not be contaminated following roughening. Otherwise, the stage is repeated for 10 seconds. After the application tip is removed from the interdentium, it is immediately rinsed off with water for 30 seconds then dried with oil-free and water-free air spray.

The application cannula is screwed onto the Icon-dry syringe. The half amount of ethanol in the syringe is applied to the lesion area and left for 30 seconds (Figure 4c). It is dried oil-free and water-free air.

Then, the approximal tip is screwed onto the Icon-infiltrant syringe, and the application strip is placed in the interdentium so that the green side will face the lesion side. A different tip is inserted for the application of resin infiltrant to the vestibular regions. A sufficient amount of resin infiltrant is applied to the lesion area by turning the syringe shaft by 1.5-2 turns. It has waited for 3 minutes for approximal surfaces, whereas the tip is applied

to the teeth surface by circular movements for the vestibular surfaces for better penetration of resin infiltrant (Figure 4d). In the approximal region, the application strip is removed from the interdentium, and any residues are removed using dental floss. The resin infiltrant is polymerized for 40 seconds (Figure 4e). It is recommended that the light device's wavelength is kept at a fixed value of at least 450 nm, and its light intensity is kept at a fixed value of 800mW/cm2. A new tip is screwed onto the resin infiltrant syringe, and the material is applied for the second time. It is waited for 1 minute to infiltrate material into pores and polymerized by light for 40 seconds.

Rubber dam and wedge are removed, and surface finish is carried out by fine-grained polishing strips and interface polishers (Kim et al., 2011; Mount & Ngo, 2000).

The purpose of performing the conditioning of the lesions using 15% hydrochloric acid gel is to remove the highly mineralized surface layer and superficial discoloration that may inhibit resin penetration. After acidification, the lesion is dried using ethanol, and thus, the resin penetrates the rough tooth structure more easily. As a result of the studies, it was reported that 15% of hydrochloric acid gel provided more effective surface erosion compared to 37% phosphoric acid gel (Meyer-Lueckel & Paris, 2010).

In the resin infiltration method, the enamel lesions lose their white appearance due to the filling of the microporosity and appear similar to healthy enamel (Figure 4f) (Shivanna & Shivakumar, 2011). This mechanism is based on the light distribution within the lesion. As a result of the studies, the refractive index (R.I) of the enamel was found to be 1.62. In subsurface lesions, the pores are filled with water with an R.I. of 1.33 or air with an R.I. of 1.0. The differences in R.I. of the enamel and water/ air environment affect the light distribution and causes the lesion to appear more opaque. The infiltrated lesions' micro porosities are filled with resin with an R.I. of 1.46, and this resin does not evaporate, unlike water. This difference in R.I. between the resin infiltrant and enamel is negligible and enables the lesion to appear similar to the surrounding healthy enamel (Kim et al., 2011; Shivanna & Shivakumar, 2011).

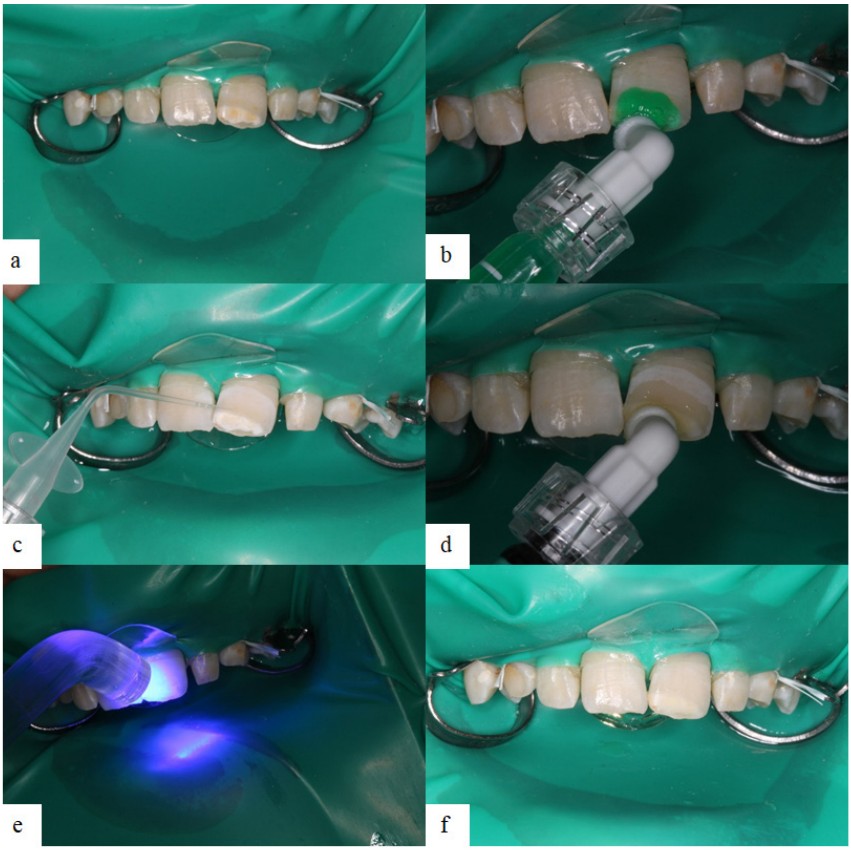

Figure 4. *Icon® procedure (a) preoperative photo of the white spot lesions, (b) application of Icon Etch for 2 minutes, (c) application of Icon Dry for 30 seconds, (d) application of Icon resin and allowing penetration by circular movements for 3 minutes, (e) light curing the resin for 40 seconds, (f) postoperative photo.*

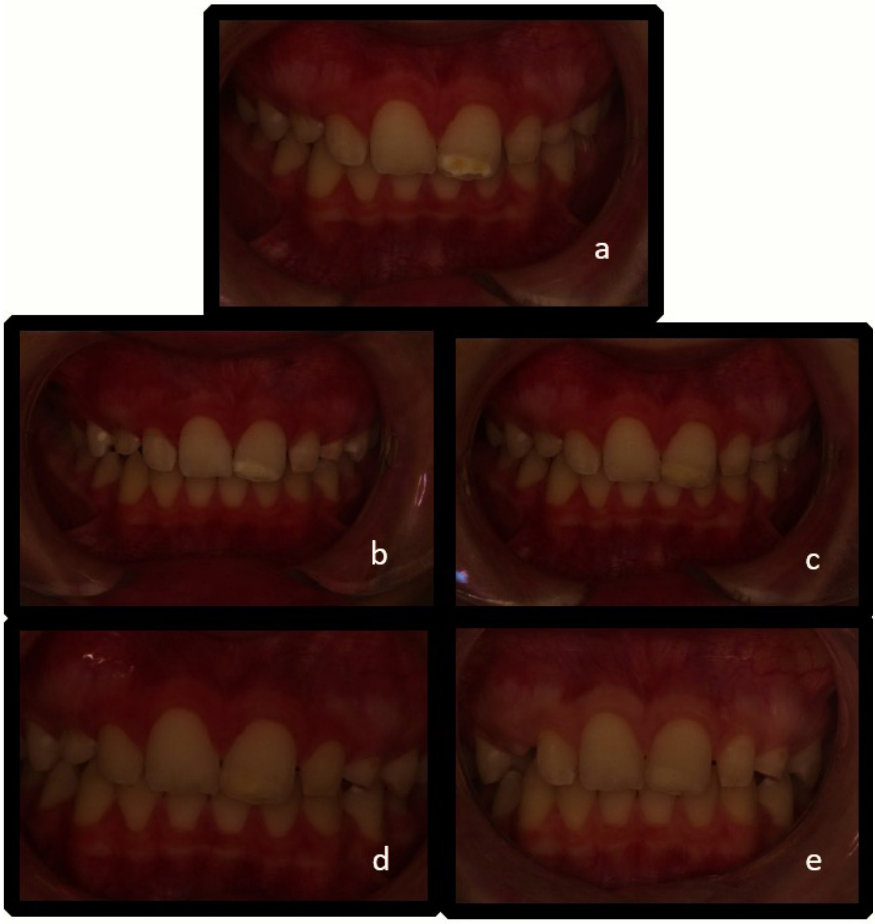

Figure 5. *Intraoral photographs of a patient with MIH lesion in tooth #21 obtained using cross-polarization photography (a) before treatment, (b) immediately after Icon® application, (c) one month control, (d) three months control, (e) six months control.*

CONCLUSION

Today, the prevalence of MIH has gradually increased. Anterior teeth affected by MIH are among the situations in which patients suffer from their aesthetic appearance and complain. This causes physical and social problems in patients (Altun, Esenlik, & Tözüm, 2009).

Diagnosis of MIH by dentists and the knowledge of conservative treatment approaches applied in hypomineralized teeth are essential. The treatment to be applied should be the least invasive option for children patients. Concerning this approach, the Resin infiltration technique

provided a micro-invasive way of treating early non-cavitation, superficial, and limited demineralized lesions (Sebastian Paris, Meyer-Lueckel, Coelfen, & Kielbassa, 2007).

The resin infiltration technique aims the diffusion pathways obstruction and arrests further progression of superficial and limited enamel hypoplasia lesions. Resins with low viscosity, the low contact angle with the enamel, and increasing the refractive index of demineralized enamel penetrate the lesion body's layer, so opaque lesion image can be reduced (Kim et al., 2011).

Resin infiltration technique has several advantages such as preservation of healthy teeth structure, requiring no local anesthesia, completion of treatment procedure in a single session, causing no sensitivity or pulpal inflammation after the application, reducing the risk of gingivitis or periodontitis. (Kielbassa et al., 2009; Phark, Duarte Jr, Meyer-Lueckel, & Paris, 2009). On the other hand, the same effect cannot be expected deeper MIH lesion with resin infiltration procedure (Guerra et al., 2016). The resin technique faces limitations in such lesions.

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Chapter 18

THE EFFECT OF SUNFLOWER OIL ON SOME BLOOD METABOLITES AND MILK FAT CONJUGATED LINOLEIC ACID LEVEL IN SAANEN GOATS

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1. Introduction

Conjugated linoleic acids (CLAs) are fatty acids that are present in foods obtain from ruminants (Wahle, Heys, & Rotond, 2004). The term conjugated linoleic acid (CLA) make reference to a mixture of positional and geometric isomers of linoleic acid, which is characterized by having conjugated double bonds (Kritchevsky, 2000; Churruca, Fernández-Quintela & Portillo, 2009).

CLA is greatly found in dairy products, meat and meat products, certain vegetable products, and in some seafood. Animal sources normally have a higher amount of CLA than plant sources, and in general, food from ruminants contains more CLA than non-ruminant (Chin, Liu, Storkson, Ha & Pariza, 1992; Lin, Boylston, Chang, Luedecke & Shultz, 1995; Fritsche, Rickert, Steinhart, Yurawecz & Mossoba, 1999). The CLA isomers are constructed during the biohydrogenation of linoleic acid in the rumen and also through the transformation of vaccenic acid in the mammary gland (Sieber, Collomb, Aeschlimann, Jelen & Eyer, 2004). Human production of CLA from linoleic acid does not become evident to occur at any significant level. The amount of CLA in human adipose tissue is consideration to be directly connected to dietary intake (Jiang, Wolk & Vessby, 1999)

It has been reported that CLA has a positive effect on health with its anticarcinogenic, antidiabetic, antiadipogenic and antiatherogenic and antimutagenic properties (Pariza, 1999; Kim, Kim, Kim, & Park, 2016a). The National Academy of Sciences (National Research Council, 1996) stated that CLA is the only fatty acid that has been clearly shown to inhibit carcinogenesis in experimental animals. Furthermore, the cis-9, trans-11 isomer is the major CLA isomer found in milk fat and is considered to be a biologically active form with anticarcinogenic properties (McGuire.& McGuire, 2000). The CLA-containing diet has been determined to reduce in plasma total cholesterol, triglyceride and cholesterol-induced atherosclerosis in thoracic aorta and aortic arch in hamsters (Nicolosi, Rogers, Kritchevsky, Scimeca & Huth, 1997).

Goat milk has been determined as an alternating for infants and adults who are either sensitive or allergic to cow milk (Dixit, Appu Kuttan & Singh, 2012). The goat's dairy products are easily digestible than cow's dairy products (Chilliard, Rouel, Ferlay, Bernard, Gaborit, Raynal-Ljutovac, & Leroux, 2006). Caseins from goat milk tended to be more efficiently digested compared to caseins from cow milk (Hodgkinson, Wallace, Boggs, Broadhurst & Prosser, 2018).

In dairy goat feeding, different oils as canola (Mir, Goonewardene, Okine, Jaegar & Scheer, 1999), soybean (Bouattour, Casals, Albanell, Such, & Caja, 2008), flaxseed oil (Kholif, Morsy & Abdo, 2018) and sunflower (Bernard, Bonnet, Leroux, Shingfield & Chilliard, 2009;

Atikah, 2018; Hartanto, Cai, Yu, Zhang, Zhang, Sun & Qi, 2019) have been analyzed. In addition, (Morsy, Kholif, Kholif, A. E., Matloup, Salem, & Elella, 2015) reported that the addition of sunflower seed or whole sunflower seed oil to the goat (Damascus goat) ration, without detrimental effects on animal performance, increases the milk content of healthy fatty acids (CLA and omega 3), and has beneficial effects on milk yield and milk composition. (Razzaghi, Valizadeh, Naserian, Mesgaran, & Rashidi, 2015) have shown that dietary addition of sunflower oil had increased the supply of total trans-fatty acids and polyunsaturated fatty acids to milk in Saanen goats.

Sunflower oil from standard cultivars is featured by its high linoleic acid, moderate oleic acid, and low linolenic acid concentration (Sobrino, Tarquis, & Díaz, 2003). Linoleic acid is the main component in sunflower oil, which may increase CLA in the animal product (Morsy at al. 2015). Interest in functional foods has increased in recent years, being the enhancement of milk with CLA one of the targeted products. The aim of this research was to investigate the effect of 5% sunflower oil supplementation on performance and blood metabolites lactating goats and their kids.

2. Materials and Methods

2. 1. Animal care

This study was approved by the Ethics Committee of Adnan Menderes University (ADÜ-HADYEK) (05004/2011/089). The present study was performed in the Research Unit of the Adnan Menderes University Çine Vocational School in the Çine district (28°06′N, 37°61′E) of Aydın Province, Turkey.

2. 2. Animals and dietary treatments

Twenty seven Saanen goats (47.25±2.52 kg initial body weight) and their kids (39 kids) were used as study material. Before the experiment, the goats were treated with the internal and external antiparasitic drug with 0.2 mg/kg ivermectin (Merial, France) and oksfendazole 250 mg and oksiklozanid 750 mg (Ceva, Turkey). All goats were subjected to an oestrus synchronization program consisting of 11 days of progestagen (20 mg FGA, Intervet, France) impregnated sponge placement plus PMSG and prostaglandin injections 48 hours prior to sponge withdrawal. The goats were fed with rations consisting of protein and energy at the level recommended by NRC (1985) before and after parturition for goats which included rations consisting of barley, sunflower seed pulp, corn, alfalfa hay, wheat straw, salt, DCP, premix and sunflower oil.

The experiment was continued for 90 days, 30 days before parturition and post-partum 60 days.

In this study, pregnant goats were randomly divided into three equal groups. The first group was determined as the control group. In the second group (Diet I), sunflower oil was added at a rate of 5%, taking into consideration the balance of ration, including the period between from prenatal 30 days to postnatal 60 days. In the third group (Diet II), sunflower oil was added to 5% in the postnatal period for 60 days.

Compound feed as a concentrate, and alfalfa hay and wheat straw as roughage were used in the experiment. The composition and analytical values of the rations given to the goats before and after the parturition on a dry matter (%) were given in Table 1 and Table 2, respectively.

Table 1. *Ingredients of the experimental rations (DM %) and analytical values offered to the goats before the parturition*

Ingredients	Control Group	Treatment Groups
Alfalfa hay	12.97	13.16
Wheat straw	48.65	53.72
Barley grain	8.11	0.65
Sunflower seed pulp	20.55	24.65
Corn	7.56	0.65
Sunflower oil	-	5
Salt	0.54	0.54
DCP	1.08	1.09
Premix*	0.54	0.54
Analytical Values		
ME (kcal/kg)**	1952.25	1966.30
CP (g/kg)	109.7	109.20

Premix* : Eeach 1kg contains 16 000 000 IU vitamin A, 3 200 000 IU vitamin D_3 , 32 000 mg vitamin E, 640 mg mangan, 1 120 mg iron, 16 mg iode, 3,20 mg cobalt, 6,40 mg selenium, 16 mg molybdenum, 640 mg zinc, 224 mg copper ve 256 mg magnesium.

ME** : This value was found to with calculation using NRC (1985).

Table 2. *Ingredient of the experimental rations (DM %) and analytical values offered to the goats after the parturition*

Ingredients	Control Group	Treatment Groups
Alfalfa hay	14.57	13.10
Wheat straw	28.35	30.88

Barley grain	8.86	6.02
Sunflower seed pulp	39.70	42.50
Corn	6.03	-
Sunflower oil	-	5
Salt	0.53	0.53
DCP	1.43	1.44
Premix*	0.53	0.53
Analytical Values		
ME** (kcal/kg)	2125.76	2138.25
CP (g/kg)	162.5	162.7

Premix* : Eeach 1kg contains 16 000 000 IU vitamin A, 3 200 000 IU vitamin D_3 , 32 000 mg vitamin E, 640 mg mangan, 1 120 mg iron, 16 mg iode, 3,20 mg cobalt, 6,40 mg selenium, 16 mg molybdenum, 640 mg zinc, 224 mg copper ve 256 mg magnesium

ME** : This value was found to with calculation using NRC (1985)

Compound feed as a concentrate and roughages amounts given to groups before and after parturition is showed in Table 3.

Table 3. *Concentrate and roughage amounts are given to the goats before and after parturition*

Periods	Groups	Concentrate Feed (g/day)	Roughages (g/day)
Prenatal	Control	690	1140
	Diet I	610	1220
	Diet II	690	1140
Postnatal	Control	1610	1210
	Diet I	1560	1250
	Diet II	1560	1250

Concentrate feed and vitamin and the mineral mix were weighed separately for each group on a daily basis and given only one meal. After the concentrate feed was finished, half of the wheat straw and alfalfa hay mixture were given in the morning and the other half in the evening. After the parturition, the does were kept together with their kids. The kids were not fed during the experimental period and kept in separate from mothers during each feeding period. They were fed only with the milk, suckled from their mothers. Also, the kids were kept in separate from their mothers in each test day during 12 hours for the determining the daily milk yield of each animal. Out of the test days goats were not milked and sucked by their kids. Fresh water was given ad libitum to the animals.

2. 3. Live body weight determining

The does were weighed at the beginning, at parturition and at the end of the experiment. The kids were weighed at birth and every fifteen days after birth in the experimental period.

2. 4. Blood sampling and cholesterol and triglyceride measurements

Blood samples were obtained from jugular vein of does at the beginning of the experiment, at the parturition and at the end of the experiment. The kid blood samples were taken only at the end of the experiment. The sera were separated by centrifugation and stored in a freezer at -20 °C. The cholesterol and triglyceride levels in the serum were determined using a commercial kit (Dialab, Austria) on a spectrophotometer (Shimadzu Corp. UV 1601, Australia).

2. 5. Milk sampling and milk fat and cla in milk fat measurements

On the 15^{th}, 30^{th}, 45^{th} and 60^{th} days (test days) after parturition, the does were kept separate from their kids during 12 hours (between 20^{30} - 08^{30}) before morning milking and, their milk was milked by the same person and then the milk amount was determined with recording jars. Partial milk yield was determined with once-daily milking during the 60 days and calculated according to Holland method (Kaymakçı M., 2006).

To determine the amount of milk fat and CLA levels in milk fat, individual milk samples were taken on the 5^{th} day of lactation and at the end of the experiment. The determination of milk fat in milk was performed using the method of Gerber (Richardson 1985). CLA levels in milk fat were analyzed by gas chromatography. For CLA levels in milk fat, 0.2 ml of 0.2% KOH solution with methanol was added to 0.1g milk fat and 2 ml of heptane was added to rinse for 30 minutes. Then 1 µl of the fatty acids on the remaining saponified parts were taken and injected to the device (GC-2010 Shimadzu Corp.2006). Gas chromatograph conditions were set as follows: capillary column (60 m × 0.25 mm i.d. × 0.2 m film thickness, Süpelco, Bellefonte, PA, USA), Inlet and detector temperatures were 240 and 250 ◦C, respectively. The hydrogen carrier gas flow rate was 1,2 ml/ min.

2. 6. Statistical analysis

SPSS 10.0 statistical package program was used to evaluate the data. One-way analysis of variance (ANOVA) was used for the difference among the groups in terms of the parameters examined, and the Duncan test was applied for the significance control of the differences among the groups (Tekin, 2003).

3. Results

The mean live body weights were determined at the beginning of the experiment, the parturition and at the end of the experiment and are shown in Table 4.

Table 4. *The goat mean live weight of goats ($\bar{x}$±$S\bar{x}$)*

Live Body Weight (kg)	**Diet I** (n=9)	**Diet II** (n=9)	**Control** (n =9)	**P**
Beginning of the experiment	45.98±4.33	48.33±3.37	47.43±5.59	NS
Parturition	46.48±3.67	48.96±2.73	48.79±4.55	NS
End of the experiment	47.05±3.45	48.50±2.86	48.47±4.42	NS

NS.: Non-significant (P>0.05)

There was no difference among groups in terms of live weight of does.

Mean birth weight and the mean live weights determined by 15-day intervals of the kids are given in Table 5.

Table 5. *Mean live body weight values of the kids (kg) ($\bar{x}$±$S\bar{x}$)*

Live Body Weight of Kids	**From Diet I** (n= 13)	**From Diet II** (n= 14)	**From Control** (n= 12)	P
Birth weight	2.91±0.21	2.82±0.14	2.88±0.23	NS
15th day	5.04±0.25	5.06±0.25	4.87±0.29	NS
30th day	7.87±0.40	8.31±0.33	8.85±0.62	NS
45th day	11.17±0.64	10.99±0.37	11.10±0.77	NS
60th day	13.54±0.55	13.37±0.53	12.99±0.57	NS

NS: Non-significant (P>0.05)

There was no difference among the groups.

In all three groups, the mean serum cholesterol and triglyceride values were determined at the beginning of the experiment, at the parturition and at the end of the experiment, and were given in Table 6.

Table 6. *The goat serum average cholesterol and triglyceride values at the beginning of the experiment, at the parturition and at the end of the experiment (mg/dl) ($\bar{x}$±$S\bar{x}$)*

Cholesterol Levels	**Diet I (n=9)**	**Diet II (n=9)**	**Control (n=9)**	**P**
Beginning of the treatment	169.55±3.12	167.24±5.50	168.46±2.47	NS
Parturition	141.22±2.51	150.91±3.25	152.08±3.05	NS (P=0,52)

End of the treatment	117.66±2.32[a]	132.04±2.62[b]	143.35±3.02[c]	***
Triglyceride Levels				
Beginning of the treatment	60.79±1.04	62.01±1.70	62.68±2.30	NS
Parturition	48.18±1.54	51.93±1.60	52.49±1.56	NS
End of the treatment	37.23±1.30	39.81±1.39	41.93±1.21	NS (p=0,55)

a, b, c: Different letters within a row indicate statistically significant differences (P<0.05).

***: P<0.001,

NS: Non-significant

Serum cholesterol levels at the end of the experiment were less in both experimental groups (P <0.001) than that of the control group. There was no difference among the groups in terms of triglyceride. Average triglyceride level was least in Diet I group as numerically at the end of the treatment but it was found not significant statistically (Table 6).

Mean of serum cholesterol and triglyceride values determined in kids of the groups were given in Table 7.

Table 7. *Serum means cholesterol and triglyceride levels of kids (mg/dl) ($\bar{x}$±$S\bar{x}$)*

	From Diet I (n= 13)	**From Diet II** (n= 14)	**From Control** (n= 12)	P
Cholesterol	120.02±9.73	147.17±6.94	148.96±11.5	NS
Triglyceride	41.68±3.57	51.69±4.63	53.39±8.43	NS

NS: Non-significant

Average cholesterol and triglyceride levels were least in the Diet I kids group as numerically at the end of the experiment but it was found not significant statistically (Table 7).

Sixty days of average milk yield of the groups was given in Table 8.

Table 8. *Average milk yield (ml), milk fat (%) and mean CLA (%) in milk fat. ($\bar{x}$±$S\bar{x}$).*

		Diet I (n=9)	**Diet II** (n=9)	**Control** (n=9)	**P**
Sixty days of milk yield (ml/ day)		2340.27±96.61	2400.00±139.29	2210.83±80.7	NS
Milk fat (%)	5th day of lactation	3.76±0.19[b]	4.93±0.21[a]	4.86±0.14[a]	**
	end of the treatment	2.90±0.28	3.22±0.16	3.68±0.21	NS

Milk fat CLA (%)	5th day of lactation	4.73±0.22 [a]	2.81±0.19 [b]	2.50±0.25 [b]	***
	end of the treatment	5.86±0.20 [a]	4.38±0.29 [b]	2.44±0.41 [c]	***

a, b, c: Different letters within a row indicate statistically significant differences (P<0.05).

**:P<0.01, ***: P<0.001, NS: Non-significant

It was not found the difference in milk yield. Average milk fat (%) and CLA in milk fat (%) levels were determined in milk samples at 5th day of lactation and at the end of the experiment. On the 5th day of lactation, milk fat was found to be the least in the 5% sunflower oil group (Diet I) during the experiment (P <0.01). The average milk fat level was least in Diet I group as numerically at the end of the experiment but it was found not significant statistically (Table 8).

The level of CLA in milk fat at 5th days of lactation was determined higher in the group (Diet I) treated with 5% sunflower oil per day throughout the experiment at 5th days of lactation (P <0.001) than that of the other groups. When the milk fat samples collected at the end of the experiment were examined, it was seen that the CLA level was the lowest in the control group and the highest in the 5% sunflower oil group (P <0.001) (Table 8). Similarly, the level of CLA in the milk of Diet II goats was found high than the control group, too.

4. Discussion

At the end of the experiment, there was no difference (p>0.05) among experimental groups in terms of live weight neither of the does nor their kids. This result was similar to that obtained by Morsy et al. (2015) who not found different in live body weight among treatments have been reported in lactating Damascus goats fed with sunflower seeds, either as whole or as oil. Moreover, feeding whole sunflower oil seed at in two levels (7.5 or 15%) of lactating Holstein cow's diets (Mansoori at al. 2011) had no effect on body weight. In contrast, Abdel-Gawad & El-Emam (2018) reported that sunflower oil added to the control diet at level 3% increased the body weight by 9.82 % than weight of control at male Zaraibi goats.

In this study, there was no difference among the groups in terms of cholesterol at the beginning of the treatment. In parturition time, serum cholesterol level was least in Diet I group as numerically but it was found not significant statistically. Moreover, serum cholesterol levels at the end of the experiment were lower than the control group in both experimental groups (P <0.001) and it was found that the cholesterol levels were least in Diet I

group. Nestel et al. (1978) supported that dietary fat addition did cause a marked decrease in the excretion of acidic steroids which may have been due to the decreased formation of sterols in the liver. Fats in the diet encourage the production of lipoproteins in the intestine which is the major site of de novo cholesterol synthesis in ruminants (Espinoza, Ramirez-Godinez, Simental, Jimenez, Ramirez, et al. 1997). On the other hand, the researcher revealed that cholesterol was increased with soybean oil level but decreased with sunflower oil in Awassi ewes (Titi & Fataftah, 2013) and Shami goats (Titi, Hasan, Al-Ismail, Zakaria, Tabbaa et al. 2011). Furtermore, Delavaud, Fougère, Chilliard & Bernard, (2019) suggested that dietary addition of sunflower oil plus starch increases cholesterol esters, cholesterol, and free fatty acids levels compared to the control in the goats.

In this study, there was found no difference among the groups in terms of triglyceride at the beginning and at the parturition time of the experiment. Serum triglyceride level was least in Diet I group as numerically at the end of the experiment but it was found not significant statistically. In another study, Titi & Fataftah (2013) investigated the effects of the supplementation of soybean oil and sunflower oil on the performance of early lactating Awassi sheep. Similarly, they determined that blood triglycerides decrease with fat supplements independent of the fat source. In contrast, Roy, Mandal & Patra, (2013) revealed that oils (soybean oil and sunflower oil) in Black Bengal goats feeding did not affect ($P>0.05$) on level of serum glucose, cholesterol, and protein, but increased ($P<0.05$) serum triglyceride concentration compared with control.

Average cholesterol and triglyceride levels were least in the Diet I kids group as numerically at the end of the experiment but it was found not significant statistically in this study. In another study, Yeom, Schonewille, Van Trierum, Kappert & Hovenier et al. (2004) studied the effects of dietary α-linolenic acid (ALA) and linoleic acid (LA) on growth performance and fatty acid status of goat kids. They found that the concentration of plasma triglycerides decreased with high ALA and LA uptake. Moreover, Titi et al. (2011) revealed that serum triglyceride was reduced following soybean oil and sunflower oil treatments in the serum of the kids and the researchers reported that to be found higher value in control group than that of the treatment groups.

Different opinions have been put forward on the effect of the additional fat in ration on milk yield. Morsy et al. (2015) determined that especially sunflower oil had significantly increased ($p<0.05$) the milk yield compared with the control in lactating Damascus goats. In contrast, Titi et al. (2011) reported that daily milk production was reduced ($P<0.05$) soybean oil and sunflower oil supplemented in Shami goats. Mansoori et al. (2011) recorded that supplementation with 7.5% raw sunflower seed tended to

decrease milk yield compared with the control in lactating Holstein cows. In the present study, when compared to sixty days of average milk yield of the groups, it was not found a difference in milk yield. Similarly, Mir et al. (1999) revealed that milk yields were not ($p > 0.05$) affected by feeding canola oil in non-pregnant Alpin goats in late lactation period.

In this study, milk fat was found to be the least in the 5% sunflower oil group (Diet I) ($P < 0.01$) on the 5th day of lactation. The average milk fat level was least in Diet I group as numerically at the end of the experiment but it was found not significant statistically. This situation presumably caused by the anti-lipogenic effects of rumen biohydrogenation intermediates, such as trans-10 cis-12 CLA (Suárez-Vega, Gutiérrez-Gil, Toral, Hervás & Arranz et al. 2019). Gómez-Cortés, Toral, Frutos, Juarez & Fuente et al. (2011) reported that incremental amounts of dietary sunflower oil did not affect milk production nor the milk's fat in Assaf ewes. Besides, abomasal infusion of CLA supplement resulted in a dramatic reduction of milk fat synthesis of dairy cows (Chouinard, Corneau, Barbano, Metzger & Bauman DE. 1999). In contrast, in a study conducted on Alpine goats by adding 6.1% sunflower oil and 6.2% flaxseed oil to dry matter based on corn silage, it was reported that the average level of milk fat in the group that added flaxseed oil was higher than the control group (Bernard L at al. 2009). Similarly, Mir et al. (1999) determined that percent fat increased linearly in response to feeding canola oil in Alpin does.

In the present study, the level of CLA in milk fat was found to be higher both at 5th day of lactation ($P < 0.001$) and at the end of the experiment ($P < 0.001$) in the Diet I group. Similarly, the level of CLA in the milk of Diet II does was found higher than that of the control group. In several studies, it has been reported that supplementation of sunflower oil or linoleic acid to ration increases total CLA content in milk fat (Titi & Fataftah 2013, Titi at al. 2011, Gómez-Cortés at al. 2011). In addition, Ivan, Mir, Koenig, Rode & Neill et al. (2001) concluded that sunflower seed oil as a supplement to barley silage-based diet reduced rumen fauna and the C16:0 proportion of fat, while increasing C18:2 and CLA content in the muscle and fat tissues.

In conclusion, the addition of 5% sunflower oil to their rations significantly reduced the cholesterol and significantly increased milk fat CLA levels of Saanen goats, and lowered the cholesterol and triglyceride levels of their suckling kids.

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Chapter 19

UROLITHIASIS AND PERCUTANEOUS NEPHROLITHOTOMY

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Epidemiology

Urinary system stone disease does not show a clear prevalence due to geographical and racial differences, and it has been reported to vary between 1-20% (A, 2006). It is most common in the 30-40 age range and is more common in men than women. It is suggested that the reason for this is that high testosterone levels in men increase the production of endogenous oxalate from the liver. When regional causes are examined, prevalence increases in mountainous, desert or tropical regions. It is thought that the factor in this is also heat (Chen MH, 2016). However, as a common view, increasing fluid intake and increasing urine amount causes a dramatic decrease in the incidence of stones in patients with factors that increase the formation of urinary system stone disease. The link between fluid consumption and the occurrence of stone disease is important in two aspects. The first is the excessive loss through sweating and respiratory tract despite the amount of fluid taken, and the second is the mineralization nature of the fluids taken. Therefore, fluid consumption with a diuretic character may act as a catalyst for stone formation mechanisms (Chen MH, 2016). While there was an inverse proportion between the sociocultural and educational status of the patients, there was no significant difference between urban life and rural areas.

Risk Factors in Stone Formation

Although many theories have been put forward about stone formation in the urinary system, it is still not fully elucidated today. For this reason, it is accepted that it is formed as a result of the interaction and relationship of a wide variety of factors. The factors mentioned in stone formation mechanisms can be grouped into 2 main subgroups as personal and environmental factors. While gender, age and genetic predisposition are shown among personal factors, environmental factors include climate, geography, quantity of fluid taken, diet, profession. Studies have found that the family history of stone history is 25% of those with a family history, and those with a familial history of stones have a higher risk of disease than the normal population, although environmental and dietary factors are prevented (Hemminki K, 2018). Several genetic studies in the literature show that a large number of genomic structures are effective in the development of the disease (Taguchi K, 2017). While it is seen equally in boys and girls in childhood, an increase of 1.5-3 times was observed in men compared to women in adulthood (Jobs K, 2018), it has been suggested that the etiology of this is an increase in endogenous oxalate production in the liver and indirectly an increase in the amount of urine oxalate (Jobs K, 2018). Again, in a study with similar results, it was concluded that low serum testosterone level may have a protective effect in terms of stone formation

in childhood and women (Gupta K, 2016). Kidney stones are most common in the 4th and 5th decades (A, 2006). Increased temperature, excessive fluid loss due to respiration and sweating, dehydration and conditions that cause an increase in urine density can accelerate stone formation. There are many studies in the literature showing the inverse ratio between the amount of fluid taken and stone formation (DS, 2019). In addition to the amount of water consumed, the mineral and element content of the water may have an inhibitory or catalyst effect in stone formation (Liu Y, 2018). For example; While waters with a high content of NaHCO3 in biochemical analyzes, which are described as "hard water" among the public, have a lithogenic effect, waters with high magnesium and citrate content have a preventive effect on stone formation (Jung H, 2017). When the adhesion of UPJ (Ureteropelvic junction), medullary sponge kidney or collecting tubule epithelium increases, crystal accumulation also increases.

Stone Formation Mechanisms

Theories such as supersaturation-crystallization, absence of urine inhibitors, matrix-nucleation and epitaxy theory have been developed to explain the mechanisms of stone formation. However, combining theories such as intranephronic and fixed nucleation, extranephronic and free particle nucleation have been proposed with the combined theory created by combining these theories (A., 2018).

Supersaturation-crystallization theory is the basic mechanism in stone formation. When a crystallizable substance is added to water at a constant pH and temperature, it remains in solution. However, as the amount of this substance increases, it reaches a certain saturation point after a while. While it was in the molten state up to this point, the substance in the solution that has reached the supersaturation then begins to crystallize. These substances at the saturation point are called thermodynamic soluble products (Ksp). If the ambient temperature and pH do not change, these crystals will precipitate when more crystals are added to the saturated solution. Precipitability of crystals is prevented by the inhibitor and some other substances in the urine with a pH value of 37ºC close to the average body temperature. For example; salt components that cause stone formation such as calcium oxalate (CaC2O4) may not crystallize although they exceed their Ksp. In this case, urine is called metastable. This situation continues for a while. However, when the amount of salt is increased after a certain point, crystals form and begin to collapse and this situation is called the formation product (Kf). Depending on the density of these crystallizable substances in the urine content, 3 forms are formed in the urine. These; Sub-saturation region, metastable region and unstable / supersaturated region.

Although the substances in the sub-saturation region are Ksp and may be crystals, the ability of these crystals to become molten is preserved. In concentrations above Kf, the solution is in the unstable / supersaturated area and crystals are formed and recurrence is initiated. Inhibitors are ineffective at this stage. Even if the urine is saturated in the Kf-Ksp area, that is, in the metastable region, the substances do not precipitate and nucleation is not passed. In this region, inhibitory factors control the formation of stones and it is the place where therapeutic intervention is performed in terms of stone formation (R., 2016).

Classification of Urinary System Stones

Recent studies on urinary system stone disease have developed new classifications in the light of minimally invasive interventions with developing technology and intended new treatment modalities. The European Association of Urology (EAU) has published a guideline with new definitions on the classification of stones, and this guideline is updated annually. It is that urinary tract stones can be classified according to size, location, X-ray characteristics, formation etiology, composition and recurrence risks.

Stone size: If the stone size is given as a unit of length, the longest axis measure is usually taken. In the light of these measurements, the stones are divided into three groups as> 5 mm, 5-10, 10-20 and the widest length> 20 mm. Coraform and staghorn type stones are the terminology used in terms of clinical approaches and generally refer to stones> 20 mm in total size.

Stone localization: Stones as localization; They are classified as lower, middle and upper calyx and renal pelvis stones. The localization of the stones is very important as it will change the form and course of the treatment. In the EAU Urinary system stone disease guideline, the location of the stone was differentiated as lower pole / calyx stones and renal pelvis or middle-upper calyceal stones and multiple (coraliform) or staghorn stones and a treatment algorithm was created.

X-ray characteristics: The minerals contained in the stones and the X-ray permeability of these minerals are grouped as radiopaque, semiopaque and non-opaque (Table 1). It has been reported that non-contrast computed tomography (CT), which is accepted to be superior to IVP in the diagnosis of stone disease, can classify stones in terms of density, internal structure and components, and this is also effective in treatment selection.

Classification according to the formation etiology: The stones are renewed according to etiological reasons with the renewal of terminology and classifications; are classified as infection, non-infection, genetic and drug-related stones (Table 2).

Mixed stones which are CaOx and CaOx-CaPO4 are the most common. While pure CaOx is present in 65-70% of adult stone patients, CaOx is found in 80% of all stones as a mixed, and magnesium ammonium phosphate stones take the second place (Jung H, 2017). Uric acid stones constitute 10% of all stone disease cases. Often they are called "staghorn" stones because they are large enough to take the shape of the collecting system even at the time of diagnosis and resemble a "deer horn". Cystine stones constitute only 2% of all stone cases (Sahota A, 2019). Indinavir, thyroid hormones or handle diuretics used in AIDS treatment, and long-term use of antacids are also predisposing factors in stone formation. It is interesting that stones formed as a result of using indinavir cannot be detected in non-contrast CT.

Table 1. X-ray characteristics of stones

Radyopaque	Semi-opaque	Non-opaque
• Calcium oxalate dihydrate • Calcium oxalate monohydrate • Calcium phosphate	• Magnesium ammonium phosphate • Apatite • Cystine	• Uric acid • Ammonium urate • Xanthine • 2,8-dihydroxyadenine • Drug-stones

Recurrence Risk Factors in Urinary System Stone Disease

In 50% of those with recurrent stone disease, this relapse occurs only once in a lifetime. Except for this 50% section, many recurrent stone diseases are seen in approximately 10% of the cases. Risky groups for recurrent stone disease are presented in Table 3.

Table2. Classification of stones according to their etiology

Non-Infectious Stones	• Calcium oxalate • Calcium phosphate (including burshite and carbonate apatite) • Uric acid
Infection stones	• Magnesium ammonium phosphate • Carbonate apatite • Ammonium urate
Genetically caused	• Cystine • Xanthine • 2,8-dihydroxyadenine
Drug stones	• Indinavir and triamteren

Diagnostic Methods in Urinary System Stone Disease

Kidney-Ureter-Bladder (KUB) Radiography

Direct urinary system radiography is the first step imaging method in urinary system stone disease. Since the majority of the stones are radiopaque calcium oxalate or calcium oxalate-phosphate combinations, they can be easily seen in KUB radiography. Due to its sulfur content, cystine stones are seen as semiopaque in the form of ground glass in KUB radiography. Struvite stones are another semiopaque stone and typically have a "deer antler" appearance. Pure uric acid stones, xanthine, dihydroxyadenine, indinavir, triamterene or matrix stones are non-opaque stones and cannot be visualized on direct radiographs. The sensitivity of KUB radiography varies between 44-77%, and its specificity varies between 80-87% (Kennish SJ, 2008). If CT is planned, a KUB radiography examination is not recommended. However, KUB radiography is helpful in the diagnosis of radiopaque stones. It is used for the follow-up of previously determined stones.

Table 3. Conditions with high risk for recurrent stone disease

General factors	Early onset urolithiasis (especially children and adolescents) Familial stone disease Brushite containing stones (calcium hydrogen phosphate; CaHPO4.2H2O) Stones containing uric acid and urate infection stones Solitary kidney (from the necessity of protection)
Genetic factors	Cystinuria (type A, B, AB) Primary Hyperoxaluria (PH) Renal tubular acidosis type I 2,8-dihydroxyadenine Xanthinuria Lesch-Nyhan syndrome Cystic fibrosis

Diseases associated with stone formation	Hyperparathyroidism Metabolic syndrome Nephrocalcinosis Polycystic Kidney Disease Gastrointstinal diseases (jejuno-ileal bypass, intestinal resection, Crohn's, malabsorption, enteric hyperoxaluria after urinary diversion, bariatric surgery) Sarcoidosis Spinal cord injury, neurogenic bladder
Drugs related with stone formation	Indinavir Triamteren Antibiotics (TMT-SMX, Amoxicillin, ampicillin, Ceftriaxone, Ciprofloxacin, Sulfonamide)
Anatomic abnormalities	Medullary sponge kidney (tubular ectasia) Ureteropelvic junction (UPJ) stenosis Caliceal diverticulum or cyst Ureteral stricture Vesiko-uretero-renal reflux Horseshoe kidney Ureterocele

Intravenous Pyelography (IVP)

Providing very detailed information in determining the anatomy of the kidneys and pelvicalyceal system, IVP also allows the detection of anatomical disorders such as concurrent stenosis and rotation anomalies. However, today it loses its popularity due to the nephrotoxic effects of the radiopharmaceuticals used today and the long exposure times.

Ultrasonography (USG)

It is a non-invasive method that provides important information about the collecting system of the urinary system and parenchymal diseases. It is very safe, especially since nephrotoxic contrast material is not used and it is free from radiation. It also helps in the diagnosis of indinavir stones. However, it could not take its place as a definitive diagnostic tool due to the fact that it varies according to the person making the evaluations

and because of the inability to detect ureteral stones. In one study, the sensitivity of ultrasound in the diagnosis of ureteral stones was 45%, the specificity was 94%, and in another study, its sensitivity was reported as 45% and specificity 88% in the diagnosis of kidney stones.

Computed Tomography (CT)

It gives detailed and precise information about the size and location of the stone, the structure of the pelvicalyceal system and the relationship of the kidney with neighboring organs, ureteral obstruction and mass lesions, and stone-skin distance. With the exception of indinavir stones, all stones can be visualized with CT. In addition, the sensitivity and specificity of CT in the diagnosis of ureteral stones are quite high. In a meta-analysis, the sensitivity of low-dose CT in urinary system stone disease was determined as 96.6% and specificity as 94.9%. In addition, dual-energy CT examination can differentiate uric acid stones from calcium stones (Zheng X, 2016).

Treatment in Kidney Stone Disease

Treatment of kidney stone disease is based on two main principles; these are the location of the stone (lower pole / renal pelvis - middle - upper calyx) and the size of the stone (<1cm, 1–2cm, > 2cm). ESWL is the first choice treatment for stones located in the renal pelvis and middle or upper calyx and <2 cm. PNL is recommended for the treatment of stones ≥2 cm, as multisession treatments may be required in the treatment of ESWL, as well as the risk of treatment-related conditions such as ureteral obstruction (renal colic, steinstrasse). Flexible URS is not recommended for the treatment of stones larger than 15 mm located in the renal pelvis and middle or upper calyx. Although low stone-free rates have been reported with flexible URS, other alternative treatment modalities are recommended for stones located in the renal pelvis and middle or upper calyx. Treatment recommendations of EAU guideline for stones other than lower pole stones between 10-20 mm are summarized in Figure 9 (Türk C, 2016).

Stone-free rates for ESWL in lower pole stones are reported between 25-85%. While PNL and flexible URS are the recommended treatment methods for stones with 15 mm, the effectiveness of ESWL is limited. Factors not suitable for ESWL are; ESWL-resistant stones (calcium oxalate monohydrate, brucite, cystine), steep infindibulo-pelvic angle, narrow infindibulum (<5 mm) and long lower pole calyx (> 10 mm). In recent studies with new URS equipped with developing technologies, flexible URS has been shown to be more advantageous compared to ESWL, although it is expensive and more invasive. EAU guideline treatment

recommendations for approach to lower pole kidney stones between 10-20 mm are summarized in Figure 10 (Zanetti SP, 2016).

Medical Treatment

The stones that medical treatment is most effective are uric acid stones. In the medical treatment of uric acid stones, potassium citrate or potassium bicarbonate and allopurinol are administered, which provide urine alkalization. Captopril, thiopronine, α-mercaptopropionylglycine, N-asteylcysteine and D-penicillamine are used in the preventive treatment of cystine stones (Andreassen KH, 2016). Citruvite stones are stones that are caused by infection and contain magnesium ammonium phosphate. In preventive treatment, first appropriate antibiotherapy and then urine acidification treatments are given to dissolve the stone. Thiazide diuretics are used in calcium oxalate and calcium phosphate stones. The chemical dissolution process is based on the method of dissolving the stones formed by the oral or percutaneous administration of chemicals into the kidney. Two nephrostomy catheters are required for percutaneous thawing, one for instillation and the other for drainage. Suby G solution, Hemiacidrin and Trihydroxymethyl aminomethane (THAM) chemicals are used for this work (Heimbach D, 1995). Stones in which it can be effective have been reported as struvite, carbon apatite, brushite, cystine, and uric acid stones.

Extracorporeal Shock Wave Lithotripsy (ESWL)

ESWL is a form of treatment based on the method of modifying sound waves obtained from a source into shock waves and fragmenting these waves by focusing on the stone. Sound waves were transformed and focused for the first time in 1959 by Eisenmenger. It was first tried on kidney stones by Chaussy in the urology clinic of the University of Munich in 1980, and two years later the first ESWL center was established at the University of Munich. The first machine used is Dornier HM3. The main components of ESWL devices are energy source, focusing system, contact medium (complete water bed, partial water bed and water pad + gel) and imaging system providing stone localization (Ultrasonography and / or fluoroscopy).

Kidney stones <2 cm in diameter and proximal ureter stones <10 mm in diameter can be treated with ESWL. While calcium oxalate dihydrate, uric acid, calcium apatite stones are treated with ESWL with higher success, cystine and calcium oxalate monohydrate stones are relatively resistant to ESWL therapy. However, steep infindibulo-pelvic angle, long lower pole calyx (> 10 mm), narrow infindibulum (<5 mm) are factors that negatively affect success in ESWL.

Absolute contraindications of ESWL are pregnancy, uncontrolled hypertension and bleeding diathesis. Relative contraindications include cardiac pacemaker and renal artery or aortic aneurysm. In addition, anatomic or functional problems such as untreated urinary tract infection, active tuberculosis, obstruction or stricture in the distal of the stone, horseshoe kidney, calyx diverticulum, ectopic kidney, duplicated system, and high stone burden are shown as other contraindications. Treatment of ESWL is difficult in children with morbid obesity and skeletal system abnormalities, and children <100 cm in height. With ESWL, a stone free rate of 75% is achieved. After ESWL, 20% of residual fragments with no clinical significance are detected, and the remaining fragments at a rate of 5% may require intervention. Multisession ESWL requirement is required in 13% of the patients. It has been reported that routine use of ureteral stents before ESWL does not contribute positively to the stone-free rate (Khanna A, 2019). It has been reported that the risk of obstruction and renal colic decreases with DJS, but there is no significant reduction in the risk of infection and stone path (steinstrasse) formation. Increasing the energy applied during the treatment gradually decreases the subcapsular perinephric hematoma rate from 4.4% to 0.45%.

Retrograde Intra Renal Surgery (RIRS)

With the development of flexible ureterorenoscopes, retrograde endoscopic approach to the kidney stones has increased rapidly after the 1990s, and RIRS / PNL ratios are found to be 3/2 in many centers today. With its success better than ESWL and low perioperative complication rates, RIRS has become a minimally invasive outpatient procedure (Rodríguez-Monsalve Herrero M, 2018). Success rates of 90 to 98% have been reported for lower calyceal stones, 2 cm in diameter. According to the cumulative results obtained in recent studies involving stones> 2 cm, the ratio of the procedure performed to the number of patients was 1.45, stone-free rates were found to be 91%, and only 4.5% of complications had a Clavien score ≥3. Due to the lack of postoperative incision scar, besides the cosmetic advantages, the rapid return to daily life can be shown as a positive gain, while cost-effectiveness and multisession may be required, as well as disadvantages.

Laparoscopic Surgery

With the increasing adoption of minimally invasive treatment methods, nowadays open surgery rates can be seen at only 1–5.4%. However, in these rare open surgery indications, laparoscopic surgery is used alone or as an adjunct method. The first laparoscopic treatment of urinary stone disease was performed as laparoscopic ureterolithotomy by Wickham et al. In

1977, and nowadays all open surgeries can be performed laparoscopically. Laparoscopic surgery has advantages such as less postoperative pain, shorter hospital stay, less bleeding and low morbidity compared to open surgery due to its mini incision scar.

Open Surgery

As stated in the title of laparoscopic surgery, although open surgery does not have much place in the treatment today, complicated stone burden, failure with ESWL and / or PNL, infundibular stenosis, intra-calyx diverticulum stone, ureteropelvic stricture, anatomical disorders such as stricture, morbid obesity, skeletal deformity, contracture or fixation deformity of the hips and legs, concomitant medical disease, need for simultaneous open surgery for other reasons, non-functioning lower pole (partial nephrectomy), non-functioning kidney (nephrectomy), patient preference after unsuccessful minimally invasive procedure, PNL or in cases of stone cases in the ectopic kidney where ESWL cannot be performed, and in centers with little experience in endourology / laparoscopy, open surgery is performed. Posterior lumbotomy, anterior transperitoneal and flank approach can be chosen for access to the kidney. Methods used in kidney surgery includes pyelolithotomy, pyelonephrolithotomy, anatrophic nephrolithotomy, multiple radial nephrotomy, partial nephrectomy, and renal surgery under hypothermia.

Percutaneous Nephrolithotomy (PNL)

Great improvements in endourology were confirmed by the technology developed after the process that started with Goodwin et al.'s first implantation of a percutaneous nephrostomy to the hydronephrotic kidney in 1955 and continued with the use of bronchoscope as a nephroscope by Harris et al. In 1975. Fernstrom and Johansson first described PNL as a surgical technique by creating a nephrostomy tract for stone extraction in 1976. Smith and Clayman's long series studies in the 1980s paved the way for the PNL technique to be a viable method. Compared to open surgery, the shorter hospital stay, low cost and rapid return to daily life made PNL advantageous and has replaced open surgery in almost all centers today. The discovery and implementation of ESWL coincided with the early 1980s, and although it pushed the PNL, which had a new development in the treatment of kidney stones, to the background, even if a little, PNL gained its importance again due to the limits of ESWL's success in treatment. Lingeman and Newman reported the stone-free rate of ESWL as 95% for stones <1 cm, 87% for 1-2 cm stones, 48% for 2-3 cm stones and 35% for stones> 3 cm. They showed that ESWL may not be the preferred method of treatment for stones> 3 cm [114]. Pearle et al. Reported that

patients with BMI> 30 kg / m2 do not differ in PNL success from the normal population.

PNL Indications and Contraindications

For PNL; Uncontrolled bleeding diathesis, pregnancy, presence of tumor in the accessory line, potential tumor on the side of the stone, untreated urinary tract infection can be shown as contraindications. Anticoagulant therapy of patients should be discontinued at the appropriate time before PNL. The indications of PNL are summarized in Table 4.

Table 4. PNL indications.

Stone burden	**Staghorn stone** **Renal pelvis stone > 2 cm** **Lower pole stone > 1 cm** **Stones associated with upper system foreign body**
Anatomic abnormalities	**Ureteropelvic obstruction** **Distal ureteral obstruction** **Infundibular stenosis** **Calyx diverticulum**
Patient characteristics	**Obesity** **Scoliosis** **Renal artery and aortic aneurysm** **Patient preference**
Failure of previous treatment	**ESWL failure** **URS failure**
Stone composition	**Cystine** **Calcium oxalate monohydrate**

Preopertive Preparation

In order to avoid the harmful effects of radiation, the source that creates the X-ray in the fluoroscopy device should be under the table, and the device that creates the image should be above the patient. Thus, the amount of light scattered around is minimized. The use of protective lead shirt and thyroid protector together with the surgical team by all staff in the room is absolutely necessary. It is also recommended that the surgeon wear protective lead gloves and goggles. Special attention should be paid to the pediatric age group. Measures such as covering the genital areas

of the patients with a lead shirt can be taken. Fluoroscopy time should be kept as short as possible. Another point that should not be forgotten is that since the effect of the radiation will decrease inversely with the square of the distance, it will be beneficial to move away even for a few steps during fluoroscopy.

Patient Preparation

Systemic physical examination of the patient before PNL and a detailed history constitute the first approach. Serum electrolytes (sodium, potassium, chlorine, bicarbonate) should be examined for the exclusion of distal renal tubular acidosis, serum calcium and phosphorus for primary hyperparathyroidism, and serum uric acid values for hyperuricemia. Serum creatinine values should be considered in terms of providing preliminary information about renal functions, as well as being a benchmark for postoperative follow-up. If the patient has a history of stones, analysis results should be questioned. The routine laboratory tests including preoeprative coagulation factors are evaluated for the operation. All patients should be questioned about the use of antiaggregant agents (ASA, clopidogrel), warfarin, and heparin, and if any such drug use should be discontinued 7 days before PNL (Siev M, 2015). Routine urine test and culture should be taken. Detection of urease (+) bacteria in urine culture suggests that it may be a citruvite stone. In such a situation, preoperative 2 weeks antibiotherapy should be given. The success of the surgery during PNL depends on a good understanding of the anatomy of the kidney. Intravenous pyelography (IVP) and computed tomography (CT) enable the evaluation of fusion or malrotation anomalies, retrorenal colon, ectopic kidney, scoliosis or vertebral deformities and obese patients. Three points are important in the inspection of calyx in IVP; as the relationship with the 12th rib, the degree of hydronephrosis and the presence of any malrotation. Due to the risk of developing pneumothorax, hemothorax or hydrothorax, subcostal intervention should be preferred whenever possible. It is more appropriate to access a dilated and wide calyx. It is recommended that pre-operative imaging containing contrast material or retrograde intervention should be performed, and the placement of the stone and the anatomy of the collecting system should be displayed in order to reach the kidney stone safely.

CT is a more useful imaging method than IVU in patients with previous urinary system surgery history, diverticulum stone, ectopic kidney, horseshoe kidney, transplanted kidney, adjacent organ size (hepatomegaly, splenomegaly), radiolucent stone (uric acid, xanthine), intestinal interposition (retrorenal colon). It is a useful examination for determining the internal structure of the stone, its density and the distance

between the stone and the skin before PNL. However, CT seems to be disadvantageous when comparing CT and KUB radiography radiation intensity. Low-dose CT is recommended for patients with a BMI of <30kg / m2. PNL can be applied safely in obese patients if appropriate sheaths and nephroscope are used (Zhou X, 2017). PNL can be performed under general, epidural and rarely under local anesthesia. Local anesthesia is usually used with sedation and when general anesthesia is contraindicated.

Operation Steps in PNL

Ureteral catheterization is performed endoscopically in the lithotomy position in the urinary tract with the stone. Afterwards, percutaneous intervention can be performed in the supine or prone position. Many studies have not fully demonstrated the advantages of the supine position due to the long operation time. Despite the long operation time in some series, it was found that the stonefree rates of the supine position were lower than the prone. The prone posion offers more options for puncture and is therefore preferred for upper pole or multiple access. On the other hand, the supine position provides simultaneous retrograde access to the collecting system using flexible URS (Giusti G, 2020). Recently, the procedure has been started using the Galdakao Modified Supine Valdivia (GMSV) position instead of the complete supine position. In this position; The lower extremity on the side of the stone is extended, and the contralateral lower extremity is abducted and flexed. Thus, retrograde interventions can be performed simultaneously. The ipsilateral upper limb is taken to the opposite side by crossing the rib cage and removed from the operation area. The ipsilateral lumbar region is raised 20 degrees with an auxiliary device. Thus, the lower calyx moves laterally and becomes perpendicular to the operating table.

Retrograde pyelography (RGP) is performed by administering opaque material from the ureter catheter to visualize the pelvicalyceal structures and to determine the entry site. Percutaneous access can be performed with fluoroscopy or USG. Sometimes it can be performed under direct vision using flexible URS, especially in PNL operations performed in the supine position. Preferably, access should be made through the posterior calyx. Thus, the risk of injury of major vascular structures around the renal pelvis is minimized. However, in the presence of calyceal diverticula and localization of stones, anterior access may be required. Direct puncture to the renal pelvis should be avoided. Because the posterior branch of the renal artery can be injured. Pleural injury may occur during intercostal intervention. Subcostal interventions should be preferred whenever possible, and if possible, upper pole subcostal approaches should be tried in the deep insprium. Nevertheless, if intercostal interventions are

required, it should be checked with anterior-posterior lung radiography in the postoperative period.

Situations that require upper pole intervention;

- If the stone burden is in the upper pole, especially if the bifid pelvis or calyx neck is long

- Aiming to have a large number of stones in all three poles

- Multiple entry situations

- Presence of Staghorn stones

- In patients with horseshoe kidneys

- Morbid obese patients

- If there is a stone in the UPJ and proximal ureter

Lower pole interventions are the most convenient and safe place for access to the pelvicalyceal system. For this reason, it is the most preferred area. The rate of vascular injury is 13% in the procedures performed from the lower pole infundibulum. Arterial injury does not occur only in procedures performed in this area. There may also be venous injuries, but these are spontaneously controlled. In conclusion, situations such as interlobar or arcuate artery injuries in infindibular entrances, and entering the anterior calyx through the posterior calyx are unreliable due to the risk of occurrence.

The pelvicalyceal system is visualized under fluoroscopy by administering opaque material from the previously placed ureter catheter. Although the ideal entry point is variable, the shortest path is determined from the inferior of the 12th level to the targeted calyx. The image taken at 90º of the scope's C-arm determines the medial vertical plane after entering the calyx. In cases where the surgeon's entry experience is not sufficient, the scopy C-arm is tilted towards the 30º surgeon, and an image is obtained parallel to the long axis of the calyx to be inserted, with this image under the scope, when the tip of the needle is superposed with its body, the image formed is called the "bull's eye sign". In the postoperative period, 0.25% bupivacaine can be injected locally at the entrance site in order to prevent the pain that the patient will feel (Khan SA, 2018). After determining the entry point, an approximately 1 cm incision is made to the skin and an 18-gauch needle is entered. After the introduction, the soft tip of the 0.038 inch guidewire is pushed through the needle, allowing it to lean against the wall of the pelvicalyceal system or go to the ureter. Then the tract is dilated over the guidewire. Balloon dilators or amplatz dilators are used for the dilatation of the entrance tract. While the advantage of balloon dilation is shown to be homogeneous power distribution and less traumatic, it is an expensive method that constitutes its biggest disadvantage. Due

to the mechanical force applied in Amplatz dilatation, collecting system perforation and bleeding are likely to develop. However, it provides an advantage because it is reusable in experienced hands. Metal telescopic dilators are used in some centers. Complication risk is much higher in these types of dilators, which are more rigid compared to Amplatz dilators.

Percutaneous Removal of Stones

Making a proper entry in PNL surgery is the first step to success. Thus, it provides easy maneuverability during fragmentation and subsequent extraction, as well as facilitating access to the stone. After dilatation, a 30 Fr sheath is placed as a standard. Smaller diameter sheaths can be used in pediatric age groups and weak patients. The advantage of large diameter sheaths is that they allow the removal of larger diameter stone fragments as well as lower working pressure. Using a large diameter (24-27 Fr) rigid nephroscope provides a clearer visualization with the high pressure of the supplied water and the convenience of the instruments used for stone fragmentation and extraction with its wide lumen. It states that there is no significant difference between mini and standard PNL in the EAU guideline, although lower blood loss is detected in mini PNL, the operation time is longer, the stone-free rates are close to each other, and current literature information is not sufficient to make this comparison.

Lithotriptors in PNL

A wide variety of lithotriptors and their combinations can be used for fragmentation of stones in PNL, depending on the preference, experience and physical facilities of the surgical team. These are holmium laser, pneumatic and ultrasonic lithotriptors.

Use of Flexible Nephroscope in PNL

Although rigid nephroscope is mostly preferred in PNL, flexible nephroscope may also be needed. These situations;

- To see if there are residual stones in non-opaque stones at angles that cannot be rotated with a rigid nephroscope.

- To see the UPJ more clearly and to see if stones have escaped into the ureter by taking an antegrade nephrogram after the procedure.

It can be used to place a guidewire in the collecting system in cases of narrow renal pelvis.

In order to enter all calyceal structures, opaque material should be given and imaging should be done with fluoroscopy. A ‘nitinol stone basket’ can be used to remove small stone fragmentations.

Post-PNL Nephrostomy Insertion

Until recently, the standard method as urinary diversion in PNL surgeries was nephrostomy placement, and this condition was called PNL with tube. Applying nephrostomy has advantages such as providing urine drainage after PNL, monitoring bleeding, providing easy recovery and providing convenience when a second nephroscopy procedure is required. Different diameters of nephrostomy can be chosen. Small-diameter nephrostomies are now considered to be advantageous for postoperative pain. In addition, PNL operations without nephrostomy performed by keeping the ureteral catheter inserted preoperatively for 1-2 days are called tubeless PNL and operations performed without leaving the ureteral catheter are called total tubeless PNL. Tubeless PNL stands out due to the short hospital stay and the lack of a reported disadvantage (Xun Y, 2017). In the EAU guideline, it is stated that it is a safe alternative in uncomplicated cases, tubeless or total tubeless PNL is recommended. Again in the same guideline, it is stated that nephrostomy can be applied in the following cases:

- The presence of residual stones
- Possibility of second look
- Intraoperative bleeding
- Urine extravasation
- Ureteral obstruction
- Persistent bacteriuria due to infected stones
- Solitary kidney,
- Bleeding diathesis,
- Planned percutaneous chemolysis

Complications of PNL

It is possible to encounter intraoperative and / or postoperative complications in PNL operations despite the increasing experience and technique and rapidly developing technology. The most common intraoperative complication is bleeding. Bleeding is more common in patients with hypertension, chronic renal failure (CRF), urinary tract infection, previous kidney surgery or a history of ESWL. In the literature, the mean hemoglobin decrease level has been reported as 1.2 mg / dl and the frequency of transfusion as 3%. Clamping the nephrostomy tube is beneficial in cases of excessive bleeding. Perioperative fever can occur even with sterile preoperative urine culture and perioperative antibiotic

prophylaxis, as renal stones themselves can be a source of infection. Intraoperative kidney stone culture may therefore aid postoperative antibiotic selection. Sepsis can be seen in 0.3-2.5% of patients. The existing urinary system infection should be treated in the preoeperative period and urine sterilization should be provided. If infection stones are suspected, the removed stones should be sent for analysis and confirmed, and long-term antibiotic treatment should be given to infection stones. Intraoperative irrigation fluid pressure should be less than 30 mmHg. Preventing obstruction of postoperative urinary drainage is an important factor to prevent postoperative sepsis. Other rare complications are urosepsis, unsuccessful entry, inability to reach the stone, pneumothorax, adjacent organ injury, vascular injury and death. Extravasation occurs when the retroperitoneal area is filled with irrigation solution, urine or opaque material and is one of the most common complications of PNL. The reason is the collecting system laceration caused by percutaneous entry, tract dilatation or stone manipulation. When the laceration is large, a nephrostomy tube is placed at the end of the operation to provide drainage. Nephrostomy is kept between 2 and 7 days, and the patient is followed up with antegrade pyelography. Most of the time, laceration heals without additional intervention. In some cases, urinary catheterization, such as DJS, or even more rarely, open surgery is required in clinically unstable patients. Hemothorax, hydrothorax or pneumothorax may develop secondary to pleural damage after intercostal access, and if it impairs respiratory functions, a chest tube placement may be required. For this reason, patients with intercostal access must be checked in the postoperative period, and chest x-ray should be taken after respiratory system examination (Ajib KM, 2017). In the study by Hopper and Yake, they reported that 86% of the pleura and 29% of the lung were injured in intercostal entrances after full expiration, and 79% of them were seen on the right side and 14% on the left side. Another rare complication in PNL is intraabdominal organ injury. Mostly, colon injuries are retroperitoneal. Conservative follow-up option is available for the patient in extraperitoneal colon injuries. Pelvicalyceal system pressure is reduced by providing urinary drainage with ureter catheter or DJS. The nephrostomy tube, which is in the intrarenal position, is placed in the intracolonic position and it is ensured to function as a colostomy. The colostomy tube is kept for a minimum of 7 days and removed after a nephrostogram or retrograde pyelogram demonstrates that there is no fistula tract between the colon and the kidney (Maghsoudi R, 2017). Exploration is required in cases of intraperitoneal and colon injury with large defects. Liver and spleen injuries are much less common. While minimal lacerations are followed conservatively, exploration should be performed in large lacerations and life-threatening bleeding. Bleeding that continues in the late postoperative

period should suggest arteriovenous fistula or pseudoaneurysm. This type of bleeding is treated with superselective embolization of the segmental artery from the renal artery branches and occurs in less than 0.4% of all patients. Unsuccessful entry is seen less than 5% and is especially encountered during the first PNL experience. The mortality rate for PNL ranges from 0.05% to 0.3%. There is no specific standardized classification of perioperative complications. The classification made by Clavien et al. In 1992 was updated in 2004 and 2009. According to this classification; Grade 1 complications include abnormal changes in the postoperative period that do not require any invasive intervention. Diuretics, antiemetics, antipyretics, anti-inflammatories and balanced electrolyte solutions are used in the treatment of 1st degree complications. Second degree complications are situations that require the use of medications and blood products such as total parenteral nutrition (TPN) products, blood transfusion or other antihypertensive drugs. Third degree complications include conditions that require invasive intervention such as open surgery or endoscopic intervention. This has two subclasses. 3A are complications that can be intervened under local or regional anesthesia, while 3B are cases that require intervention under general anesthesia. Grade 4 complications include organ disorders. Single organ disorder is included in the 4A subclass, while the multiple organ disorder is included in the 4B subclass. The 5th degree complication is the loss of the patient resulting in death.

Conclusion

Urinary system stone disease is one of the most common urological diseases. Especially the treatment of kidney stones is a difficult situation for the physician and the patient. With the developing technology, minimally invasive methods have replaced traditional methods for kidney stone treatment. The gold standard minimally invasive method in kidney stone treatment is still PNL, and it can be safely applied to all types of kidney stones in experienced centers.

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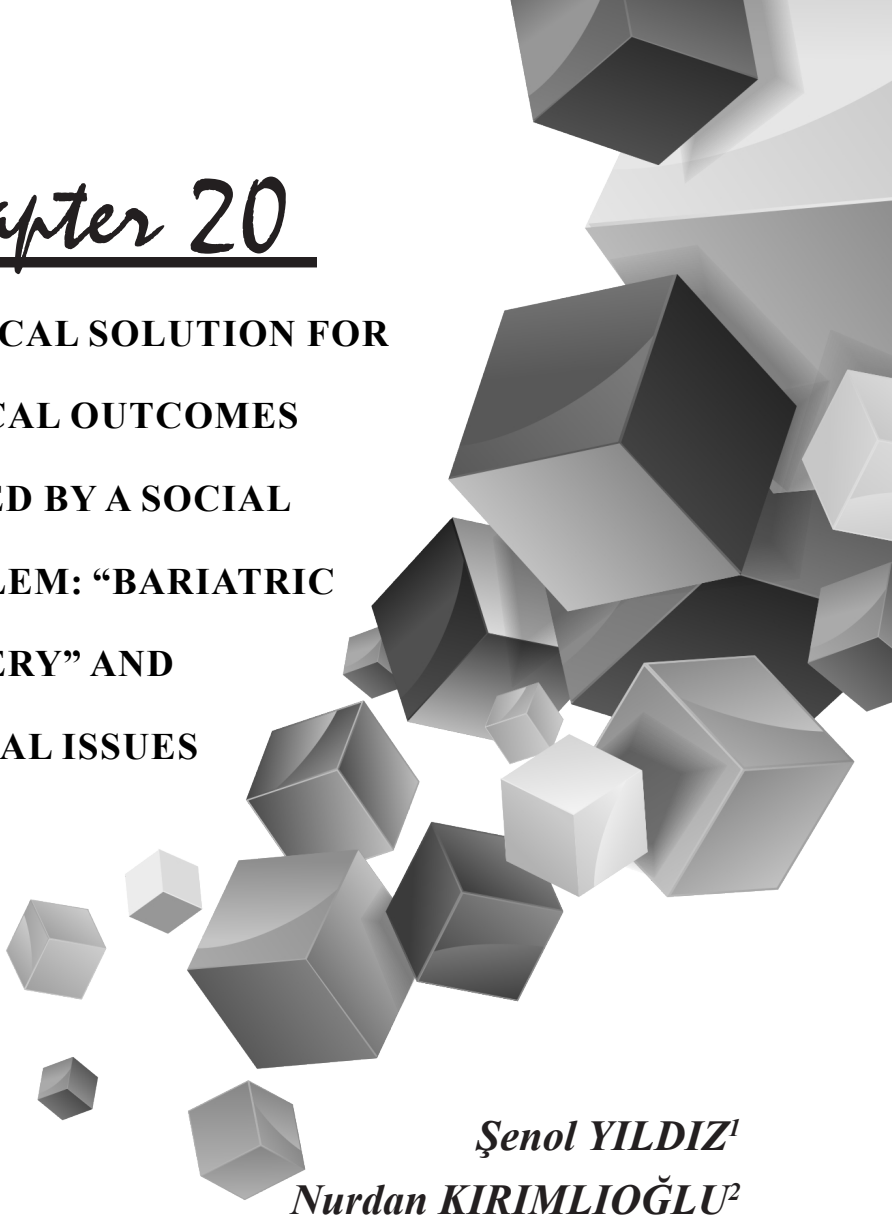
Chapter 20

SURGICAL SOLUTION FOR MEDICAL OUTCOMES CAUSED BY A SOCIAL PROBLEM: "BARIATRIC SURGERY" AND ETHICAL ISSUES



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Obesity, derived from the word "*obesus*", that means "fat" in Latin (Alphan, 2013), is referred with the words such as strength, potency, sovereign, and majestic, as well as fertility, abundance and plenitude throughout the history.

Perspective of the society on obesity, which is the symbol of the continuity of life and power in ancient gods, is completely changed with the industrial revolution. The search for individuals having physical qualities to adapt this movement in the fast working pace of the day has caused individuals to live with obesity that will be referred with the words such as "bulky, problematic, slow, and unhealthy" (Bozbora, 2002).

Obesity is an important public health problem that concerns all the age groups, affects the health of individuals negatively in various aspects, has an increasing prevalence, can be treated, and has chronic, progressive, social, and psychological aspects (Yıldırım, Akyol & Ersoy, 2008; Baysal, 2009). The concept of obesity is closely related not only to the diseases but also to the concepts of self-control and body ideals (Hoffman, 2010).

Body weight, that is defined as the sum of bones, teeth, muscles, organs, body fluids, and adipose tissue, varies with growth, reproduction, physical activity, and age. The body fluids that make up approximately 60% of the body weight of an adult vary about 1-2 kg depending on water intake and dehydration. While age-related changes are observed in the bones and muscles, adipose tissue varies depending on energy intake and the physical activity level (Baysal et al., 2011).

Obesity is a chronic disease characterized by the increased mass of adipose tissue (Akbulut, 2016). World Health Organization (WHO) defines obesity as: "*abnormal or excessive fat accumulation in the body that may impair health"* (WHO, 2017).

Obesity is increasing day by day throughout the world and it is predicted that this increase will continue. According to 2016 data, while the rate of individuals with obesity living in Turkey is 19.6%, it is specified as 15.4% on average in EU countries as given in 2014 data in the most recent study of European Union (EU). Among the countries examined, Malta takes place on the top, and Turkey follows Latvia and Hungary. (TURKSTAT, 2017). In case the current trend continues, it is predicted that 60% of the world population will be affected by obesity in 2030, and there will be 2.2 billion overweight and 1.1 billion obese people in the world (Kelly, Yang, Chen, Reynolds & He, 2008).

Classification of Obesity

Various methods are used for the classification of obesity. The most common method is *body mass index* (BMI) (Merdol, Başoğlu & Örer,

2011; Akbulut, 2016). BMI is calculated by dividing the body weight (kg) by the square of the height (m^2) (Orhan-Bozbora, 2008).

Different definitions of obesity make the results obtained from the studies complicated. Although '*overweight'* and '*obesity*' concepts are used interchangeably (Hinds, 2005), overweight should have a body mass index (BMI) between 25.0 and 29.9 kg/m^2, however obesity has a BMI value of 30.0 kg/m^2 and over (Secretariat of the Pacific Community, 2002).

Classification of BMI (Bozbora, 2002), which is a practical method for the evaluation of the weight of individuals with obesity, is given in Table 1.

Table 1. Classification of BMI *(WHO, 2016)*

Classification	**BMI (kg/m^2)**
Weak	**<18.50**
Severely weak	*<16.00*
Moderately weak	*16.00-16.99*
Mildly weak	*17.00-18.49*
Normal	**18.50-24.99**
Slightly obese	**>25.00**
Pre-obese	*25.00-29.99*
Obese	**>30.00**
Class I obese	*30.00-34.99*
Class II obese	*35.00-39.99*
Morbid obese	*40.00-49.99*
Super morbid obese	*≥50.00*

Etiology of Obesity

The reasons of obesity may include bad feeding habits, environmental factors (chemical and obesogenic environment), familial and ethnic factors, genetic factors, adipose cells, drugs, stress, central nervous system damage, infectious diseases, endocrine and metabolic diseases, disorders in the regulation of body weight, and the changes in intestinal microbiota (Akbulut, 2016). Although there is no definite information about the mechanisms that cause obesity in the "only true" nature, it is stated that the complex interaction of genetic, environmental, and behavioral factors play a role in the development of obesity (Voigt-Schmidt, 2011).

Treatment of Obesity

Obesity treatment is a specialized situation because the body perceives weight loss as a catabolic condition and tries to take precautions (Arslan, Dağ & Türkmen, 2012). Treatment of obesity requires a multidisciplinary teamwork. As the authorities and responsibilities of the team members are different, it is essential to share information and experiences. Considering that the quality of life of the individuals with obesity decreases both in relative and absolute terms and their health is under risk, the importance and priority to be given to the obese population in health services is obvious (Persson, 2014).

Different methods are used in the treatment of obesity. These are:

1. Medical nutrition therapy
2. Exercise therapy
3. Psychosocial support and behavior therapy
4. Pharmacological treatment
5. Surgical treatment (Turkish Endocrinology and Metabolism Society, 2014).

When a selection is done between these treatments, a personal approach should be used to balance the harm of obesity on the patient and the risks that will occur during the treatment.

Many individuals think that traditional treatment, including lifestyle changes such as diet and exercise, is superior to surgical intervention. The reason is that traditional approach is economic and safe as well as it has no adverse effects (Persson, 2014).

Lifestyle changes are the cornerstone of obesity treatment and also pharmacological treatment and bariatric surgery are becoming more common every year (Hoffman, 2010).

Medical nutrition therapy

Medical nutrition therapy aims to reduce body weight to the targeted level, to meet all the nutritional requirements of the patient in an adequate and balanced manner, to ensure that the client gains correct nutritional habits instead of bad eating habits, and to preserve the current weight without regaining the weight lost.

The first step of the planned changes is to understand the current situation of the patient and to develop a collective consciousness environment. In order to initiate training on new eating habits, it is required to evaluate the existing nutritional habits and food consumption status of the person.

The changes to be made should be acceptable by the patient in terms of taste and applicability as much as possible. Otherwise, the patient may not adapt the recommended nutrition therapy. Thus, it is necessary to consider the nutrition therapy specified by the dietitian and to perform the controls of the patient at regular intervals (Ministry of Health, 2013).

Exercise therapy

Exercise, which is an important tool in the treatment of obesity, increases energy expenditure and reduces the loss of lean tissue caused by diets with limited energy. Exercise together with reduced energy intake provides more body weight loss than diet alone and muscle mass is preserved. Therefore, it is recommended to include exercise in the programs aimed at reducing body weight (Ministry of Health, 2013). It is very difficult for obese individuals, who only do exercise, to maintain the weight after they fell to their ideal body weight (Rinsho, 2013).

Psychosocial support and behavior therapy

Combination of the lifestyle changes such as diet and physical activity with cognitive-behavioral interventions in the treatment of obesity increases the effectiveness of the treatment and ensures the persistence of body weight. The therapy includes self-monitoring, stimulant control, eating control, reinforcement and strengthening, cognitive restructuring, correct nutrition training, and increasing the level of physical activity (Oğuz, Karabekiroğlu, Kocamanoğlu & Sungur, 2016).

Medical (pharmacological) treatment

Drug treatment in obesity means using certain drugs to prevent fat accumulation and increase fat burning by reducing appetite and fat absorption.

Despite it has been stated that the effectiveness of the drugs used in the treatment of obesity is quite low when compared to surgical treatment, all the drugs to be used in medical treatment are more risky than diet and exercise, so the benefit-loss ratio should be considered when deciding about the medical treatment (Adaş & Mert, 2014; Hoffman, 2010).

Surgical treatment

Treatment of obesity via surgical methods was first reported by *Kremen ve Linner* in 1954 as *"jejunoileal bypass method"* . As of this date, developments in bariatric surgery have gathered momentum.

Bariatric surgery is the most controversial, probably the most effective,

method in the treatment of obesity (Persson, 2014). Bariatric surgery, that has been ever-increasingly applied in many countries, especially in the United States (USA), is seen as a surgical solution to the medical consequences caused by a social problem (Arslan, Dağ & Türkmen, 2012; Hoffman, 2010).

Although bariatric surgery can be performed by using open and laparoscopic techniques, laparoscopic methods are preferred more. Surgical treatment methods are classified in three ways as the methods limiting the amount of foods that can be consumed, the methods reducing the absorption of nutrients, and the methods having both effects (Dikmen & Ersoy, 2016). Gastric restrictive procedures are generally accepted as safe and fast for application however, long-term results and quality of life have been examined (Korenkov, Sauerland & Junginger, 2005). Table 2 shows the treatment methods in bariatric surgery and the ratios of reducing body weight.

Table 2. Treatment Methods in Bariatric Surgery and Ratios of Reducing Body Weight *(Dikmen & Ersoy, 2016, s. 23; Sauerland, et al., 2005).*

Primary Restrictive Procedures	**Primary Malabsorptive Procedures**	**Primary Combined Procedures**
• Laparoscopically adjustable gastric band *(45-55%)* • Vertical band gastroplasty • Sleeve gastrectomy *(55-65%)*	• Biliopancreatic diversion *(65-75%)* • Biliopancreatic diversion and Duodenal switch	• Roux-en-Y gastric bypass *(60-70%)*

Bariatric surgery has some side effects such as cardio-respiratory insufficiency, venous thromboembolism, wound infections, anastomotic leakages, and chronic gastrointestinal symptoms (Hoffman, 2010). Besides, there are studies showing that there are no or very low rates of deaths caused by surgery, even they reduce obesity-related mortality rates (Puila, Puila & Cristea, 2016; Hoffman, 2010; Christou, Sampalis & Liberman, 2004; Buchwald, Avidor & Braunwald, 2004; Lee, Huang, Wang, 2004; Schauer, 2007).

It is stated that bariatric surgery both provides a reduction in body weight and significantly increases the quality of life (Persson, 2014). There are findings that bariatric surgery provides 77% recovery in type 2 diabetes, 66% in hypertension, and 88% in sleep apnea syndrome (Hoffman, 2010). Controlled and observational studies show that surgery provides significant and sustained weight loss when compared to other forms of treatment (Maggard, Shugarman & Suttorp, 2005).

Indications of bariatric surgery

- Having BMI >40 kg/m² and over,
- Having BMI >35 kg/m² and inability to control obesity-related comorbidities (type 2 diabetes, obstructive sleep apnea syndrome, severe hypertension etc.) with medical treatment and lifestyle changes,
- Previously applied non-surgical methods with unsuccessful outcomes (Turkish Endocrinology and Metabolism Society, 2014).

Bariatric surgery in Turkey

Bariatric surgery is applied in different ways depending on the health policies of the countries. In Turkey, conditions required for the implementation of bariatric surgery are adjusted via the 13th article of the Communiqué related to Making Amendments in the Health Implementation Communiqué (HIC) that is published in the Official Gazette dated February 4, 2018 with no. 30322.

ARTICLE 13 – 2.4.4.O – Bariatric Surgery

(1) Below-mentioned rules are adapted in the bariatric surgery procedures specified in HIC and its attached lists.

a) For people with BMI> 40 kg/m²; a medical board report is required that consists of the general surgeon, endocrinology specialist (internal medicine specialist in the absence of an endocrinology specialist), mental and mental health diseases specialist, and an anesthesia and reanimation specialist and also the medical indications.

b) For people with BMI 35-40 kg/m² and with accompanying disease (coronary artery, diabetes mellitus, hyperlipidemia, hypertension, sleep apnea, degenerative osteoarthritis that causes movement restriction and vertebral disc degeneration) a medical board report is required to be drawn up by doctors of general surgery, endocrinology (in case of no endocrinology specialist, an internal medicine specialist) mental diseases, anesthesia and reanimation and by the relevant specialist of the existing accompanying disease.

c) For the medical board report for people with BMI 35-40 kg/m² and with accompanying disease (coronary artery, diabetes mellitus, hyperlipidemia, hypertension, sleep apnea, degenerative osteoarthritis that causes movement restriction and vertebral disc degeneration) ukları) it has to be indicated that there has been no weight loss with a minimum of 6 months life - style change under the supervision of a specialist and/or with medical treatment.

ç) The Medical Board consists of the physicians working at the health care service provider and is valid at the relevant health service provider.

d) Health care facilities, where bariatric surgery will be performed, should have a third-level adult intensive care unit and an endoscopy unit, and an operating room having the necessary infrastructure and surgical equipment for the conditions of obesity (Official Gazette, 2018).

Ethics in Bariatric Surgery

Bariatric surgery, which is seen as an effective treatment of morbid obesity, does not eliminate the social causes of obesity and does not solve the epidemic of obesity at the public health level. Although the number of surgical operations in obesity has significantly increased, the number of obese individuals, who can potentially benefit from surgical treatment, exceeds the capacity and available resources in many countries. It is not clear yet how bariatric surgery will affect the obese individuals, who are subject to discrimination in many ways (Saarni et al., 2011).

In recent years, significant developments have provided in bariatric surgery. These developments have occurred in the form of increasing the quality assurance of institutions, keeping the records, training the surgeons, and certification of the institutions. Despite such developments, no major development has gained ground in developing ethical standards in this area (Dixon, Louge & Komesaroff, 2013). However, bariatric surgery is an important form of treatment that should be evaluated ethically with its intervention, conditions, and outcomes.

Bariatric surgery reveals various ethical questions such as *"Is obesity a disease, does individuals living with obesity get better with surgical treatment, what does" good "mean here, does bariatric surgery harm the autonomy and integrity of the patient, is bariatric surgery a result of social prejudice and does prejudice prevent the treatment efficacy, is there an unfair distribution of bariatric surgery, does the surgery option fully provide obesity treatment, for whose interests the bariatric surgery provide service, does the surgery option question the accuracy of the patient and physician relationship?"* (Hoffman, 2010). These questions regarding bariatric surgery will be addressed in principle.

Autonomy and the principle of respect for autonomy

Surgical treatment will directly affect the daily life of the individuals with obesity by limiting the choices for lifestyle. This revives the question of whether surgical treatment reduces the autonomy of the patient.

Obesity, which is often referred to as a *"lifestyle disease"*, affects the autonomous choice of the individual and the action of the individual

autonomously in terms of the level of meeting their nutritional needs. This situation causes obesity to be considered as *"an indicator of frailty and character defect"* . However, it is suggested that obesity may have a genetic origin and impair the autonomy of the individual. In both cases, it can be specified that the need for bariatric surgery may be caused by the lack of autonomy.

A potential lack of control over nutrition does not reduce the ability of the individual to consent to bariatric surgery. Within this scope, obesity is not a sign of impaired autonomy.

Although surgery seems to be the most effective intervention for the treatment of obesity, submission of surgery and a specific method as the only option prevents the autonomy of the patients (Hoffman, 2010). It is extremely important to inform the patients and actively support their autonomy, since the balance of benefit and loss is sensitive in bariatric surgery (Saarni et al., 2011). In order to protect the autonomy and the principle of respect for autonomy, the individual should meet the qualification criteria and be informed (Moreira, 2017).

Considering that there is a asymmetrical knowledge between the patient and the physician, it is likely to have the surgeon convince the patient in the desired direction. In order to minimize this situation as much as possible or to equalize the asymmetrical knowledge level, it is essential to obtain the consent of the patient (Dikmen & Ersoy, 2016). Consent is one of the prerequisites of good medical practice and is based on the principles of autonomy and respect for autonomy, which are the basic principles of medical ethics (Turkish Medical Association, 2013). Pursuant to this principle, patients have the right to obtain information about their health status, medical procedures that are required to be applied, related outcomes, the consequences that may arise in the absence of medical intervention, and the development of the disease (Dikmen & Ersoy, 2016).

Consent of the patient should include the realistic projections about the short- and long-term risks, benefits, and outcomes of surgery as well as the alternatives to bariatric surgery (Wee et al., 2007). The patient should be informed about the surgical procedure, and the postoperative monitorization protocol, and most of all, the patient and the physician should talk about the postoperative expectations (Yüksel, 2016). It should be explained that the operation will not create a miracle and the outcomes desired are only possible with the compliance of the patient with the follow-up protocols in the postoperative period (Orhan & Bozbora, 2008). Informed consent, detailed preoperative patient evaluation, and selection of the appropriate procedure are mandatory for obtaining good results in bariatric surgery.

In order to have the patient make decisions about the option of surgery, it is not only necessary to obtain the information given by the health care professional, but also to be able to realize this information (Wee et al. 2007). Risks can vary up to 20 times depending on the patient population. Understanding the risks, benefits, and consequences of surgery weakly leads to unrealistic expectations, non-optimal decision making, and also legal problems (Kaufman, McNelis, Slevin & LaMarca, 2006). Instead of just serving as a legal barrier, consent provides the patients with easy-to-understand and missing information required to provide authorization for the proposed surgery (Kaufman et al., 2006).

Knowledge and attitudes towards bariatric surgery play a key role in accepting surgery as an option in the treatment of obesity (Sikorsky et al., 2012). Regarding bariatric surgery, patients receive information from the internet, television, radio, books, newspapers, magazines, or friends. Information on bariatric surgery in printed and visual media has different and conflicting content with a low quality (Hoffman, 2010). It is clear that people frequently use internet to learn about bariatric surgery and that the information obtained affects their attitudes and behaviors. According to a study, information on internet regarding bariatric surgery has low- and medium-quality regarding the DISCERN criteria (Akbari-Som, 2014).

Information having low reliability and inaccurate information about bariatric surgery increase the anxiety of the patients and prevent them from accessing treatment, and lack of information and inadequate communication with the patients before the surgery lead to unsuccessful post-operative outcomes (Dagan et al., 2018).

In a study conducted with obese (33.6%) and non-obese (66.4%) individuals, despite 70.8% of the participants specified that surgery resulted in weight loss, only 16.2% stated that surgery was the best option in the treatment of obesity (Aldawqi et al. 2018). The current study draws attention to the lack of knowledge and prejudice regarding surgery. Among bariatric surgery candidates, briefing activities are effective on providing nutritional information for surgery, learning the risks, optimizing the expectation for weight loss, reducing the anxiety level, and the patient's decision to operate as an autonomous individual.

Studies show that the patients, who had undergone bariatric surgery, had no knowledge about possible complications or forgot the information given in the preoperative period, before they gave a decision about surgery. In a study conducted, only 20.3% of the participants had a positive opinion about bariatric surgery and they thought that deaths occurred after bariatric surgery are caused by surgical procedures (Sarwer et al., 2013). The level of knowledge about the options of surgical treatment for

obesity among the individuals providing the indications is far from being satisfactory. Individuals, who have not tried the surgery option yet, also have incomplete and erroneous information about bariatric surgery, their autonomy becomes damaged, and the death news, especially on the media, leads to the approach to surgery with bias (Hoffman, 2010; Madan et al., 2007; Sikorsky et al., 2012).

Non-Maleficence

Failure to achieve the desired results and the complications as a result of the surgery underline the necessity of non-maleficience in bariatric surgery (Caniano, 2009). Innovations in bariatric surgery have the potential to cause much maleficence far beyond the postoperative period. There are uncertainties regarding the effectiveness, the need for revision surgery, or the irreversibility of surgery, nutritional deficiencies, sustainability of body composition, and also functional and psychosocial outcomes. Bariatric surgery, that aims to create permanent changes by its nature, requires clinical studies in order to ensure the necessary confidence in the collective use of a new procedure related to the principle of non-maleficence (Dixon, Logue, Komesaroff, 2013).

The issue that medical treatments and preventive measures may cause discrimination, stigmatization, or excessive medicalization of obese patients is considered within the principle of non-maleficence (Torres, Valderrama, Serrano, 2019).

Since the body is seen as "an important part representing the individual" in Western society, obesity gets far beyond a medical problem. A thin body is considered as beautiful and interpreted as normal and healthy. Owing to the control of eating habits is seen as a way of disciplining the body (Moreira, 2017), overweight and obese individuals are seen as lazy, unmotivated, maladaptive, imprecise, careless, weak-minded, and deprived of self-discipline (Hoffman, 2010). Such prejudices cause discrimination by attributing the body integrity and dignity of obese individuals to their external appearance, and the individual feels himself/herself under pressure in order to undergo a surgical operation, and they tend to have surgery as if it is the only option without volunteering, although it is not necessary or the conditions are not appropriate.

Obesity is a psychological, physiological, and social health problem. On the other hand, it is obvious that a study should be conducted regarding the striking prejudiced, discriminatory, and stigmatizing behaviors of the members of society against obesity (Hoffman, 2010; Ercan, Ok, Kızıltan & Altun, 2015). Individuals with obesity are approached with prejudice due to their weight (Voigt-Schmidt, 2011) and this discrimination is

rapidly increasing in the society. Some anti-obesity campaigns even use stigmatization of individuals with obesity as a public health strategy. (Vartanian-Smyth, 2013).

Unfair access to bariatric surgery may increase prejudice and discrimination against obese individuals. Discriminations against obese individuals are characterized by the inequalities in employment, health care, and educational institutions. Prejudice is common against obese individuals, including health care professionals involved in the management of obesity (Rouleau, Rush & Mothersill, 2016; Hoffman, 2010).

Bariatric surgery will help to reduce inequalities and discrimination when it is only considered as being more than a simple surgical procedure that shapes the body of the individual.

Principle of beneficence

Beneficence supports the well-being of the patient, partial or complete recovery of health, and increase in the quality of life. Bariatric surgery supports the principle of beneficence in alleviating the comorbidities of the patient (Caniano, 2009).

As in the management of other chronic diseases, obesity is a health problem that should be kept under control by losing weight and maintaining the final weight (Blüher & Kiess, 2011). The most important indication of bariatric surgery is to have BMI 40 kg/m² and above. In fact, this is the peak value at the point where the risks that may arise for the benefit of the surgery to be performed rather than a necessity are considered. It is an ethical problem whether surgery is necessary for any patient (Puila, Puila & Cristea, 2016). The beneficence to the provided for the patient is possible by choosing the right patient and the right surgical technique (Dikmen & Ersoy, 2016; Turkish Endocrinology and Metabolism Society, 2014).

Surgical procedures, care, and monitorization in obesity will prevent the benefit to be at the intended level if it is not planned specifically for the patient. The long-term and successful outcomes of bariatric surgery depend on the changes in the life style of the patient such as adequate-balanced diet and physical activity. It is inevitable to evaluate and monitor the eating habits, social, environmental, and clinical conditions of the patient with an ethical approach before the surgery (Yüksel, 2016). The patient should be followed up by an interdisciplinary team that includes an experienced bariatric surgery specialist, endocrinology specialist or an internal medicine specialist, dietitian, psychiatrist or a psychologist, nurse, and a coordinator working in cooperation with the clinical ethics committee (Ministry of Health, 2014). Interdisciplinary approach in bariatric surgery is an indispensable principle for maximizing the benefit to be provided to the patient.

Nutrition is a basic requirement having strong social and cultural aspects. As bariatric surgery changes the medication regimes and socialization skills of the patients, it deeply affects the eating habits and consumption preferences. Within this scope, bariatric surgery will provide an extensive intervention in the daily life of the patient. A decrease in body weight will increase the sense of control of the individual on his/her own body and will also increase the quality of life.

Principle of Justice

The health care system is constantly faced with lack of resources (Persson, 2014). Due to the high costs of bariatric surgery, collection of resources from other departments of health care services is in question (Hoffman, 2010). Due to the limited resources allocated to health care, the possibility of transferring resources from basic health care services to expensive technologies or reducing the resources allocated to basic health care practices bring disputes in terms of medical ethics. Also, the insufficiency of primary and preventive health care services can cause a vicious circle that will result in an increase in the prevalence of obese patients (Dikmen & Ersoy, 2016).

Strict indications oriented to bariatric surgery, surgical guidelines, advertisements, gender and differences in sexual orientation, age and ethnicity discrimination can cause an unfair distribution in bariatric surgery (Puila, Puila & Cristia, 2016). Studies indicate that the causes of inequality between the individuals, who can benefit from bariatric surgery and those who had undergone bariatric surgery, are the "lack of knowledge" about insurance coverage and also the surgeries (Sarwer et al., 2013). Efficient use of limited resources in health care will be prevented by the inequalities associated with both cultural and income differences.

In a study investigating the attitudes of the Danish people regarding bariatric surgery, 33% of the population stated that bariatric surgery used the public financing unfairly, while more than 46% of the participants indicated that the surgery costs should be covered by the individual (Lund, Sandoe & Lassen, 2011).

Since discrimination, which is more common in the groups with socioeconomical disadvantages, may cause problems for the obese individuals in accessing treatment, the principle of justice should be considered in the regulation of the surgical treatment of obesity (Saarni et al., 2011).

Conclusion

Increasing interest in bariatric surgery makes it necessary to inform the patients. Bariatric surgery should be realized by evaluating the risks and considering the benefit-loss balance, and this operation should not be done with aesthetic concerns.

In bariatric surgery, the health care professional should support the fair and transparent allocation of resources by supporting the autonomy of the patient actively.

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Chapter 21

ROLE OF THE SALIVA AS A DIAGNOSTIC FLUID

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1. Introduction

The diagnostic value of saliva has an old history which dates back to centuries. In ancient China saliva used as a lie detector known as the Rice Test which judges that the difficulty during swallowing dry rice was the sign of the guilt. The pathophysiology of this folktale phenomenon based on the assumption that guilt leads to nervousness often result in reduced saliva production. (1-3) In scientific literature usage of saliva for diagnostic purposes initiate in the early 1960s for screening cystic fibrosis. (4, 5) Despite its old history the physiological importance of the saliva is underestimated because of the technological limitations. However, the emergence of new technologies has led to overcome technological barriers in question and to raise awareness of the value of saliva as a diagnostic bio-fluid, in the last 50 years. (6-8)

Over the years; urine, blood, and cerebrospinal fluid have been used for diagnosis and monitoring of various conditions and diseases. (5, 9-11) However, current diagnostic techniques have shortcomings such as the requirement of invasive sampling and trained personnel, the need for specialized equipment and high costs. Saliva includes a similar organic and inorganic biologic elements like serum; and has several advantages over serum. (5) The advantages of saliva led this bio fluid to be preferred as a new noninvasive diagnostic approach that overcomes the shortcomings of current diagnostic techniques. (12, 13) Nowadays, including proteomics, metabolomics, genomics, bioinformatics and transcriptomics approaches opens a new avenue in salivary diagnostics. With the growing discovery of various disease-specific biomarkers salivary diagnostics become a part of patient care in terms of diagnosis, clinical monitoring, and decision making in medicine and as well as in dentistry.(6, 12, 14, 15)

2. Physiology of Saliva

2.1. Composition and Functions

Saliva is odourless, colourless, bio fluid which has a relative density of 1.004–1.009 and a pH of 6.6 – 7.1.(16) The whole saliva represents a mixed fluid derived predominantly (90 %) from the parotid, the sublingual, and submandibular the glands. The remainder whole saliva is originated from approximately 450-750 minor glands located on various sites of the mouth such as buccal mucosa, tongue, palate, and gums.(17-19) Contribution to whole unstimulated saliva of salivary glands is different for submandibular, parotid, minor-Von ebner and sublingual glands and approximately 65%, 23%, 8% to 4% for each gland respectively.(20) Each gland secretes a fluid that has a specific protein composition. Parotid glands are entirely serous, while sublingual and submandibular glands have a sero-mucous content.

Minor glands are majorly mixed with higher serous content. Differences in the concentration of organic and inorganic contents among glands can be observed. (20, 21) Although major glands produce the majority of the whole saliva, minor glands have a critical role in protection due to protective elements in its content.(21)

According to secretion is unstimulated or stimulated salivary composition changes.(20) Parotid gland secrete bicarbonate-rich watery fluid and include amylase, phosphoproteins, and proline-rich proteins. Submandibular and sublingual glands are the main source of the mucins that have several protective and regulatory functions, and mainly active at rest.(22-24) Whole saliva is not the only production of the glands but also has non-glandular components originating from oropharyngeal mucosae, serum and blood derivatives, desquamated epithelial cells, crevicular fluid, cellular components, and food debris.(19, 20, 25) Moreover, by means of passive transfer, diffusion and/or ultrafiltration various hormones and drugs can reach saliva.(26)

The formation of saliva is a complex process which takes place in different stages. In first stage sterile isotonic plasma like primary saliva which includes the majority of the organic components and water formed. In the second stage content of primary saliva further modifies and a final fluid hypotonic in nature produced.(27, 28) The hypotonicity of unstimulated saliva prevent plasma sodium levels to mask perceptiveness of taste. The hypotonic environment required for the taste perception is promoted with the bicarbonate, glucose, and urea in saliva.(21, 29) The medulla is the center that control the saliva secretion. (21) The serous and mucous secretions thereby output controlled by autonomic nervous system. The sympathetic division of the autonomic nervous system controlled serous part whereas the mucous part is under the control of parasympathetic and sympathetic divisions. The branch of facial nerve and glossopharyngeal nerves provide the parasympathetic supply of sublingual-submandibular glands and parotid gland, respectively. The sympathetic supply is provided through the fibers originating from the superior sympathetic ganglion. Depend on the stimulation high volume saliva constitute low levels of inorganic and organic elements created with parasympathetic stimulation while lower amount protein and potassium-rich saliva secreted as a result of sympathetic stimulation.(12, 19, 20, 28, 30, 31) Specific stimuli like mechanical, gustatory, and olfactory stimulus affect the secretion. Other factors affecting secretion include pain, some medications, and several pathologic conditions affecting the glands.(21)

Saliva is constituting approximately 99 % water, 0.3 % proteins, and 0.2 % inorganic and organic substances. Typically, normal daily salivary flow ranges from 0.5 to 1.5 L, at a rate of 0.5 ml/min.(19-21, 32) At rest,

0.25 to 0.35 ml/min saliva secreted from mainly the submandibular and sublingual glands. Any type of stimuli can increase the secretion rate to 1.5 ml/min. The secretion of the maximum saliva occur around 12 a.m. and least amount of secretion observed during sleeping at night.(33) Several conditions like stimulation of smell or taste, chewing, sychological and hormonal status, medications, age, heredity, oral hygiene, circannual rhythm, climate, exercise, and circadian flow variations can affect quantity and quality of saliva secretion.(20, 21, 30, 31, 34)

Saliva is a complex bio fluid composed of various elements that include inorganic, organic, non-protein, hormone, protein/polypeptide, lipid, and >700 microorganisms. have important functions, inthe maintenance health. (16, 20, 31) A compositional and functional analysis of saliva and as well as the contents may be important for discovering the place and implication of saliva in diagnosis, screening or decision making in healthcare or other eras (Figure 1, Table 1).(20, 21, 23, 32)

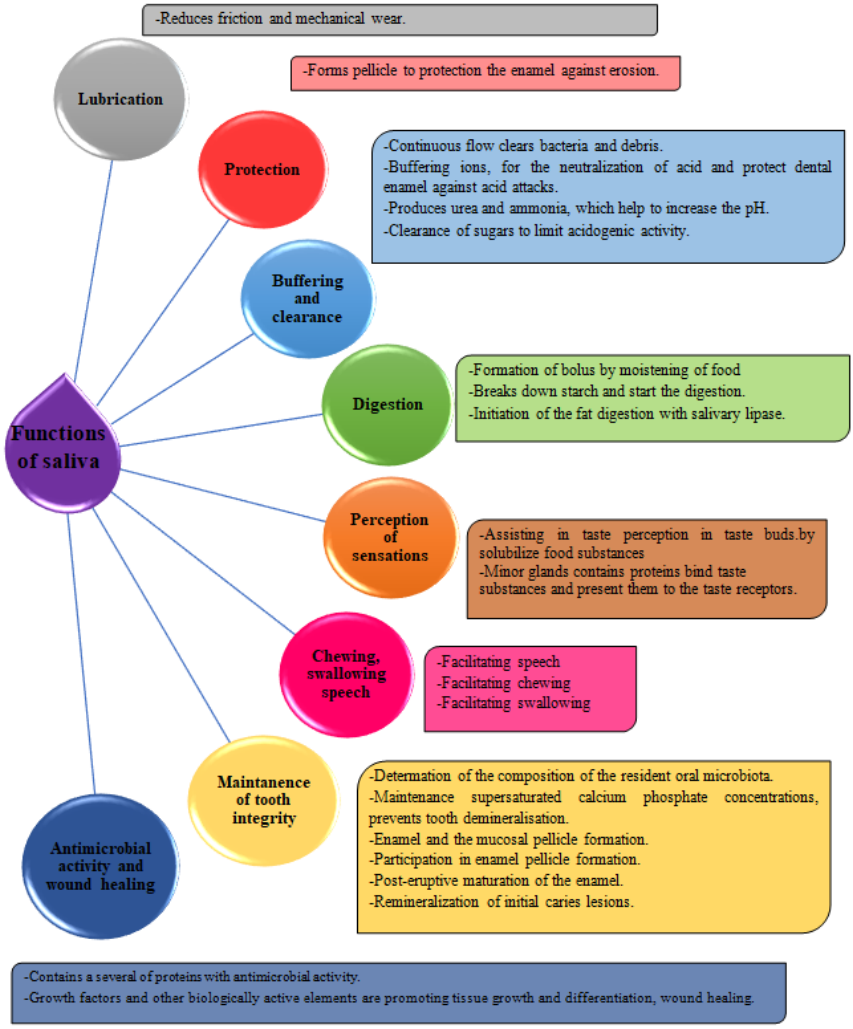

Figure 1: Functions of the saliva

Table 1. Components of saliva and their functions(6, 21, 25, 28, 35, 36)

Component			Function
Water			• Lubrication • Lavage, cleaning • Mucosal integrity • Taste • Speech and Phonation
Organic Elements	**Enzyme**	Amylase	• Anti-microbial • Tissue coating • Digestion • Lubrication
		Maltase	• Digestion
		Lingual lipase	• Digestion
		Lysozyme	• Anti-microbial • Inhibition of glucose uptake
		Phosphatase	• Anti-microbial
		Carbonic anhydrase (gustin)	• Taste
		Kallikrein	• Regulation demineralization and demineralization balance
		Peroxidase	• Anti-microbial • Protect mucosa from H_2O_2
		Lactoferrin	• Anti-bacterial • Iron binding
		Protease	• Digestion
		Catalase	• Anti-bacterial
		DNAse, RNAse	• Digestion
	Proteins and peptids	Mucin	• Inhibition of demineralization • Lubrication, elasticity, stickiness and viscosity • Bolus formation • Anti-microbial • Mastication, speech, and swallowing • Preserving mucosal integrity • Regulation intercellular calcium levels • Initiation of commensal oral flora • Thermal/chemical insulation
		Transferrin	• Transfer of iron
		Proline rich protein (PRP)	• Remineralization • Lubrication, and viscosity • Anti-microbial • Formation of pellicle • Tissue coating • Binding tannins
		Lactoferrin	• Anti-microbial
		Immunglobulins (A, G, M)	• Anti-microbial
		Glicoproteins	• Antimicrobial
		Statherin	• Remineralization • Anti-microbial • Tooth integrity • Formation of pellicle • Lubrication
		Cystatin	• Anti-microbial • Formation of pellicle

		Histatin	• Anti-microbial • Wound healing • Formation of pellicle • Binding tannins • Pore formation in microbial membranes
		Chromogranin A Sialin Agglutinin	• Antimicrobial
		Defensin	• Anti-microbial • Pore formation in microbial membranes
		Matrix metalloproteinaz-8	• Anti-microbial
		Interleukin-8	• Anti-microbial
		Phoshpolipaz a2	• Degradation of phospholipid
		Uric acid	• Antioxidant
		Urea	• Buffering • Modulate pH
		Ammonia	• Buffering • modulate pH
		Lipids	• Salivary protein binding • Bacterial absorption to appetite • Plaque microbial aggregation
		Tyocianide	• Antimicrobial
		Other organic elements	• Carbohydrates, Vitamins, Blood group antigens, albumin, amino acids
	Hormones	✓ Insulin ✓ Cortisol ✓ Testosterone ✓ Oestrogen ✓ Thyroxin ✓ Triiodothyronine ✓ Parotin ✓ Dehydroepiandrosterone ✓ Progesterone ✓ Aldosterone ✓ Growth hormone ✓ Prolactin ✓ Melatonin	
Inorganic Elements		Sodium	• Buffering
		Calcium	• Remineralization • Buffering • Anti-caries activity
		Potassium	• Buffering • Contribute to the osmolarity
		Bicarbonate	• Buffering • Modulate pH
		Bromide	• Buffering
		Chloride	• Buffering • Contribute to the osmolarity
		Floride	• Buffering • Anti-caries activity
		Phosphate	• Remineralization • Buffering • Anti-caries activity • Modulate pH
		Zinc	• Taste
Cellular components		• Epithelial cells • Exosomes and microparticules • WBCs • Microorganism (bacteria, virus, candida)	
Gases		• Oxygen • Carbon dioxide • Nitrogen	
Growth factors		EGF, IGF, TGF A, B, IGF –I, II, NGF, FGF	• Wound healing

2.2. Saliva collection methods

Saliva is a complex fluid that shows wide intra- and inter-individual variations in terms of composition, and whole saliva contains components from different sources. Thus, standardization of saliva collection is the crucial step for the usage of saliva as a research material.(6) One of the important aspects which could influence the results and need to be considered when obtaining saliva is origin of the saliva either whole saliva or gland specific.(17, 28) Whole saliva collection is and not requires specialized armenterium.(17) Glandular saliva can be collected by cannulation of the ducts or specific devices that placed into the glandular ducts emergence. However, collection of gland specific saliva is more complex, invasive and would require skilled personnel, special equipment and longer collection time.(12) Another important factor which needs to be considered during collection of saliva samples is the level of stimulation.(17, 28) By masticatory stimulus triggering with paraffin or gum or gustatory action initiated by citric acid stimulated saliva can be collected. Stimulated collection affect the composition of saliva and especially preferred in patients who could not salivate enough saliva. (3, 8, 25) The diluting effect of stimulation with gustatory agents cause interferences in analysis and reveal differences in flow rates. Although, contamination with exogenous materials is out of question, in mechanical stimulation method, standardization need to be performed during the collection period, by controlling the frequency of mastication by means of device like a metronome at about 70 chews/min in this method. Practice effect which may affect salivary flow and composition, is an important phenomenon that must be considered when collecting whole saliva. The unstimulated salivary flow rate tends to increase in an individual familiar with salivating about 15 %, compared to the first time.(37) Flow rate of unstimulated saliva is commonly affected by the light, hydration, olfactory stimulation, posture, seasonal changes and diurnal factors.(8, 25)

Generally whole saliva containing both lo¬cal and systemic sources is preferred for diagnosing and screening purposes. In conditions require information about diseases in specific glands then gland specific saliva is preferred. However, in some instances obtaining of samples both whole and glandular saliva may be preferred.(17) In terms of stimulation, it has been reported that with stimulated saliva more precise analysis can be performed especially for cancer markers. Also, cases with low salivary flow stimulation may be required.(17) Because of the effect circadian rhythm, fasting saliva sample is generally preferred. The timing of collection also important in salivary research for specific elements analyzed.(12) Also it must be considered that content of saliva reveal daily variations due to the events takes place in the body.(17)

2.2.1. Collection methods for Whole Saliva

Common methods used are the draining, spitting, suction, and swab (absorbent) methods.(8, 28, 37)

Draining method

The draining method is one of the most recommended ways of whole saliva collection. In this method unstimulated saliva is drip off from the lower lip into a test tube. To ease salivate a funnel can be used. The final saliva quantity measured by weighing the tube or reading from the graduated tube.(25, 37, 38) In this method a low amount of saliva can be collected.(12, 20)

Spitting method

In this method saliva is collected from the floor of the mouth. The saliva then spits out into the test tube. To ease salivate a funnel can be used. The final saliva quantity measured by weighing the tube or reading from the graduated tube. In spitting method less evaporation occur during the collection. Thus, this method is more preferred than draining method. (37, 38)

Regarding collection of stimulated whole saliva stimulants like chewing paraffin or flavorless gum at a fixed rate are applied. Similarly, the accumulated saliva from the floor of the mouth spit out into a test tube. Because of contaminating effect gustatory stimulation with citric acid is not preferred.(37, 38) Collection with this method reported containing 14-fold more bacteria than those collected with draining method.(20)

Suction method

Saliva is aspirated from the floor of the mouth by means of a saliva ejector or aspirator into a test tube. The final saliva quantity measured by weighing the tube or reading from the graduated tube. This method is not recommended for collecting unstimulated saliva because of causing some degree of stimulation.(37-39)

Absorbent method

Saliva is collected by pre-weighed substances such as cotton roll, swab, or gauze sponge which placed in orifices of the major salivary glands. These substances reweighing after collection to determine the amount of saliva collected. Degree of hyposalivation can be estimated with this method.(37, 38, 40) However, it is least recommended method, because of the possibility of permanent absorption of some analytes.(31)

2.2.2. Gland Specific Collection of Saliva

Parotid Gland Saliva

A Lashley cup, personalized plastic intraoral device and a snail collector can be applied to the orifice of the canal to collect saliva. Blunt lacrimal probe can be used to aid access to the duct and. from the orifice a thin tube can be placed. Parotid gland saliva is generally collected with stimulation and commonly citric acid used as a stimulant.(37, 38, 41, 42)

Submandibular/Sublingual Gland Saliva

Collection of secretions from submandibular and sublingual gland separately is difficult because of that the secretions from these glands enter the mouth from same duct. Because of the thin nature of Wharton's duct cannulation must performed carefully. Individual prosthesis, a custom-made segregator, can be used for the collection of submandibular/ sublingual saliva. However, it is not practical for daily routine. Suction method is suggested as an easier method for collection of saliva from these glands. Citric acid can be performed for stimulation of the glands. Wolff apparatus is another collecting system for submandibular and sublingual saliva.(37, 38, 43, 44)

Minor Salivary Glands

Pipette, special collectors or absorbent filter papers can be used in order to collect minor salivary gland saliva. Because of small amount of saliva obtained secretions may also be measured by means of periotron. A paper strip absorption can be used for labial and buccal situatrd glands. Palatine saliva can be collected by pipette, filtration paper, impression of palate and individual collection prosthesis.(37, 38)

Furthermore, commercial devices also present to facilitate collection. However, it should be carefully chosen by considering of the analyte to be evaluated. Some examples are: Salivette method, Orapette, Saliva Collection System, Uplink, Toothette plus, transorb wicks, and oral diffusion sink.(12, 28)

Important points for the standardization of saliva collection

- Collecting saliva in a constant flow rate and enough period is important for standardization of the samples. A period of 10 min has been suggested.
- Sugar and/or caffeine increase flow rate and decreased pH levels. Patients must be instructed to not to eat and drink as or performed any

hygienic procedures at least 90-120 min before to the saliva collection, and to stop oral movements during salivate.

- Multiple collection in the same patients must be performed at the same time of the day.
- Saliva collection preferable performed preferably between 9:00 and 11:00 a.m. to minimize the effects of diurnal variation.
- Medications should be stopped according to the half-life of drug when applicable.
- Collection should be performed in a separate room, in a sitting, relaxed position with head slightly bent down, and minimal movement of the face and lips, after 5 min of adaptation.
- Rinsing mouth with water/distilled water useful to eliminate debris that effect the results.
- Especially in patients with very low flow rates conditions should be organize in order to minimize the evaporation.(12, 17, 20, 28, 37)

3. Saliva processing and storage

The way of the sample processed and stored directly associated with the stability of the sample. Temperature changes and bacterial action affect the composition thus storage conditions and the amount of time frame from collection to analyse of the sample have crucial importance. (20, 28) Exogenous substances such as desquamated epithelial cells, microorganisms and debris should be removed to refine saliva samples. If this cannot perform then, the sample recommended to be stored at -80ºC to prevent decomposition.(17) Some salivary analytes can have a very short half-life so that required to be analyzed immediately, while others can remain stable until analysis. Thus, storage procedure before the analysis should be chosen by considering type of molecule analysed.(20, 28)

After collection of saliva handling process has crucial importance in order not to affect the nature of the sample. Changes in the structure of sample usually occur associated with bacterial metabolism and proteolysis. To prevent or minimize bacterial action samples recommended being collected on ice and/or centrifuged. Centrifugation clear saliva from debris and refines the saliva however the turbidity during centrifugation can affect analytical techniques.(6, 28) Commercially available protease inhibitors useful agents to prevent proteolytic reactions after collection. (6, 37) Transported should also be in controlled temperature to obviate bacterial contamination.(12)

Thawing of a frozen sample must be performed very quickly in order not to affect the structure of the proteins and prevent cryoprecipitation of mucin-protein complexes. 100 μL aliquots of samples may be prepared to avoid multiple freezing and thawing cycles. Also, storing the saliva sample in 1:1 diluted in glycerol at −80◦C, may obviate effects of freezing and thawing that hamper the analyses.(45)

For DNA, pre-clearing of samples is not recommended. Appropriate storage conditions, DNA samples can be stored at least one year at room temperature. Before or after extraction saliva DNA samples can also be stored at -200C or -800C.(28, 46) For RNA, saliva sample centrifugation after collection and the use of RNAase inhibitors are recommended. RNA can be stored until use by freezing at -800C.(28, 46) Regarding protein analysis, pre-cleared saliva with centrifugation and/or microfiltration can be stored on ice for only few hours. Protease inhibitors are also recommended for protein analysis. For few days saliva samples can be stored at -200C but long term stroge is not recommended since it may lead to significant precipitation of protein analytes.(28, 46)

To obviate degradation of salivary components;

- Immediate storage by not performing any processing if;
- Analyses perform in 30-90min stored at room temperature;
- Analysis after 3 to 6 hours stored at +40C
- Analysis after more long period stored at -200C or better at -800C
- In liquid nitrogen snap freezing
- Using enzyme inhibitors
- Using sodium azide to prevent bacterial growth
- Using trifluor acetate to denature certain salivary enzymes.(20, 28)

Processing of saliva for multipurpose use.(45)

- Standardized collection with ice-cooled vials with draining, spitting, and glandular saliva collection methods.
- Vortexing (2 min, maximal speed).
- Centrifugation (5 min, 10,000× g, or 20 min, 3,000× g).
- Storage at −20◦C or below (better at −80◦C).

4. Advantages and Limitations of Saliva

Advantages

- Saliva collection is a non-invasive method and obviates the risk of cross-contamination.
- Collection of saliva can be performed at home.
- In individuals who have compromised venous access, has special diseases cause advance bleeding, were older or child, do not cooperate in obtaining blood or urine samples saliva pose an efficient alternative.
- Saliva collection is easy and very little armamentarium is needed.
- Saliva is cost effective than serum and applicable for screening large populations and suitable for epidemiological researches.
- Saliva is not required trained personnel for collection.
- Multiple collections can be performed easily.
- There are commercially available screening assays.
- Saliva collection is free of stress and appropriate for privacy issues as in urine collection
- Saliva has many advantages over other diagnostic fluids in terms of collection, handling (not clot like blood), storage, transport.
- Most of the biochemical parameters in both saliva and blood show high accuracy. Thus, saliva can reflect the current health status of an individual.
- Saliva can use for diagnosis of pathologic conditions and monitoring health. (5, 8, 16, 17, 25, 32, 47, 48)

Disadvantages and Limitations

- Contamination of whole saliva with other biologic materials like blood is a potential source of error that can affect the results in salivary research. Thus conditions like history of oral surgical procedures or any other dental procedure that may have cause bleeding within the past 6 weeks, history of dental prophylaxis within the past 2 weeks, brushing or flossing within the past hour, blood disorders associated with enhanced bleeding, history of desquamative gingivitis, pemphigus, pemphigoid, or erosive lichen planus should be taken into account.
- Some proteins in saliva show diurnal variability; therefore, each protein that is tested for diagnostic purposes in saliva must be evaluated to determine whether its concentration or presence is dependent upon this form of biologic variability.

- Handling protocols and storage of saliva immediately after collection is affect the composition thereby the results. The most appropriate methods to process saliva prior to analysis still needs to be determine.
- Certain proteins may have intraoral bacterial analogues that can lead to erroneous salivary readings.
- Some biomarkers in saliva do not always show correlate with serum.
- In unstimulated collection methods it is not possible to obviate of stimuli of minor oral movements
- In stimulated collection methods it is impossible to standardize the intensity of stimulation completely.
- Flow rate changes significantly affect the pH and quantity/quality of biomarkers.
- Saliva secretion is effected by parameters that include thermal and seasonal changes, hydration, and mood of the individual. Variability in flow rates can be observed between individuals and in the same individual.
- Many biomarkers of serum reach saliva using different pathways. This affects diagnostic efficiency.
- Pathologic conditions may affect salivary gland function and components of saliva.
- Proteolytic enzymes in saliva hamper stability of certain biomarkers.
- Collected whole saliva is not the total amount since some of it will remain on the oral surfaces.
- Some amount of saliva not collected because of evaporation and retention in devices.
- It is difficult to distinguishes true salivary constituents and components of cellular or bacterial origin.
- With time composition of the saliva changes as a result of bacterial metabolism.
- Turbidity during centrifugation and also cellular debris may cause interferences during the application of analytical techniques. (6, 19, 47, 49)

5. Diagnosis of Systemic Diseases

Early diagnosis of disease has an important role in terms of reducing severity of pathologic conditions, prevent complications and thereby in increasing the success of treatment.(5) Diagnostic ability of saliva has been investigated widely over the years, however early salivary diagnostic studies have difficulties because of the lack of knowledge about biomarkers entrance ways to saliva, low levels of some marker in saliva when compared to serum, method of collection and storage of saliva samples. However, the subsequent advances in salivary diagnostics researches led molecular approaches called saliva 'omics' that blanket proteomics, genomics, transcriptomics, and metagenomic approches have advanced the discovery biomarkers exponentially. Nowadays saliva used as a diagnostic fluid for many oral and systemic conditions and pathologies (Figure 2).(5, 8, 32)

Figure 2: Oral and Systemic conditions diagnosed by means of saliva

5.1. Infectious Diseases

Specific antibodies of viral, bacterial, fungal and parasitic infectious agents related to various systemic and oral diseases can be detect in saliva. (15, 19)

5.1.1. Viral Diseases

Saliva can be used to detect antibodies against viruses for diagnosis of acute, congenital and reactivating viral infections. (25, 50)

Hepatitis: Salivary diagnosis reported to be highly sensitive and specific for hepatitis viruses A, B and C regarding the presence of IgM antibodies.(25, 48, 49) Also, saliva has reported use using for large scale screening of surface antigen of hepatitis B during epidemiological researches.(25, 51)

Human immune deficiency virus (HIV): HIV targets the immune system of humans. It has shown that HIV can be detected in saliva in a similar quantity with serum, and can be used in clinical and large scale testing.(25, 48) Salivary IgA levels of HIV patients suggested being used in screening the progression of HIV infection.(25) Salivary diagnostic Kits for HIV detection available are Aware BSP, OraQuick Advance Rapid HIV 1/2 antibody and Aware OMT test.(5, 48)

Herpesviruses: Using saliva samples as a diagnostic medium herpesvirus–8, Epstein-Barr virus and cytomegalovirus, have been reported to be detected. Also, reactivation of HSV-1 in Bell's palsy cases reported to be detected with salivary diagnostics.(25, 47, 52, 53)

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2): The ideal specimen for the detection of SARS-CoV-2 still needs to be determined and regarding diagnostic role of the saliva in detection of SARS-CoV-2 limited evidence present in literature. However, according to the results of most published studies on this topic saliva seems to be a reliable diagnostic tool for diagnosis SARS-CoV-2.(54-56)

Moreover, for other viruses that include but not limited to humanpapilloma virus, dengue, rotavirus, parvovirus, rubella, measles, zika, and mumps viruses saliva can be also used as a diagnostic biofluid. (17, 20, 25, 32, 48)

5.1.2. Bacterial infections

Helicobacter pylori: Helicobacter pylori infection leads production of a specific IgG antibody which can discriminate infected or noninfected patients. A sensitivity of 84 % has been reported for the detection of the bacteria in saliva samples.(17, 25, 48)

Borrelia burgdorferi: Borrelia burgdorferi is a spirochete cause Lyme disease and is blood-feeding ticks are transmission way. Salivary anti-tick antibody has a potential diagnostic marker suggested being useful tool for screening patients who have tick bites.(25, 57)

Pneumococcal pneumonia: The diagnosis of pneumococcal C polysaccharide reported showing low sensitivity but high specificity in saliva samples.(25, 58)

Moreover, other bacteria such as mycobacterium tuberculosis, shigella, entamoeba histolytica, pigeon breeder's disease, and taenia solium saliva can be also used as a diagnostic biofluid.(25, 33, 35, 47)

5.1.3. Fungal Infections

The oral cavity harbor an important fungal content referred as the oral mycobiome. The most frequently isolated fungi species reported are Candida, Aureobasidium, Fusarium, Cladosporium, Saccharomycetales, and Aspergillus. Detection of fungal species in is reported as difficult and need further investigation because of the variable results observed in different assays.(15, 32, 59)

5.2. Autoimmune disorders

Sjogren's syndrome

Sjogren's syndrome (SS) is an autoimmune exocrinopathy characterized by salivary and lacrimal dysfunction, ocular dryness, multiple organ pathologies and serological changes. Salivary diagnostics pose a valuable tool in terms of diagnosis of this autoimmune disorder.(5, 20, 48) The use of salivary proteins for diagnostic purpose of SS can reveal biomarkers enables to differentiation of primary and secondary variants of the disorder.(17, 20, 60) Damage occur in glandular tissue also can be screened by the use of the proteomic and genomic salivary markers(15) Elevated b2 microglobulin, thromboxane B2, IgA, lactoferrin, IgG, cystatin S-C, Na, IgM, prostaglandin E2i albumin, and lipid levels in saliva onserved in SS.(32) Especially IgA antibody can be found in the saliva samples obtained from SS patients before observed in the serum. (25, 61) In some patients SS anti-La antibody detected in saliva while is not observed in serum this may be suggest that this antibody originate from the salivary glands.(25, 62) Sialochemistry is also be useful approach in helping the detection of SS .(25)

Cystic fibrosis

Cystic fibrosis (CF), is a frequent hereditary disease which leads to respiratory complications. Increased sodium, chloride, calcium, potassium and phosphate ions and a lower saliva quantity and pH observed in these patients. Also, higher levels of uric acid. Proteins, and antioxidants in whole saliva samples of young CF patients have been detected. These

changes considered to be the result of inflammatory and oxidative activity related to the progression of the disease and thus, these changes in saliva could enable clinicians to evaluate the etiology and monitoring the progression of CF.(5, 20, 25)

Coeliac disease

Coeliac disease is a congenital disorder characterized by the malabsorption of gluten in the small intestine.(25) Serum IgA antigliadin antibodies (AGA) are increased in these patients. Salivary IgA-AGA levels reported being specific and sensitive method to screen coeliac disease.(25, 48) Moreover, tissue anti-transglutaminase (TTG) antibodies can be used to monitor celiac patients.(17)

5.3. Neurologic Diseases

The salivary biomarkers related to end-stage renal disease that include nitrite, uric pH, acid, lactoferrin cortisol, chloride, amylase, and sodium have been reported.(15, 63) Also salivary phosphate considered to be a better biomarker when compared serum phosphate for hyperphosphatemia. (15, 64) For screening renal function also salivary creatinine used as a marker.(32)

5.4. Cardiovascular Diseases

C-reactive protein (CRP) is an inflammatory mediator released when acute infection or injury occurs. Good correlation is reported in salivary and serum CRP levels. Because of it is released as a response to cell necrosis cardiac troponin (cTn) can be used as a biomarker for the detection of acute myocardial infarcts.(5, 20, 21, 65, 66) Also, salivary alpha-amylase suggested as a marker for the determination of myocardial infarction.(32)

5.5. Endocrinopathies

Cushing's syndrome

Cushing's syndrome is an endo¬crinopathy which is challenging to diagnose. Measuring cortisol in saliva is an easy and convenient method and reported to show high reliability in the detection of cushing syndrome.(17, 67)

5.6. Metabolic Diseases

Diabetes

Recently, salivary proteomics considered to be useful markers for detection and monitoring of diabetes. A report by Rao et al. reported a

unique proteomic profile in saliva in type-2 diabetic patients which include 65 proteins revealed change more than a 2-fold.(5, 20, 68) Another study demonstrated that alterations in C3 and alpha2-macroglobulin complement, nd albumin have a relation with increased levels of HbA1c. (69) Cabras et al.(70) also reported founding 120 components as a potential marker in saliva of type 1 diabetes children.

5.7. Neurological Diseases

Alzheimer disease

Current management of Alzheimer disease an lead increase in the activity of acetylcholinesterase. Sayer et al. reported based on the results of their study that salivary acetylcholinesterase activity may be used as a marker for Alzheimer disease.(19, 71) Recently González-Sánchez et al.(72) suggested that salivary lactoferrin may be a useful biomarker for this disease.

Parkinson's disease

In Parkinson disease salivary changes like lower production of saliva and amylase, nd also higher sodium, potassium, chloride concentrations were observed.(48, 73)

5.8. Cancer

The early detection of diseases like malignancies can be achieved with specific biomarkers.(15, 17) It is suggested that saliva salivary diagnostics is an effective approach for early diagnosis of cancers that include oral cancer, pancreatic cancer, breast cancer, lung cancer, or gastric cancer.(32) The first salivary biomarker HER2/neu reported for the detection of breast cancer.(17) Moreover, CA 125 reported being a biomarker for ovarian cancer and c-erbB-2 (erb) and cancer antigen 15-3 were found to be elevated in the saliva of female breast cancer patients. (17, 20, 25)

Oral squamous cell carcinoma

Regarding oral squamous cell carcinoma p53 antibody reported to be detected in the saliva and can be useful tool for early detection, and screening. (25) It has found that 13 % of patients with oral squamous cell carcinoma had antibodies against p53 in the saliva. In patients with oral carcinomas 3.5 fold higher levels of a particular 7 mRNAs reported to be found. Also, salivary RNA biomarkers were IL8, SAT, IL1B, HA3, S100P, OAZ1, and DUSP1 reported to show highly sensitive and specific in distinguishing

squamous cell carcinoma. Presence of human papillomavirus suggested as another biomarker for oral squamous cell carcinoma.(5, 19, 20, 48, 74) Elevated levels of salivary defensin-1 suggested as a marker for oral squamous cell carcinoma and represent high correlation with serum levels. (25, 48) In patients with premalignant lesions higher salivary quantity of TNF-α, IL-1, IL-6, and IL-8 have been observed.(17) In a current met analysis study salivary MMP-9, Chemerin found highly sensitive and specific markers for oral squamous cell carcinoma.(75)

5.9. Drug Monitoring

Saliva has recommended as a useful clinical tool for the screening of systemic levels of medications. Drugs that can be monitored in saliva are; therapeutic drugs such as caffeine, carbamazepine, sulfanilamide, lithium, diazepam, cyclosporine, metoprolol, digoxin, methadone, paracetamol, tolbutamide, phenytoin, quinine, theophylline, cisplatin, barbiturates, cocaine, and benzodiazepines. And abused drugs that include opioids, amphetamines, ethanol, marijuana, phencyclidine, nicotine.(17, 19, 20, 25, 48)

5.10. Hormones

Detection of the hormones in saliva require some points take into consideration like timing of collection since most of the hormones exhibit circadian variations. Also, variables like salivary flow rate, enzymes in saliva, and pH can affect the quantity of some hormones. Steroid hormones, particularly cortisol those reach saliva by diffusion usually high enough in saliva. However, the quantity of hormones which use ultrafiltration way, are more affected by salivary flow rate.(25) Salivary catecholamines reported having a poor correlation and thyroxin and triiodothyronine seem to have a correlation with serum levels.(20) Currently, cortisol, dehydroepiandrosterone, aldosterone, testosterone, estradiol, prolactin, estriol, growth hormone, insulin, melatonin and progesterone can be assessed in saliva.(19, 20, 25, 47, 48)

5.11. Sialochemistry analysis

Monitoring of heavy metals can be performed by means of sialochemistry. Cadmium and lead reported being detected in saliva. Thus, saliva can be a good medium for screening of exposure to heavy metals.(20, 48)

5.12. Forensic science

Important number of buccal epithelial cells present in saliva is an excellent sample for DNA analysis. Samples from the buccal cells can

be easily obtained by untrained personnel.(15, 48) Samples can also be obtained from sources like cigarette butts, envelopes, drinking glasses, then used to detect blood-group substances or salivary genetic proteins. 85% of individuals reported to present salivary blood group antigens which are used in both criminal and paternity cases.(15) Even dried saliva can be used from bite wounds suffered by the victim.(17) Further more saliva used in this field to differentiating from dead bodies from alive individuals.(32)

5.13. Oral diseases

Caries

Streptococcus mutans and Lactobacillus in saliva is directly associated with the incidence of dental caries. In literature it is demonstrated that the Streptococcus mutans have a role in initiating caries, while Lactobacilli active in the progression of caries.(5, 16, 20) Aalpha-defensins HNP1-3 in low levels suggested to contributing to caries vulnerability in children whereas mucins reported promoting streptococci agglutination. (32) Risk assessment of caries can be also be provided by evaluating saliva buffer capacity, flow rated, as well as the pH.(20, 32)

Periodontal diseases

Saliva is a valuable tool for periodontal evaluations. The most common periodontal pathogens include Treponema denticola, Porphyromonas gingivalis. and Tanerella forsythensis. Host responses to periodontal disease include inflammatory mediators, bone-specific markers, 8-hydroxy-deoxyguanosine, prostaglandin E2, host enzymes, matrix metalloproteinase-8, dipeptides, as well as the fatty acids.(32)

Positive correlations have been reported between periodontal disease and salivary toll-like receptor-2 and interleukin-4. Pyridinoline cross-linked carboxyterminal telopeptide of type I collagen, suggested as a potentially effective diagnostic biomarker. Increasing diagnostic sensitivity and specificity observed when a panel of biochemical markers such as elastase, osteocalcin, macroglobulin, collagenase, PGE2, and alkaline phosphatase was evaluated.(20, 48)

Orthodontics

Saliva may reflect the pathophysiology of many conditions as well as orthodontic treatment. Flórez-Moreno et al. reported that Deoxypyridinoline dominantly occur the early stages of orthodontic tooth movement while bone-specific alkaline phosphatase might be

a marker for bone formation when the tooth movement finalize.(76) Salivary interleukin-1b receptor antagonist, proteoglycans, interleukin-1b, anti-HDE sIgA antibodies and regulatory subunit of type II (RII) of cyclic AMP-dependent protein kinase 150 reported to revealing changes in patients undergoing orthodontic treatment. Thus, analysis of saliva considered providing the evidence to minimize root resorption.(32)

Medication Related Osteonecrosis of the Jaws

Medication-related osteonecrosis of the jaw (MRONJ) is a side effect originate from the usage of antiresorptive, and antiangiogenic drugs. (77, 78) Although still no consensus has been reached regarding salivary biomarkers in MRONJ some salivary biomarkers reported as a candidate in the detection and screening of MRONJ. Kolokythas et al.(77) reported that bone-specific alkaline phosphatase and N-telopeptide of type I collagen explored as a potential biomarkers and significant difference in salivary levels of these biomarkers observed among control and MRONJ patients. Also, salivary Interleukin 1 alpha (IL-1a), interleukin 1 beta (IL-1b,) interleukin-1 receptor antagonist (IL-1RA), and Interleukin 6 explored as possible biomarkers for biphosphonate-related osteonecrosis of the jaw in other studies and it is suggested as useful for monitoring biphosphonate-related osteonecrosis of the jaw.(79, 80) In a recent systematic review, it is concluded that there is no clear clinical evidence present to use these biomarkers for MRONJ cases.(78)

6. Conclusion

Salivary diagnostic research has been evolved with a high acceleration in parallel to the bio technological advancement, and today being used for the diagnosis and screening of the various oral and systemic diseases and conditions. Continuous progression in this era enable salivary diagnostics to become the key element globally for monitoring and decision making purposes in healthcare settings in near future.

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Chapter 22

THE IMPORTANCE OF ACRYLAMIDE FORMATION IN FOODS

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In order to improve the sensory properties of foods and increase their safety, heat treatments have been applied in domestic and industrial sizes from past to present. The high temperature applied to foods causes the formation of new compounds in foods through chemical reactions. While some of the new compounds formed have positive effects on health, it is reported that some (such as acrylamide) have toxic effects (Studer et al., 2014).

The International Agency for Research on Cancer (IARC) named acrylamide in the Group 2A class '' possible carcinogenic to humans '' in 1994 (IARC, 1994). Acrylamide was considered an environmental contaminant until the Swedish National Food Organization, together with Stockholm University, reported in 2002 that they found evidence of acrylamide in starch-based foods that underwent heat treatment (such as baking, roasting and frying) (Tareke et al., 2002).

The health effects of acrylamide taken into the body are genotoxic, carcinogenic, neurotoxic and toxic on the reproductive and developmental system. These metabolic effects are evaluated in different classes (WHO, 2002).

The health effects of acrylamide taken into the body through food-borne and non-food-borne ways are divided into toxicological and carcinogenic. Studies have shown that metabolites cause adverse effects on the central nervous system and oxidative stress, as well as mediate the toxic effects of acrylamide (LoPachin, 2004).

In addition, as stated in different sources, the neurotoxic effects of acrylamide were observed both in humans and animals, and genotoxic, carcinogenic and toxic effects on the reproductive system were observed only in animals. (Shipp et al., 2006).

Acrylamide does not have a legal limit in our country and in the European Union legislation. However, the level of acrylamide in food is controlled by the European authorities, and it is considered to set maximum limits for acrylamide in the future. (EFSA, 2012).

The genotoxicity of acrylamide and its metabolite, glydamide, has been investigated in various studies. There are studies proving that acrylamide damages DNA (WHO, 2002). While it was observed that acrylamide did not cause gene mutation in bacteria, it was determined that glicidamide caused gene mutation in the absence of metabolic activation. There are some studies in the literature showing that the risk of kidney, uterine and ovarian cancer increases with acrylamide exposure (Wilson et al., 2012; Kumar et al., 2018).

Acrylamide (2-propenamide) is a reactive unsaturated amide containing double bond. Due to the double bond in its structure, it has the ability to bond with amino (-NH2) and sulfhydryl (-SH) groups. (Friedman, 2003). It is an odorless, white, crystalline solid compound with a molecular weight of 71.08 g/mol, a melting point of 84.5°C, a boiling point of 192.6°C. Acrylamide, a polar substance, is easily soluble in polar solvents (such as water, methanol, and ethanol), (Figure 1).

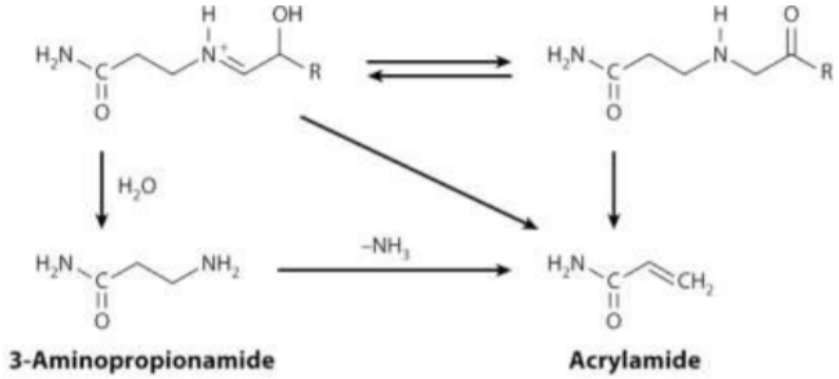

Figure 1. Formation of Acrylamide

There are two different forms of acrylamide as monomer and polymer (polyacrylamide). It has been reported that the monomer form of acrylamide is generally formed in foods (Vattem and Shetty 2003).

The monomer form of acrylamide is thought to be carcinogenic in humans, as it resembles vinyl chloride, which is known to be carcinogenic. Acrylamide formation in foods; It has been reported that it is associated with the Maillard reaction between the asparagine amino acid in food and reducing sugars, especially due to cooking at high temperatures. The temperature to be exceeded for acrylamide formation is 100 °C. It has been stated that the temperature at which the formation of this chemical structure begins is 120 °C, and the highest level is seen at 180 °C. When the reports are examined, it is stated that the acrylamide formation levels of foods with high carbohydrate content are high. It has been emphasized that foods with high protein content have lower acrylamide levels (Yaylayan et al., 2003; Paul et al., 2016; Muttucumaru et al., 2017).

People's exposure to acrylamide varies according to the age, body weight, consumption habits and preferences of the person. In a study, tolerable daily acrylamide intake value was determined in terms of neurotoxicity. This value is calculated by dividing body weight by the

daily intake level. As a result, it was stated as 40 µg / kg body weight / day (Tardiff et al., 2010).

Maillard reaction is a type of non-enzymatic browning such as caramelization, and it usually takes place between the free amino group (-NH2) of free amino acids, proteins or peptides in foods and the carbonyl group (C=O) of reducing sugars (Arusoğlu, 2015). Acrylamide is one of the best known products of Maillard reaction (Yıldız et al., 2010). Acrylamide formation mechanism scheme by maillard reaction is showed that Figure 2.

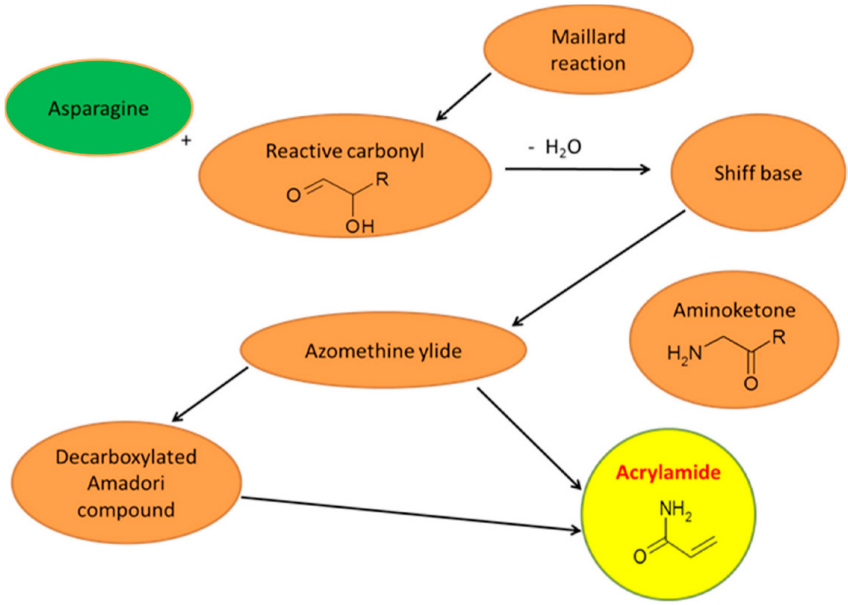

Figure 2. Acrylamide formation mechanism scheme by maillard reaction.

To express the formation of acrylamide, it occurs as a result of a series of chemical reactions. That is, it is assumed that asparagine, an amino acid present in food during baking and frying processes, is derived from the Maillard reaction between the amino group and the carbonyl group of the reducing sugars in the food content (Manini et al., 2001).

The formation of acrylamide by the Maillard reaction mechanism is Schiff base formed between a carbonyl source and the amino group of asparagine and then decarboxylated into acrylamide with the high temperature effect of Schiff base (Alpozen, 2012). The stated carbonyl source can be reducing sugars or lipid oxidation products (Vural, 2016) (Figure 3). In processes where there is no asparagine, acrolein and ammonia play a role in the formation of acrylamide in foods with high

fat content. Acrolein and acrylic acid are formed by degradation of fats (triglycerides) at high temperatures.

Major factors of acrylamide formation in food are asparagine and reducing sugars or reactive carbonyls. Their content depends first on the species and variety of popular food products, methods of their growing, harvesting, and storage.

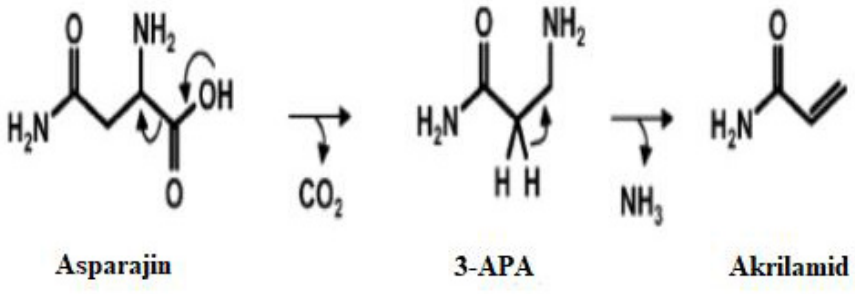

Figure 3. Acrylamide formation from asparagine (Granvogl and Schieberle, 2006)

There are many instrumental analysis methods available to determine the level of acrylamide present in food. The majority of acrylamide analyzes are based on chromatographic methods such as GC (Gas Chromatography) or HPLC (High Pressure Liquid Chromatography) combined with MS (Mass Spectrometry). In recent years, in addition to all these instrumental analysis techniques, liquid chromatography has been added to the measurement method. The essence of this method; LC-MS/MS method that does not require pre-derivatization (Geng et al., 2008).

Acrylamide levels in foods are a concern for the consumer. Scientific studies and developing technological opportunities determine this formation. In vivo and in vitro studies have determined and reported acrylamide levels in foods.

Food authorities have announced the acceptable limits with reports. When the literature studies are examined, many studies are found to determine the acrylamide level of foods. Foods with high exposure to acrylamide when studies are generally evaluated; French fries, potato chips, coffee, bread, biscuits and breakfast cereals. (Svensson et al., 2003; Konings et al., 2003). Acrylamide content of different food products are showed that Table 1 (Lingnert et al., 2002).

Table 1. Acrylamide content of different food products (Lingnert et al., 2002)

Product/product group	***Acrylamide range (µg/mg)***
Potatoes (Raw)	<10-<50
Potato chips/crips	117-4.215
French fries/chips	59-5.2
Bakery products and biscuits	18-3.323
Breads	<10-3.2
Bread (toast)	25-1.43
Breakfast cereals	<10-1.64
Other fruit and vegetable products	<10-70
Chocolate products	<2-826
Roasted coffee	45-935
Coffee substitute	80-5.39
Coffee extract/powder	87-1.18
Meats	<10-116
Dairy products	<10-100
Baby food and infant formula	<10-130

The United Nations Food and Agriculture Organization (FAO) and the World Health Organization (WHO) Joint Expert Committee on Food Additives (JECFA) published an opinion on the risks posed by acrylamide in the diet in 2006 (JECFA, 2006). Although the adverse neurological effects of acrylamide exposure are not high, it has been stated that morphological changes in the nerves may occur for individuals with high

exposure. In addition to all this information, exposure limits constitute a health problem for a genotoxic and carcinogenic compound. Acrylamide formation in the processes of popular foods, highly preferred by the consumer, is becoming one of the most difficult problems facing the food industry.

In the light of this information, the opinion may be that it will not be easy for the food industry to stay away from this structure. There is no doubt that it is difficult to reduce acrylamide levels in food products. The color, taste and aroma compounds of a food product differ in each food. Thus, the process stage applied to these products combines with the functionality of these compounds. The acrylamide pathway takes shape when both the consumer demand and preferred properties and the properties of the product combine. In order to emphasize the formation of acrylamide in foods and the importance of this formation for human health, we can define it with a definition specified by food authorities.

"Food security exists when all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food to meet their dietary needs and food preferences for an active and healthy life." As a result, there can be no food security without food safety.

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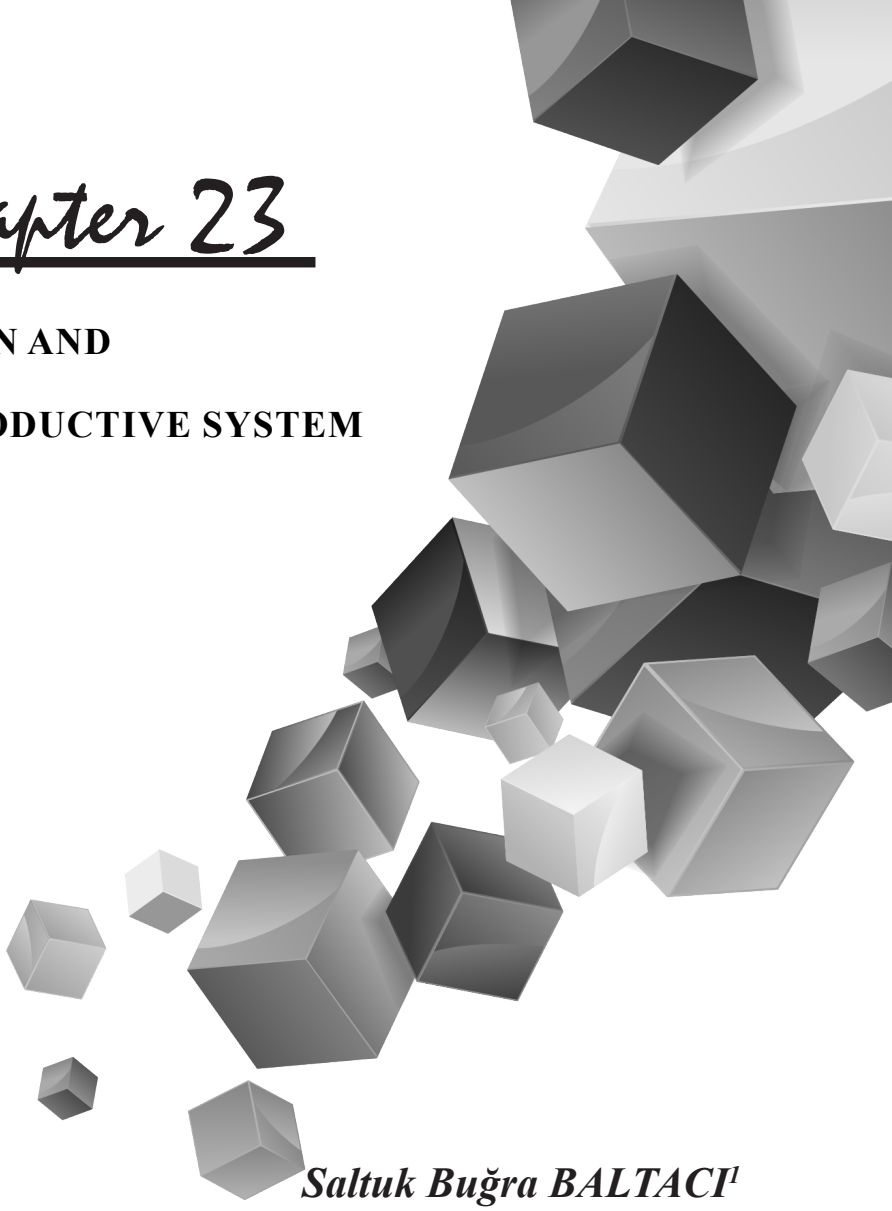
Chapter 23

LEPTIN AND REPRODUCTIVE SYSTEM



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Introduction

Leptin hormone has attracted attention with its effect on the regulation of food intake in the late 1990s. Leptin, a protein which is produced by the obese gene and also called ob-protein. Leptin is a protein structured hormone which is synthesized and secreted by adipose tissue. Although it is mainly produced from white adipose tissue, it is also produced from a very small amount of brown adipose tissue.

The main effect of leptin on the hypothalamus is that it inhibits the secretion and synthesis of neuropeptide Y. Thus, leptin reduces food intake and increases energy expenditure. As a result, it also reduces blood insulin levels. Because of these effects, leptin is used successfully in the treatment of diabetes.

The leptin hormone, which is considered a satiety factor, informs the brain about the body's energy stores. Leptin hormone is considered to be a factor preventing obesity by feedback from adipose tissue to the hypothalamus. Many studies, both in experimental animals and humans, showed that the leptin is critical in regulating nutrition and body weight. The relationship of leptin with reproductive functions was first reported at 1996. This result was demonstrated by the coexistence of obesity and infertility in male and female ob/ob mice. In this case, restriction of food intake and reduction in body weight did not affect fertility, whereas recombinant leptin supplement resulted in an improvement in fertility. Indeed, it is pointed out that leptin creates strong signals that initiate puberty in both men and women by stimulating secretion of GnRH, LH and FSH. However, there are differences between genders in terms of leptin levels. The leptin level in men begins in childhood, reaches its highest level in the early stage of puberty and then decreases, and as a result, leptin levels are 3-4 times higher in women than in men. Serum testosterone and testicular volume are inversely proportional to leptin levels following puberty, but estrogens stimulate leptin secretion.

As a result, there is a close relationship between leptin and the reproductive system. The purpose of this chapter is also is to provide information about leptin and the reproductive system.

General Informations

The most important target organ for leptin to show its effect in the reproductive system is hypothalamus. Leptin receptor localization in the hypothalamus-pituitary axis indicates that leptin may have a significant reproduction (Liu et al., 2020). It has been shown that leptin receptors, which are mostly found in gonadotrophic cells in the hypothalamus and anterior pituitary, are also commonly found in the organs that make up the

female and male reproductive system (Odle et al.2018). Leptin can acting in two ways in the HPG system (hypothalamic–pituitary–gonadal): 1. Even leptin insufficiency alone causes dysfunction in the hypothalamus-pituitary-gonadal system. 2. Low-dose leptin administration provides recovery of impaired functions (Odle et al., 2018).

It has been determined that leptin accelerates GnRH secretion, not only in pulse amplitude, but also in neurons in the arcuate nucleus in a dose-dependent manner (Petrine et al., 2020; Ziylan et al., 2009). While it has been reported GnRH secreting nerve cells are reported to describe leptin receptors, it has been shown in vitro that GnRH-releasing neurons increase GnRH secretion with leptin administration (Odle et al., 2018). Leptin also has indirect effects on GnRH secretion. Leptin may also facilitate GnRH secretion by affecting CART (Cocaine- and Amphetamine-Regulated Transcript) which regulates peptides, and/or neurons that secrete neuropeptides such as hypothalamic MSH (Melanocyte Stimulating Hormone) (Odle et al., 2018; Parent et al., 2000). Leptin may also have an additional indirect effect by stimulating the secretion of nitric oxide (NO) from adrenergic neurons. The increase in NO secretion stimulates GnRH secretion from neurons that secrete GnRH by activating both guanylate cyclase and cyclooxygenase 1 (Del Bianco-Borges et al., 2015; Kosior-Korzecka and Bobowiec., 2006; Odle et al., 2018).

Other important effects of leptin on the control of nutrition on GnRH secretion have not yet been determined. In experimental studies, the effects of the neuropeptide Y hormone on GnRH secretion can be both stimulating and suppressive. These differences may be due to the following: 1. Acute and chronic applications of NPY, 2.Type and gender differences, 3. Sexual maturation status (True et al., 2017). For example, acute in vitro exposure of NPY on prepubertal rat hypothalamus increases pulsatile GnRH pulsation (Lebrethon et al., 2000a), however, chronic intracerebroventricular (ICV) given of NPY inhibits GnRH (Pierroz et al., 1996). Accordingly, leptin may possess jointly stimulatory and inhibitory effects on GnRH release. Thus, its effect may possibly due to dose-dependent and changable with species, culture conditions and sex of species (Spicer, 2001).

Leptin-to-pituitary relationship has been shown in many studies. In addition to experimental animals, leptin receptor mRNA was detected in pituitary gland of sheep and pig. This information shows that leptin acts directly on the hypophyseal gland (Odle et al., 2018). In an in vitro study with rat anterior pituitary, it is demonstrated that leptin directly increases LH and FSH secretion (Dagklis et al., 2015; Odle et al., 2018). In addition, it was reported that LH rhythm of rats fed on a normal diet decrease with the administration of leptin antiserum to the lateral ventricles and

the estrous cycle stops (Dagklis et al., 2015). Leptin shows a rhythmic secretion and peaks between 01.00-02.00 at night. This rhythmic structure is synchronous with LH (Odle et al., 2018). Giving leptin to leptin-deficient adolescent girls with hypogonadotropic hypogonadism for 12 months stimulates nocturnal LH secretion in accordance with precocious puberty (Bouvattier et al., 1998). Based on these results, it can be said that leptin plays a critical role in regulation of LH secretion from the anterior pituitary (Bouvattier et al., 1998).

Leptin has a direct effect on the anterior pituitary as well as stimulatory effects on the HPG axis at the hypothalamic level. (Kořínková et al. 2020). Approximately 90% of gonadotropes in the pars tubaralis and 30% of gonadotropes in the pars distalis define Ob-R (Kořínková et al., 2020). Leptin can directly stimulate LH secretion and affect FSH secretion through activation of nitric oxide (NO) synthesis in gonadotropes (Del Bianco-Borges and Franci, 2015).

Leptin and the Reproductive System

The relationship of leptin with reproductive functions was first reported in 1996. This result is due to the coexistence of obesity and infertility in male and female ob/ob mice (Goumenou et al., 2003). It is emphasized that leptin is an important signal protein that triggers the onset of puberty (Nieuwenhuis et al., 2020). It is stated that the first estrous cycle, acceleration of cell division, LH and changes in estrogen levels in women are initiated by leptin (Ergün, 1999).

Differences in Leptin Levels by Gender

The leptin level gradually increases with until the age of 20. After the adolescent scoring reaches Tanner 2, it continues to increase in girls but decrease in boys, which is due to the negative feedback mechanism between testosterone and leptin and the inhibiton of leptin by androgens, while estrogens stimulate leptin formation (Wang et al., 2018).

Even after body weight and fat tissue differences are eliminated, women have higher leptin than men (Soliman et al., 2012). There may be many reasons for the gender difference in high leptin levels. 1. Women have relatively higher percent body fat and the visceral-to-subcutaneous fat ratio distribution differ greatly in women. Leptin mRNA expression in subcutaneous tissues of women is higher than in visceral tissue. 2. Leptin binding proteins may be lower in women than in men. This means that free leptin is high in women (Baltaci and Mogulkoc., 2012; Soliman et al., 2012).

Although it has been stated that gender differences in serum leptin levels can be found in the uterus at birth or in the late period of pregnancy, there is no consistent data on this. Male neonates have lower leptin levels in cord blood than females (Moschos et al., 2002).

Leptin and Puberty

Biologically, puberty offers the attainment of rapid growth and reproductive maturity and is influenced by body mass and/or body fat index. Leptin is considered the signal that initiates puberty. However, puberty still occurs with hormonal changes in the systems leptin, hypothalamus-pituitary-adrenal glands, as well as growth hormone-IGF-1 axis (Egan et al., 2017).

Reproductive disorders in leptin-deficient ob / ob mice have been reported to be treated with leptin supplementation (Cheab et al., 1996). Levels of circulating leptin show changes in the advancing adolescence. As a result, women have higher leptin levels than men (Lucka and Wysokiński, 2019; Moschos et al., 2002).

Girls have higher serum leptin concentration before, during, and after puberty than boys, even after the greater adiposity in females is taken into account (Demerath et al., 1999). It is observed that the leptin increase by about 50% before the puberty in healthy boys and then decrease to about baseline values after the initiation of puberty (Caprio et al., 2001). After puberty begins, testosterone levels in men are inversely related to leptin concentration. In contrast, there is a positive relationship between estrogen and leptin levels in pubertal girls. As a result, while leptin increases in girls with the onset of puberty, it decreases in boys. (Brann et al., 2002).

Endocrine Control of Puberty and Leptin

Leptin, defined earlier than kisspeptin, functions as adipose tissue hormone and is also involved in the regulation of puberty. GnRH (Gonadotropin Releasing Hormone) is the main regulatory hormone responsible for sexual maturation and reproduction. The onset of puberty is triggered by the decrease in inhibitory signals on GnRH neurons and the increase in stimulus signals. The role of leptin's hormonal and metabolic signals is critical in this event.

As a result, leptin stimulates LH and FSH secretion by binding to its receptor in hypothalamic neurons producing GnRH. In LH and FSH, testosterone in men increases the secretion of estradiol hormones in women and triggers the onset of puberty (Pankov, 2015). In fact, the neuroendocrine mechanisms that initiate the stimulation of the HPG (Hypothalamo-pituitary gonadal) axis in puberty have not been fully

elucidated. However, our understanding of the mechanisms responsible for the physiological control of puberty has been altered by the expression of the peptide products of the KISS-1 gene, kisspeptins and its receptors GPR54 (Cortés et al., 2015). GnRH (Gonadotropin Releasing Hormone) is the primary regulatory hormone responsible for sexual maturation and reproduction (Pankov, 2015). The onset of puberty is triggered by the decrease in inhibitory signals on GnRH neurons and the increase in stimulatory signals (Cortés et al., 2015). In addition, the role of peripheral hormonal and metabolic signals such as leptin is critical in this event (Pankov, 2015). Recent research suggests that leptin increases kisspeptin secretion from kisspeptinergic neurons, and thus kisspeptins bind to the GPR54 receptor in GnRH-secreting neurons (Cortés et al., 2015).

Leptin in the Female Reproductive System

Evidence from experimental studies on rodents indicates that leptin plays an important role in female fertility. Therefore, it is thought that the absence of the biological effects of leptin leads to infertility and absence of pubertal development (Schroeder et al., 2013). Leptin supplementation maintains the reproductive cycle and initiates puberty, preventing sterility in female ob/ob mice lacking endogenous leptin (Tena-Sempere and Barreiro, 2002). While leptin stimulates the secretion of gonadotropin, blockade of endogenous leptin impairs the estrous cycle and pulsatile secretion of LH in female rats (Tena-Sempere and Barreiro, 2002; Figure 1)

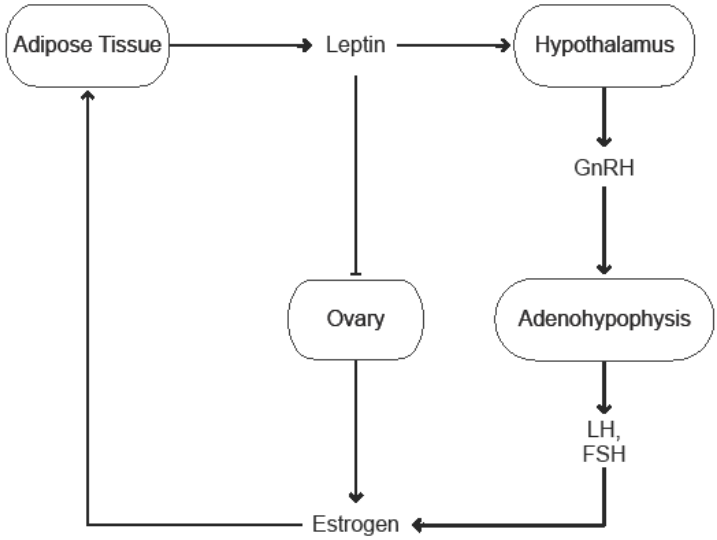

Figure 1. Leptin and Female Reproductive System

Leptin and Ovarium

Leptin acts directly on the ovary (Tu et al., 2018). Ovary, granulosa and thecal cells have high affinity receptors for leptin (Spicer, 2001). Leptin receptor mRNA has been identified in reproductive system (Tu et al., 2018; Spicer, 2001).

Insulin is needed for some of the affects of leptin on reproductive system. Some experimental studies have reported that leptin antagonizes the stimulating effects of insulin in the synthesis of steroid hormones in ovarian granulosa cells. In the absence of insulin, leptin has been shown to have limited or no effects on granulosa cells (Spicer et al., 2000). The effect of leptin on the aromatase activity of insulin-induced undifferentiated granulosa cells is stronger than its inhibitory effect on undifferentiated cells. The effect of insulin-induced undifferentiated granulosa cells of leptin on aromatase activity is stronger than its inhibitory effect on undifferentiated cells. This effect indicates that the number of leptin receptors in granulosa cells may decrease considerably in follicular growth and development, and mature graff follicles are more sensitive to the negative effects of leptin (Spicer, 2001). As a result, leptin-secretion fat cells may be more sensitive to insulin hormone or other substances in women. In this event, it causes a stimulating effect on leptin production (Krishnan et al., 2016). Therefore, many studies have investigated the relationship between leptin and ovaries (Krishnan et al., 2016; Trisolini et al., 2013). It has been shown that serum leptin levels decrease 8 weeks after ovariectomy in rats and this reverses with estradiol treatment (Shimizu et al., 1997). In similar studies, it was reported that 17ß-estradiol treatment stimulated leptin gene expression in ovariectomized rats and resulted in an increase in serum leptin levels (Russell et al., 2017). In vitro studies in both rodent and bovine models showed that leptin has a negative effect on ovarian-derived steroid hormones (Duggal et al., 2002). In particular, leptin has been reported to inhibit insulin-induced progesterone and 17ß-estradiol production in granulosa cells and prevent insulin-induced progesterone and androstenodione secretion in theca cells (Duggal et al., 2002). Leptin inhibition of androstenedione secretion can provide a control mechanism that prevents excessive estradiol production. However, it is still unclear that leptin has a positive effect on ovarian and reproductive functions in ob/ob mice, whereas it has a negative effect on ovarian hormones in vitro (Brash et al., 1996, Cheab et al., 1996). This incompatibility can be explained by the versatility of the effect of leptin on the hypothalamic-pituitary-ovarian axis in vivo (Spicer, 2001).

Leptin, Menstrual Cycle and Menopause

Changes in serum leptin levels, dramatic changes in LH and FSH and ovarian-derived steroid hormones, and other hormones known to

interact with leptin have been extensively searched at different stages of the menstrual cycle, and numerous studies have shown that there have been notable changes in serum leptin throughout the menstrual cycle, and levels are higher in the midluteal phase than the follicular phase (Baig et al., 2019; Moschos et al., 2002). In addition, several studies have shown that serum leptin levels have displayed a slight but not significant increase towards a high level (Teirmaa et al., 1998) or did not change at all at the end of the cycle (Stock et al., 1998, Lin, 1999). Decrease in leptin concentration has also been reported after menopause (Spicer, 2001). Several studies suggest that serum leptin levels in postmenopausal women are likely to be decreased in the postmenopausal period, particularly in obese (Baig et al., 2019; Shimizu et al., 1997).

Leptin in the Male Reproductive System

The true impact of leptin on male reproductive functions is debatable contrary to its known effects on female fertility (Tena-Sempere et al., 2002). The use of leptin for proper functions in the male reproductive system is not very clear. However, various experimental and data gathered from independent groups in recent years suggest that leptin may affect the hypothalamic-pituitary-testicular axis at different levels (Tena-Sempere et al., 2002). Hypogonadotrophic hypogonadism and infertility are common in male ob/ob mice as in female mice. Leptin treatment without calorie restriction, which normalizes body weight in male ob/ob, can rearrange reproductive capacity (Mounzih et al., 1997). In addition, systemic addition of leptin or its active fragment (leptin $_{116\text{-}130}$ amide) leads to FSH and LH secretion in male mice and rats (Barash et al., 1997).

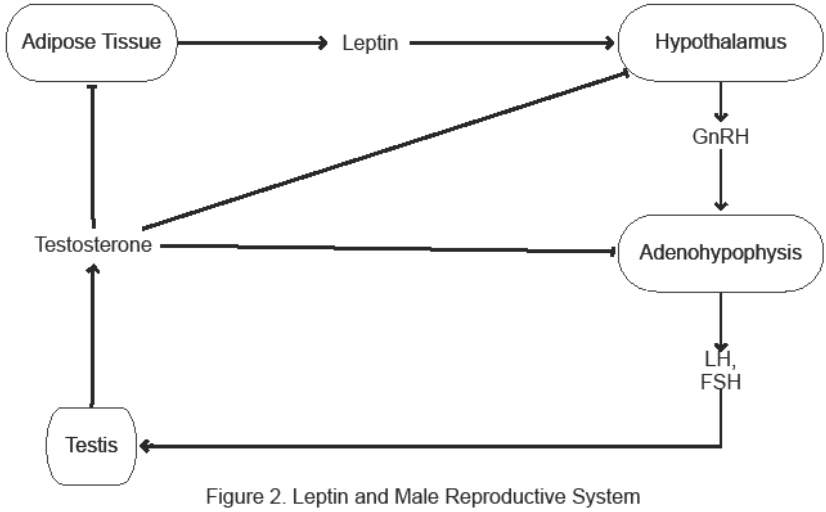

Figure 2. Leptin and Male Reproductive System

The Role of Leptin in Testicular Function

Ob-R expression in testes was first described by Hoggard in murine spermatic cells and Leydig cells while extracting mRNA for the general extracellular domain by in situ hybridization (Hoggard et al., 1997).

Leptin receptors and/or their mRNAs are found in the testes of rats (Tena-Sempere et al., 2001) and mice (El-Hafnawy et al., 2000) and rat leydig cells (Caprio et al., 1999). There are studies indicating that testicular leptin receptor gene expression is developmentally regulated and sensitive to regulation by LH and FSH. Similar to theca cells, leptin is reported to inhibit hCG-stimulated testosterone secretion from testicular tissue and Leydig cells at a dose of approximately 20 ng/ml (Spicer 2001).

In vitro studies indicated gonadal hormones such as testosterone may be an important regulator of leptin secretion. A strong inverse relationship between serum leptin and testosterone levels was reported in untreated and testosterone-treated hypogonadal men (Luukka et al., 1998).

Studies in rats showed the primary inhibitory effect of leptin on gonadal function in men. In contrast, in primates and mice had been showed that leptin does not affect testicular steroidogenesis (Banks et al., 1999, Lado-Abeal et al., 1999).

Male ob/ob mice treated have affected positive manner reproductive function (Barash et al., 1996).

Luukkaa et al. (1998) measured circulating leptin levels in 269 men without diabetes to explain the relation between endogenous leptin and testosterone. They also monitored circulating leptin levels with a 12-month treatment in ten healthy men to determine whether the administration of testosterone affected leptin levels. As a result, Luukkaa et al (1998) found an inverse relationship between leptin and testosterone levels. Serum leptin was inversely correlated with testosterone. This inverse correlation was also seen for body mass index and plasma insulin. Supplementation of testosterone caused to decrease in serum leptin in men. Similar findings were reported by Behre et al (1997). Results of in vitro studies also show that testosterone may plays an important regulatory role in leptin secretion. On the other hand, in a study conducted on elderly men, it was found that leptin has a negative effect on testosterone in healthy human beings, whereas testosterone does not have a determining effect on serum leptin.

The information presented in this review concludes that leptin hormone plays a critical role in the reproductive system beyond nutritional behaviors. Future research on this topic may provide us with valuable information on the relationship between leptin and the reproductive system.

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Chapter 24

NEW APPROACHES IN CALF FEEDING

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1. Introduction

The few initial weeks of life of newly born calves are very critical and important because when they are born they don't have fully developed and functional immune system that can protect them during the hard environment. The stomach of a newly born calf is similar to that of monogastric animals even the stomach is not fully developed and functional during the few initial days of life of a calf. So the feeding priorities of neonatal calves are based on increased and improved quality of liquid diets of carbohydrates, energy, fats and protein sources. The initial 2-3 weeks of calf's life are very critical because in this period lots of physiological and metabolic transformations occur in the GIT of calf. After weaning the calf has to undergo the transition phase from liquid to solid feed and in this respect the GIT of calf has to be mature and develop enough to facilitate this transition (Toullec and Guilloteau, 1989; Davis and Drackley, 1998). The dry feeding should be offered to calves at an early life because it stimulates ruminal development. The different changes in GIT of calf include digestive secretions, enzymatic activities and development of epithelial tissue of rumen which absorbs volatile fatty acids (VFA), these changes in GIT enable the calf to consume solid feed effectively which leads to better weight gain of calf (Sander, 1959).

The physical and chemical composition of starter feed is of great importance in calf nutrition (Warner, 1991), it should contain highly fermentable carbohydrates and adequate amounts of digestible fiber which are important for necessary growth and development of tissue (Williams and Frost, 1992; Greenwood et al., 1997).

During the early days of neonatal calves the rumen is not fully developed and functional and ruminal microflora is not mature enough to perform digestibility (Anderson, 1987a, b; Williams and Frost, 1992). So at this time if the long hay is fed to calves it will reduce the intake and consequently the metabolizable energy intake is also reduced (Stobo, 1966). So it is highly recommended not to offer the long hay to calves until after weaning (Quigley, 1996a; Davis and Drackley, 1998). One of the important factors is type of particles of starter feed either it is pellet, texturized or ground and it is prevents the abnormal growth and development of ruminal papillae keratinization and also to prevent impacting particles between papillae (McGavin and Morrill, 1976; Greenwood, 1997; Beharka, 1998).

There are 3 developmental phases regarding the digestive functions and these are as under (Davis and Clark, 1981)

- **Liquid-feeding phase:** This is the first phase of calf nutrition and in which almost all of the nutrient needs are fulfilled by milk or milk replacer (Orskov, 1972).

- **Transition phase:** In this phase the feeding stuff is changed or gradually changed from liquid to solid feed and both of the liquid diet and starter feed fulfilled the nutritive requirements.

- **Ruminant phase:** In this phase the solid feed is offered to growing calves, microbial fermentation occurs in rumen through which the essential nutrients are taken and used for the growth and development of animals. One of the most important practices on dairy farm is to separate the calves from their dams. During the phases of milk feeding either it is pre-weaning, weaning or post-weaning the calves are at great danger of getting diseased and can die if not properly handled and cared (USDA, 2009). At the time of birth the neonatal calves have non functional and not fully developed rumen, they don't have antibodies and so an early feeding of colostrum invigorates them to be able survive in the most vulnerable period of their life. It is an unhealthy practice done by majority of farmers that the farmers wean the young calves at very young age just to reduce the economic loss that can be obtained by selling milk instead of letting the calves to drink milk from their dams. When the weaning starts there are a lot of transitions, changes happen inside of rumen of young calves. The feed is changed from liquid to solid and entire process of feeding is changed so the entire rumination and digestion process has to be changed. The changes of GIT of young calves include development of rumen, ruminating behavior, morphological developments in gut. All of these developments enable the young calves to start digesting, utilizing forage, hay and concentrates effectively particularly in pre and post weaning periods (Baldwin, 2004; Khan, 2011).

The starter feed that is composed of highly and easily fermentable carbohydrates are supposed to expedite the development of rumen by bringing changes in the morphology of forstomach epithelium (Baldwin, 2004; Drackley, 2008). Contrary to this the young calves reared on extensive system and the calves reared in pastoral system have less access to the starter feed. On the other hand in forage-based, the quality and quantity of forage determines the supplementary feeds' requirement to expedite support growth and development of young calves. However whatever the system may be, the pasture determines the requirements of nutrients for the overall growth and development of young calves.

2. Growth Performance of Calves

2.1. Effect of Starter Feed and its composition

The commercial starter feeds are available in pellet, mash, and textured form, whereas the pellet form is made from finely ground grains, or whole grains and these forms of starter feed ensures the palatability of feed that allows the calves to eat more. The nutritive ingredients of calf starter

provide protein, minerals, energy, and vitamins which invigorates and fulfill the nutritive requirement of young calves (Tamate, 1962; Montoro and Bach, 2012). In past a lot of research trials have been conducted on how to improve and enhance the quality and palatability of starter feed, it was done by mixing various concentrations of carbohydrates (Khan, 2008; Hill, 2008a,b), by implementing various procedures (Abdelgadir and Morrill 1995; Lesmeister and Heinrichs, 2005; Bateman, 2009), by analyzing physical forms (Franklin, 2003; Coverdale, 2004; Bach, 2007), and most importantly by supplementing it with different feed additives (Bunting, 2000; Gorka, 2011; Terre, 2015).

The starch being one of the most important parts of starter feed and its sources include sorghum, corn, wheat, barley, oats, and rice. In an experimental trial it was reported that calves fed with pellet starter feeds, composed of similar concentration of starch mainly from corn source, consumed more solid feed and got high BW than that of those which were given starch from source including barley, oats, or wheat diet (Khan, 2007).

It was reported that in calf starter if sucrose, soybean hulls or molasses is replaced with corn, the body weight gain can be decreased by 10-14% after weaning (Hill, 2008b), whereas on the other hand adding oats instead of corn will increase body weight gain. During the process of digestion, the enzymatic digestion can be affected by physical and chemical attributes of ingredients i.e. corn, barley, and oats. The starch bypasses the rumen and this process is increased by slow digestion and after bypassing rumen the digestion of starch affects the performance and efficiency of feed. The source of starch and how it is processed can affect the rate of fermentation but in this regard some further investigations are required to get a deep knowledge of this (Svihus, 2005).

The commercially available calf starter feeds are composed of 18% crude protein on a dry matter basis (NRC, 2001) and if it is increased up to 22% even then it has no remarkable effects (Akayezu, 1994; Hill, 2007). The protein is an integral part of calf starter and its sources includes canola, sunflower, soybean, corn gluten and linseed meal. The protein source in calf starter is soybean meal (Drackley, 2008) and other protein sources are also used i.e. rapeseed meal (Schrama, 1986), cottonseed meal (Hollon, 1958), sunflower meal (Stake, 1973), corn gluten (Terui, 1996). Generally it is said that when other protein sources are used instead of soybean meal, the body weight gain and feed intake both are negatively affected (Schrama, 1986) which is possibly due to anti-nutritional factors, comparatively low quality poor amino acids present in other protein sources, and less palatability of other protein sources. In a trial (Suarez-Mena, 2011) mixed 39-40% DDGS in starter feed of 7 weeks old young calves and observed low body weight (6 to 10%), lower dry matter digestibility (10%).

As for as the fat concentration in starter feed is concerned, generally it is about ~3–4% because the fat contents higher than this concentration are generally responsible for low intake of feed (Doppenberg and Palmquist, 1991). Hill (2009), observed increased feed intake, improved FCR and improved BW gain when he supplemented ruminally inert fat. (Araujo, 2014) made a nutritional comparison, the calves were given restricted amounts of milk replacer (4-6 L/day), fat contents (as low as 4.1% or as high as 11.2%). It was reported that post weaning feed intake and weight gain was more when 6 L/day milk replacer and high fat contents 11.2% were fed to the calves. This could be due to fact that in post weaning phase, the growth and development was improving gradually which should have been supported by high energy contents in feed.

2.2. Effect of Forage Supplementation

During pre-weaning phase, forage should not be fed to the neonatal calves however the calves should be given access to pasture so that they could nibble the grass and learn this new thing from their dams (Tedeschi, 2009). The herbivorous animals see, smell and find the feeding stuff with the help of light, nostrils and tongue and their mother also help and educate them in this process of ingestion (Mirza and Provenza, 1994, 1990).

However the calves can initiate the forage intake slowly and gradually at young age after learning the foraging skills from their mothers. On the other hand the forage consumption gradually increases with decreased milk consumption. On certain commercial dairy farms, during the initial few weeks of life of young calves, there is a practice of providing ad libitum starter feed with almost no forage feeding. After the weaning the calves are given full access to starter feed and forage consumption (Drackley, 2008). Prior to the phase of weaning the heifers take less amount of forage when reared on a concentrate-only diet is offered to them as compared to the calves who are offered forage plus concentrate diets before weaning (Khan, 2012). (Miller-Cushon, 2013) observed an increased feed sorting behavior of calves when they are offered ration having finely ground forage and observed a decreased feed sorting behavior when ration having coarsely chopped forage was offered.

The type of solid feed provided to the calves in their early life, particularly in post weaning period, can impact their foraging, intake of feed. A behavior is seen among the young calves of not accepting the new feed, they show reluctance to eat the feed and this is called food neophobia (Chapple and Lynch, 1986) but this behavior can be overcome if many calves are reared in groups (Costa, 2014). This social grouping among the young calves particularly in post weaning phase helps them for transition from liquid to solid feeding (de Paula Vieira, 2010, 2012).

in his trial observed forage fed intake can be increased if the calves are provided with chopped hay, high sufficient quantities of milk in an early life of young calves and it also increases forage and nutrient consumption in post weaning phase.

In an experimental trial (Montoro, 2013) made a comparison between coarsely and finely ground feed, he supplemented mixed ration composed of 90% of starter with coarsely 10% chopped (3-4 cm) hay or 10% ground (2 mm) hay, he found increased intake of diet and FCR in young calves which were given coarsely forage than those offered finely ground feed. Taller and smaller bellies of heifers were resulted when the heifers were offered starter feed and hay after weaning as compared to calves which were given only the starter feed (Khan, 2012). When the various other forages were included in the diets of calves, it resulted in increase in feed intake and increased weight gain without hampering the nutrient digestibility (Castells, 2012).

Furthermore, from 2nd week to weaning, the starter feed intake can be improved if oat, hay, barley, or silage was given ad libitum. Therefore it is concluded that solid feed consumption can be enhanced when the forages are too supplemented in the diets of young calves which can ensure true BW and FE in calves, but palatable forages or low digestible forage may decrease the starter feed uptake and body weight but these can fill the gut space.

3. How nutrition impacts the immunity of calf

The neonatal calves are agammaglobulinometric which means when they are born they don't have their own antibodies and developed immune system. This lack of own antibodies is due to the fact that when the calf is developing inside the uterus the maternal antibodies cannot permeate the placenta so when the calf is born it does not have immunoglobulins. This issue is resolved by feeding colostrums to the neonatal calf immediately when it is born. Colostrums is the first lacteal secretion of mammary glands just after birth and it contains large amounts of immunoglobulins which protects the newly born calf from different diseases. The time of colostrums feeding to newly born calf is very important. The early the colostrum is fed to neonatal calf immediately after birth, the more chances of ingestion of huge number of good quality antibodies. The early ingestion of colostrum ensures transfer of the antibodies from dam to the circulation system of calf and in this way the calf is protected from lots of pathogenic microbes.

The prime and sole objective of feeding colostrum to the newly born calves is to transfer immunoglobulins from dam to their offspring and the

calves receive the passive immunity from dams which immunize them up to the level that they become strong and healthy enough to survive and any disease or stressful condition. In this context some important factors include time of colostrum feeding immediately before the birth of calves, quantity of colostrum, breed and age of the cow, etc these factors affect the efficiency of colostrum feeding. A trial was performed by (Stott, 1979) where he reported that age was very important when the first shot of colostrum was fed. The colostrum should be fed to the newly born calves immediately before they are born.

Mixing the antibodies and coccidiostat in order to give a strong and healthy start to the newly born calf is a good practice but most of the time this is overlooked by the farmers. There is a general assumption that if growth of the calf is good, the health must be good. If the decreased amounts of protein and energy are fed to calves it will not only make the calves vulnerable but it also leads to death. In recent past there is a practice to increase the concentration of protein and energy in milk replacers and/or starter feed in order to fulfill the optimum dietary requirements and to expedite or accelerate the growth and development of growing calves. These specially formulated feeds, particularly starter feed, are supposed to not only immunize the calf and protect it from diseases but these also give a strong and healthy start. It is observed that milk replacer having increased proportions of fats and energy contents affects positively and sometime negatively.

Furthermore the absence of any of the essential nutrient in feedstuff of calves, it severely affects the overall health and particularly the immune system of growing calves. The supplementation of minerals, vitamins and trace elements has a positive effect on immunity of calves. Similar kind of research studies were performed by (Gengelbach, 1998) and (Reddy, 1986) at Kansas State University.

4. Feed Additives in calf nutrition

Probiotics are various categories of live bacteria colonizing in the GIT of animal and these are supposed to generate favorable impacts and to compete, destroy, and dismantle the harmful microbes in digestive tract of animals. There are different types of probiotics have been studied, analyzed and recommended to be used as calf nutrition. These products are, a multispecies human-derived product and a calf-specific Lactobacillus product these are were efficacious by improving average daily weight gain and minimizing the chances and severity of disease in young growing calves (Timmerman, 2005). The probiotic product including mixture of *Bifidobacterium pseudolongum* and *Lactobacillus acidophilus* is given calves before weaning it resulted in improved weight gain and minimized the

chances of getting scour (Abe, 1995). In another it was reported that when a combination of probiotic without antibiotics was given to calves it resulted in increased weight gain and reduced the risk of getting diseased (Morrill, 1995). Similar types of results were obtained in study trials conducted by (Jenny, 1991; Higginbotham, 1993; Cruwagen, 1996; and Ewaschuk, 2004). Similarly when the calves having diarrhea were offered live yeast product in milk replacer or in starter feed, it not only treated the diarrheal condition but also improved the weight gain of calves (Galva˜o, 2005).

On the other hand prebiotics are nonliving and indigestible substances used by the microbiota of digestive tract and the microbes become empowered and invigorated enough to compete and destroy the pathogenic bacteria of gut. The carbohydrates i.e. oligofructose, mannanoligosaccharides, etc have shown fruitful and productive results in young calves (Webb, 1992). There is a research trial where it was observed that when mannanoligosaccharides was supplemented in milk replacer it had improved health and an increased starter feed intake as compared to the calves which were offered milk replacer or milk replacer having antibiotics chlortetracycline and neomycin (Heinrichs, 2003). The trisaccharide galactosyllactose supplemented in milk replacer showed better weight gain and also treated scour (Quigley, 2002).

The use of essential oil and phytogenics in starter and/or milk replacer has gained a lot of attention being these as an entirely natural and organic feedstuff. In this regard a trial was conducted where a combination of phytogenics was supplemented with milk replacer and offer to young growing calves (Hill, 2007) and resulted in the increase in weight gain and improved feed efficiency. Furthermore the nutraceuticals (the natural, organic and agro-based compounds used not only as feedstuff but also to treat the diseases naturally). The garlic is used as nutraceutical agent, it contains an important component, allicin, having antimicrobial, antioxidant characteristics and it is used to cure against anomalies in calves. A clinical trial was conducted and it was observed that allicin had some positive impacts on infection caused by Cryptosporidia in calves (Olson, 1998).

An experimental trial was conducted where either fresh or autoclaved ruminal fluid was offered to the young growing dairy calves; and the treatment resulted in improvement of overall health (Muscato, 2002). It was assumed that the bacterial polysaccharides of ruminal fluid extract might have stimulated the immune system of calf. The supplements extracted from serum also may have improved the overall health of growing dairy calves (Quigley, 2002, 2000).

One of the most crucial diseases of young dairy calves is coccidiosis and it results in economic losses, low weight gain and illness. This

condition can be treated by supplementing either milk, starter feed or milk replacer with any standard coccidiostats i.e. decoquinate or lasalocid, and it results in minimizing the clinical signs of coccidiosis and improvement in weight gain of growing calves (Bauer, 1992; Webb, 1992; and Quigley, 1997). The coccidiostats can be included in started feed to treat coccidiosis and it is suggested but the calves may not take starter feed before 1 month of age. However the farmers have to make the calves use to of ingesting the starter feed (Quigley, 1997).

The minerals, although they are required in small concentration as compared to rest of other nutrients, are responsible to have important physiological, catalytic, regulatory, structural and immunological role in living body (Wilson, 2016). They are required in small proportions but even then their absence in diet can affect the reproduction and productivity of dairy animals (Miranda, 2006). So therefore the feed formulations for growing calves should carefully be done, suggested and recommended mineral concentrations should be added to the diets of young growing dairy calves regardless of breed, body weight or performance of animals (NRC, 2001).

The first few weeks of newly born calves are very critical because they don't have antibodies (agammaglobulinometric) when they are born, immune system is immature, and they are weak enough that if the colostrum is not fed immediately after birth, they can die. This colostrum feeding is in fact passive immunity transferred from mother to its offspring. So an immediate feeding of colostrum should be given to the neonatal calves that will empower and invigorate them to be able to survive under stressed conditions. A healthy and strong start should be provided to newly born calves in the beginning of their lives so that they could sustain during diseases and gain considerable weight. Furthermore the optimum growth and development will make them strong enough that they will pass through the difficult transition phase of weaning (transition from liquid feed to solid feed) (Botteon, 2008).

The practice of administering the injectable vitamins to animals also proves to be effective way of improving the performance of animals (Collet, 2017). During the harsh summer days particularly in hot countries the calves become weak, vulnerable, lethargic, show less growth, reduced performance, hyperpnea, low feed intake (West, 2003; Tao, 2013; and Kargar, 2018). So if proper protection, supplementation or antibiotic cover is not given during the summer days of environmental stress, it will result in reduced weight gain, increased chances of diseases, and death of calves (Roland, 2016).

It is observed that the supplementation of chromium in either in milk or in sold feed leads to improved weight gain particularly during summer

months and it is manifested by decreased respiratory rate and improved feed ingestion (Kargar, 2018). Similar results were obtained when minerals were supplemented in diet of calves and it resulted in overall good growth and performance (Glombowsky, 2018; Tomasi, 2018; Volpato, 2018).

Productive and efficient feeding and management plan for rearing the dairy calves determines effective weight gain, overall performance of calves, and future profitability. The way and method of feeding the young calves has huge effects on behavior, performance and health of calves. On the other hand the restricted liquid feeding usually lessens the growth of calves (Jasper, 2002). It is also reported that ruminal fermentation, growth and development of GIT is delayed if the calves are allowed for ad libitum milk intake (Baldwin, 2004) due to the fact that ad libitum ingestion of milk will reduce the solid feed intake (Hammon, 2002; Jensen, 2006; Quigley, 2006).

In a comparative experimental trial more significantly increased milk feeding; more solid feed consumption, increased weight gain, and good feed efficiency were observed when the calves were fed on step down (STEP) method of feeding before and after the weaning as compared to the calves fed on conventional feeding system. The conventional feeding system included milk feeding 10% of body weight for 45 days whereas the STEP feeding method included feeding of milk 20% in first 25 days then milk feeding 10% of body weight till weaning (Khan, 2007). The process of transition from liquid feeding to solid feeding is important and necessary for calves in a way that it is an economically profitable for the farmers. In milk feeding period the nutrients are supplied through milk whereas when the animals shift to solid feed the nutrients are supplied by the grains and forages. This shift to solid feed expedites and triggers the metabolic ramifications and changing going on in the GIT. The tissues of digestive tract of calves have to convert from glucose supplied by milk to advanced metabolism of short chain fatty acids. So therefore the feed ingredients and nutrients act as catalyst and trigger the growth and development of entire GIT of calves when the calves are shifted from liquid to solid feed (Baldwin, 2004).

On majority of the dairy farms, early weaning and restricted-milk feeding plans are practiced to minimize the cost of milk feeding in raising calves. Apart from this gradual inclusion of calf starter feed to the diets of young calves is also practiced. There is another strategy of offering increased quantity of to the growing calves which has shown some positive effects regarding improved weight gain, growth and development of calf (Khan, 2011a).

The practice of offering forage to growing calves when they are on restricted milk feeding is not appreciated because this can choke the rumen

with undigested fiber and due to this choking the starter feed intake can be reduced (Drackley, 2008). If the starter feed contains irregular particles and the fibers in starter feed may produce abrasions on the ruminal wall (Greenwood, 1997). For instance the feeds contain adequate particles and high amounts of processed grains expedite acid production in rumen lower the ruminal pH (Laarman, 2011, 2012). Drackley (2008) documented that forage should not be offered if the concentrate feeds are composed of long particles i.e. whole oats, corn, beet pulp, or cottonseed hulls. These starter feeds, also known as texturized, enhance starter feeding (Porter, 2007) and also improve the overall growth performance (Bach, 2007).

5. Effects of amino acids supplementation

Small intestine development in the neonatal calf is crucial for feed ingestion, absorption of the feed nutrient, and to protect against harmful microbes (Blum, 2006). Structural and functional changes of the small intestine influence neonatal intestinal health and growth performance (Hammon and Blum, 1997).

Amino acids are a major fuel for rapidly dividing intestinal enterocytes and involved in carrying out various processes in small intestine. Arginine (Arg) and Glutamine (Gln) are conditionally essential AA for the growth of neonates (Wu, 2009). In addition to the role that Arg plays as a building block for protein synthesis, it is a common substrate for nitric oxide and polyamine synthesis and is a key signal regulating intestinal cell proliferation (Tan et al., 2010). The small intestine is dependent on Gln because it is used by enterocytes as a source of energy as well as for the endogenous synthesis of Arg (Wu and Knabe, 1995).

Glutamine supplementation has also been shown to improve intestinal morphology (villus height and crypt depth) in weaned calves (Hu et al., 2013). The effect of Gln and Arg on small intestine development in pre-weaning calves has received little attention. Increasing pre-weaning growth through provision of higher milk allowance can have lifetime effects on the first insemination and milk synthesis of dairy cows (Soberon, 2012). Thus, there is an increasing trend globally to offer calves higher milk allowances which expedites the calf growth but minimizes the intake of solid feed in pre-weaning phase and eventually it hinders the development of rumen (Khan, 2007, 2016).

6. Effects of phytogenic oils

In cases where the prices of raw milk are high and the number of calves is higher, dairy cattle farms can be given milk replacer after drinking colostrum for the first 3 days. Milk replacer is mainly obtained by drying milk and milk products with special processes. In addition to milk and milk

products, various plant sources are used to obtain milk replacer. Although calves which are offered milk replacer have a less BW as compared to the calves fed milk, this difference closes with compensatory growth after the weaning. Milk replacer should be taken at body temperature and be sure of their quality. Cold drinking or poor quality calf foods can lead to diarrhea and development disorders (Akyüz et al., 2017).

The case of diarrhea caused by infection or feeding is defined as the loss of too much liquid and mineral in the body due to the deterioration of normal fluid movement in the digestive tract. Diarrhea can cause the loss of the body weight with excessive fluid loss by disrupting the chemistry of the body, causing loss of developmental performance and death in more advanced cases (Costello, 2005). The high level of loss of calves both endangers the future of breeding herds and reduces the amount of milk and meat to be obtained from cattle and causes enormous economic losses of the enterprises.

In the neonatal period, calf loss is between 1% and 10% in developed countries, while this rate can reach up to 10% and 15% level in Turkey (Civelek, 2018). In the neonatal period, 60 % and 62.5 % of calf deaths are caused from diarrhea. Calves are very sensitive and vulnerable in the first month after birth and they are faced with many types of harmful bacteria threat. Pathogenic bacteria such as *Escherichia coli*, *Salmonella spp.*, and *Campylobacter spp.* cause diarrhea in calves, and in more severe cases, it may result in coccidiosis and death (Tüzemen and Yanar, 2013; Akyüz et al., 2017). Albeit cattle breeders take numerous measures to prevent calf deaths, pathogenic bacteria are abundant in digestive systems in the early days of calves and diarrhea cases persist and calf deaths cannot be fully prevented (Tüzemen and Yanar, 2013).

Due to the widespread use of antibiotic feed additives in recent years, the resistance of human and animal pathogens has increased as result of renewing and improving themselves. These pathogens have passed on to humans by leaving residues in animal products, and it has been a concern that antibiotics used for the treatment of these diseases do not work. Due to these concerns, antibiotics were banned in Sweden in 1986 and later in 2005 in EU countries as a feed additive which promotes growth in animal nutrition (Ünlü and Erkek, 2013). The use of antibiotics as a growth promoter in feeds for animals was prohibited in Turkey in January 1, 2006. Due to the antibacterial and antifungal effects of oregano oil, carvacrol and thymol have a lethal effect on microbes (Souza, 2007).

The dietary feed should be formulated in such a way that it should contain all the required nutrients that ensure the optimum growth and development. The diet should be capable enough to strengthen the calf to successfully

execute the transition phase from liquid feed to solid feed (Drackley, 2008). The more early the transition phase comes the more economically feasible it is for the farmers. This is because almost 40% of the expenses are utilized at milk feeding from birth to weaning period (Boulton et al., 2015). The growth rates of female calves indicate their age at first calving (AFC), as the gestation period of heifers is 23-24 months which is economically viable (Boulton et al., 2017). Two years gestation period of heifers is an optimum objective and it leads to economic viability, more number of pregnancies are possible, more number of calves can be produced, increased fertile life of animals, more milk production, etc (Cooke et al., 2013; Wathes et al., 2014; Eastham et al., 2018).

A typical Holstein-type heifer should maintain a growth rate of about 750 g/day from parturition to obtain adequate BW and calve at 24 months (Wathes et al., 2014). The optimal protein to energy ratio for growth in pre-weaned calves is estimated to be about 11.5 g of crude protein per MJ of metabolisable energy (ME) (Hill et al., 2013). Almost 325 g/day whole milk solids (2.5 L/day) or 380 g/day calf milk replacer (3 L/day), which consists of 22.5 MJ ME/kg and 19.5 MJ ME/kg respectively (Drackley et al., 2008).

The conventional milk feeding strategies include offering milk 10% of body weight of calf, and the objective was to enhance intake of solid feed which will facilitate the ruminal development to prepare the calf for an early weaning (Bleach et al., 2005). When the calves early weaned, they are not provided with the required amounts of protein, energy, fats, etc they become malnourished, their life is at stake as their immunity level is decreased remarkably that they become vulnerable and susceptible to disease and eventually die (Godden et al., 2005; Gerbert et al., 2018).

There is a phenomenon of offering the milk to calves above from their requirements for growth and development or in other words it is also known as over-feeding. This is not considered as a good practice since it does not positively affect the health of animals (Gerbert et al., 2018; Hengst et al., 2012). The calves should not be compelled to drink the milk whereas if the calves are allowed to suckle the milk voluntarily the calf consumes about 9 L/day of milk (Bleach et al., 2005), depicting the fact that the larger milk intakes are needed to satiate calves.

The results obtained in a trial recommend daily milk or milk replacer feeding 20% of body weight of calf (Khan et al., 2011) this increased quantity of milk or milk replacer will not improve the weight gain and health of calf but it will also double the weight of calf at the time of weaning (Soberon et al., 2012).

According to the European Union Directive No. 2008/119/EC and

Welfare of Farmed Animals (England) Regulations 2007, the minimum standards are set regarding the adequate quantity of daily feed intake of growing young dairy calves. According to the rules

- It is necessary for protection of calves to feed twice day milk feeding till 6 months of age.
- Milk feeding in the first month of life for once in a day can lead to complications of abomasums (abomasitis or bloat).
- Sufficient amount of fresh water should be accessible to calves round the clock particularly in hot summer days (Van der Burgt, 2013; FAWC, 2015).

Water is one of the most important nutrients and it has a very critical role regarding growth and development, although calf gets enough quantity of water from milk or milk replacer but provision of fresh water 24/7 is an important management strategy (Thomas et al., 2007). The water intake is important in a way that it initiates the ruminal development and (Govil et al., 2017) which leads to increased starter and solid feed intake and this aids in increased weight gain and performance of young growing dairy calves (Wickramasinghe et al.,2019).

7. Gut health feeding strategies

It was reported that in 2010, about 7-10% heifers, in pre weaning phase, suffered from digestion related problems and were treated with antibiotics (NAHMS Dairy Heifer Raiser Report, 2011). Now it has become a strategy to add some antibiotics or nutraceuticals in the diets of calves just to give protection to calves if they suffer from any disease particularly in the first 2 weeks of their life. This strategy is cost effective as it saves additional expenditure that could be spent on treating the calves. These strategies of giving protection through feeding are supposed to minimize the possible interaction of harmful microbes to the cells of GIT. In this context some products are used through feed and these include hyperimmunized egg protein, probiotics, prebiotics, etc. Specific antibodies can be produced by inoculating some specific pathogen in eggs and these are known as hyperimmunized egg proteins, these are then given to calf to cure the diseases of GIT. The probiotics or direct-fed microbials are live microbes either single or in combination with other microbes, inside of digestive tract they destroy the harmful bacteria. The prebiotics are indigestible dietary components; these empower and invigorate the beneficial bacteria in the GIT of calves. In 2011, Ballou performed a research trial where mixture of probiotics, prebiotics, and hyperimmunized egg proteins was used in Holstein calves to check the effects on growth and performance and it resulted in showing positive effects on GIT.

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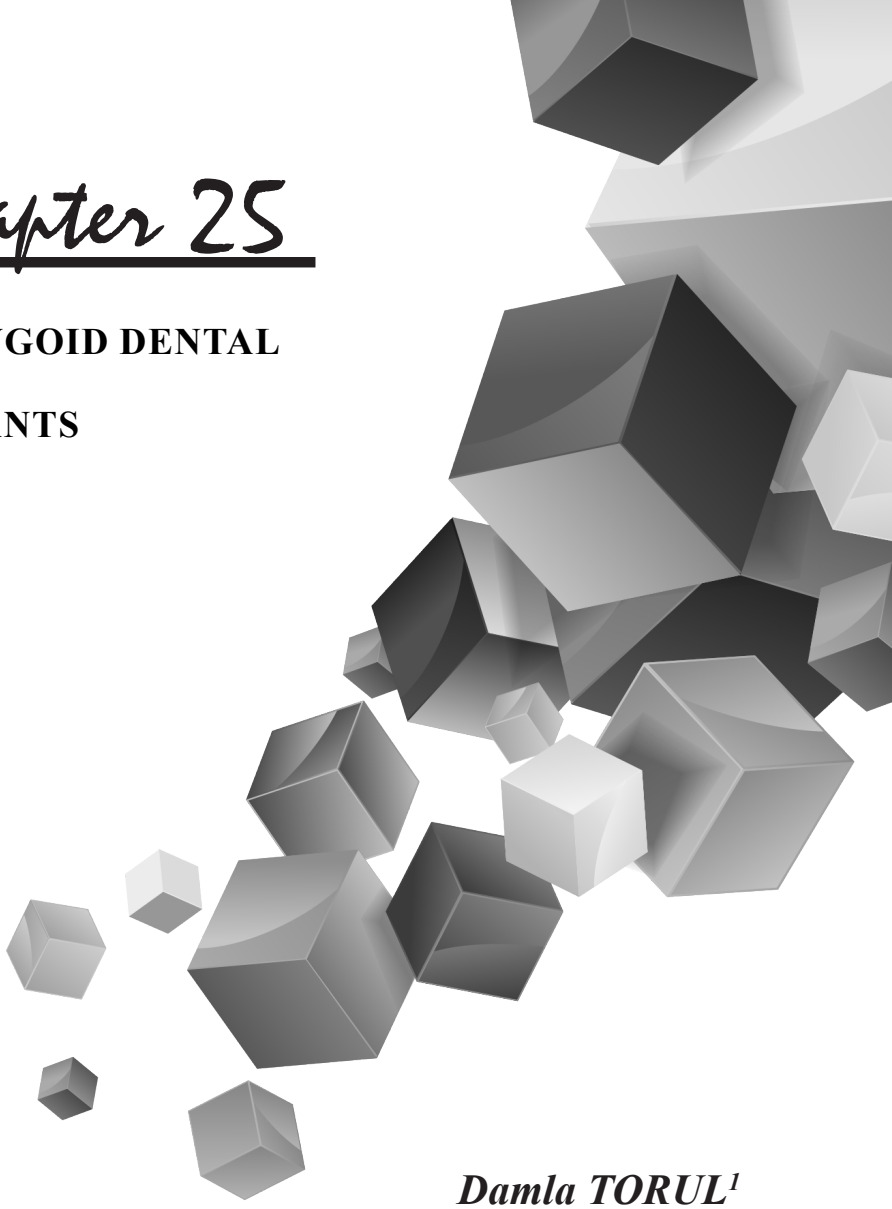
Chapter 25

PTERYGOID DENTAL IMPLANTS



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1. Introduction

Edentulism usually causes functional, aesthetic, psychological, and social limitations. Osseointegrated implants have been used for many years for the rehabilitation of the partial or total edentulism since the osseointegration phenomenon first introduced by Branemark (1, 2). It has been reported that osseointegrated implants showed 90 % survival rates over a 10-year follow-up (3, 4). However, patients who have severe posterior maxillary atrophy pose challenges in terms of placing implants because of poor bone quality, lack of adequate bone volume as a result of pneumatization of the maxillary sinus, and accessibility problems (5, 6). Several factors play a role in severe maxillary atrophy such as the long-term absence of teeth, hyperpneumatization of the maxillary sinus, infections, severe periodontal disease as well as bone defects occur due to the tumors and cysts (7, 8).

There are various approaches for the rehabilitation of the maxillary atrophy and placement of implants. These include sinus lift procedure, grafting with autogenous bone, allogeneic materials, inlay/onlay grafting, ridge expansion, guided bone regeneration, interpositional grafting performed with Le Fort I, and distraction osteogenesis (2, 7, 9, 10). However, these techniques require additional surgical procedures, a long healing period, associated with higher morbidity, and need higher costs (2, 5, 9, 11).

Utilizing different types of implants such as shorter and/or ultra-short, tilted implants, as well as zygomatic implants have been recommended to solve these problems (3, 12). It has been reported that the buttresses of the midface with cortical bone serve as regions which provide excellent anchorage for initial implant stabilization (13). Base on this the zygomatic implant which placed into the zygomatic bone was introduced by Branemark for the rehabilitation of patients with defects caused by pathologic and congenital conditions to obviate the need for sinus lifting procedures or the requirement of other grafting techniques. Despite the advantages of the zygomatic implants, the surgical procedure can be difficult and requires an experienced physician (12, 14-16).

Pterygoid implants have been recommended as an alternative treatment procedure for severely atrophic maxillary jaw, which enables inserting implant without sinus augmentation or additional grafting and, requiring distal cantilever during prosthetic rehabilitation. Linkow in 1975 first introduced pterygoid implants and Tulasne in 1992 described the surgical approach (10, 17-20). These implants have high reported success rates and obtaining anchorage from the dense cortical bone in the pterygoid process (sphenoid bone) and pyramidal process (palatine bone). Dense bone in that area provides excellent anchorage for the implants (5, 8, 21).

Confusion exists regarding the terminology of the implants placed in the pterygomaxillary region. Several terms that include 'pterygoid implants', 'tuberosity implants', and 'pterygomaxillary implants' are used in literature (2, 22, 23). Pterygoid implants described as the implant placed from the maxillary tuberosity into the pterygoid plate. 'Pterygomaxillary implant' considered to refer to the implant those apex is anchored from the pterygoid plates. On the other hand, tuberosity implants rarely placed to the pterygoid plates and usually engages the distal aspect of the maxillary alveolus that is referred to as tuberosity area. The tuberosity area is majorly composed of Type III or Type IV bone (2, 22). The length and angulation of these two types of implants show differences. Tuberosity implants were shorter implants with lower angulation of 10°–20° to simulate wisdom tooth, however pterygoid implants were longer and are placed in the pterygoid plate of 45° to 70° angulation (6, 22-24). 15-18 mm dental implants reported placing the pterygoid plates usually without complication (8).

The purpose of this review is to summarize the indications, surgical procedures, prosthetic rehabilitation, as well as the clinical implications of the pterygoid implants.

2. Quality and Quantity of Bone

Generally, the quality and quantity of the jawbones differ according to the location. The more dense bone is usually seen in the anterior mandible. This is followed by the anterior maxillary and mandibular posterior regions respectively, and the least density is typically observed in the posterior maxillary region. The cortical and the trabecular bone of the upper jaw differs from the lower jaw. When compared to the mandible the cortical bone is thicker and denser, and the trabecular bone is coarse. In 1985, Lekholm and Zarb described the bone qualities (25) (Figure 1).

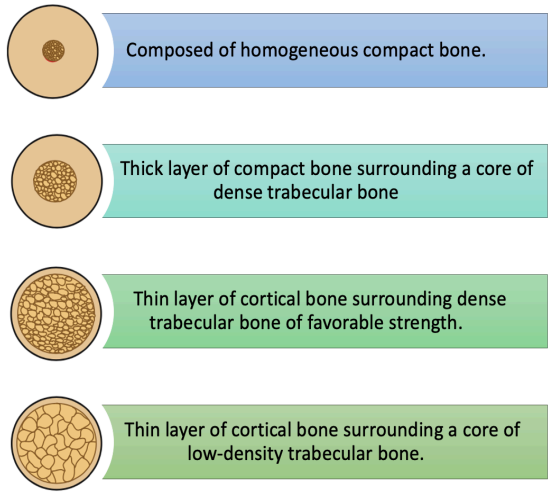

Figure 1. *Bone qualities described by Lekholm and Zarb*

Misch described four bone categories located in the edentulous areas of the maxilla and the mandible (Figure 2). D1 bone consists of cortical bone with high density and almost never seen in the maxillary jaw, usually found in the mandible anterior region. A dense and porous cortical bone-D3 bone was observed in the anterior and also posterior regions of the mandible. The bone within this cortical housing has a coarse trabecular bone. D3 bone type is observed most commonly in the maxillary arch. D4 bone is usually seen in the posterior maxilla (26).

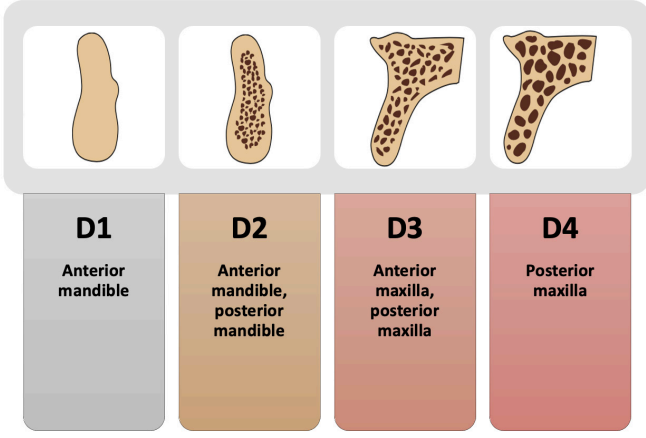

Figure 2. *Bone densities described by Misch (26)*

In 1988, Cawood and Howell proposed classification and described the changing of the shape of the alveolar process of edentulous jaws (Figure 3) (Table 1) (27).

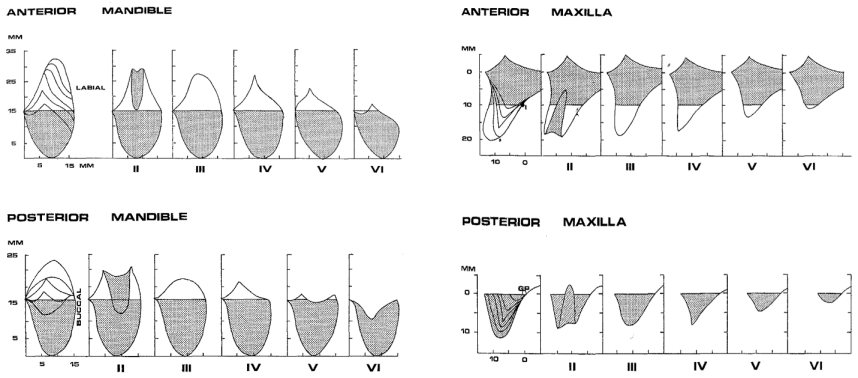

Figure 3. *Classification of edentulous jaws (27)*

Table 1. *Cawood and Howell, 1988 (27)*

Class	
I	Dentate.
II	Immediately post extraction.
III	Well-rounded ridge form, adequate in height and width.
IV	Knife-edge ridge form, adequate in height and inadequate in width.
V	Flat ridge form, inadequate in height and width.
VI	Depressed ridge form, with some basalar loss evident.

Implant success is often related to the arch location, bone density, and degree of atrophy in jaws. The bone density and the location of the bone type are a dominant factors that affects the treatment plan firstly. The dentist can predict the bone quality in the implant area to make an initial treatment plan. The D4 is most frequently seen in the posterior location of the maxillary jaw (≈40%) (28).

3. Anatomy of Pterygomaxillary Area

The lower density maxillary tuberosity, the higher density pyramidal process, and the pterygoid process formed the pterygomaxillary region. The mean thickness of cortical bone in the junction of pyramidal and pterygoid processes is 6–6.7 mm (8, 29). Ideally placed pterygoid

implants are placed from maxillary tuberosity to the pyramidal process and finally engaging pterygoid process (22, 30). The highly mineralized pyramidal and the pterygoid process provides excellent anchorage that obviates additional surgical procedures (5, 17, 30). However, tuberosity is composed of poorly demineralized cancellous type bone of type III and IV (2, 5). When compared with tuberosity it has reported that density in the pterygoid region 139.2 % higher (8).

The thickest bone is located in the middle part of the pterygoid process and located 3–4 mm medial to the alveolus, thus the implant should place slight medially to reach this region. The hamular process also can be palpated as an anatomic landmark on the medial pterygoid plate (29). The pterygopalatine ganglion, branches of maxillary nerve, fatty tissue, the Vidian nerve, pterygoid muscles, the distal branches of the maxillary artery, and a few emissary veins make up the content of pterygoid fossa (21). 1 cm superior to the pterygomaxillary suture internal maxillary artery crosses with an average distance of 2.5 cm (29). Pterygoid venous plexus and descending palatine artery are adjacent to pterygomaxillary fissure and pose a risk for hemorrhage (8).

Knowledge of anatomy and adjacent vital structures that can be damaged during surgery has crucial importance in performing pterygoid implant surgery. Especially the structures such as maxillary artery, greater palatine canal, and nerve should be taken into the consideration (18, 31). Although the insertion of pterygoid implants requires surgical experience and skills, considering the anatomical structure of the pterygoid process there is a common thought inserting a pterygoid implant in this region has a low risk of complication. It is suggested that bleeding observed during surgery usually comes from the pterygoid muscles and will be controlled by inserting the implant (6, 18, 23, 24) (Figure 4).

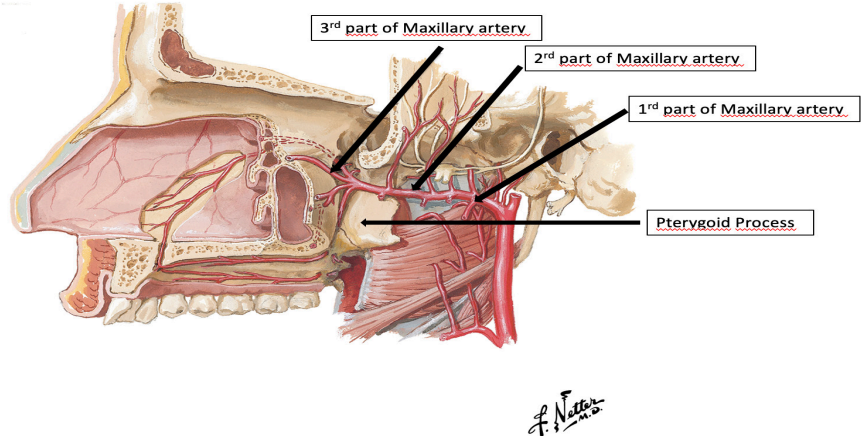

Figure 4. *Anatomy of the pterygomaxillary region (32)*

4. Indications and Contraindications of the Pterygoid Implants

For patients with partially-completely edentulous arches, or maxillectomy defects pterygoid implants are indicated. They are particularly useful when four implants do not provide enough support. The indications of the pterygoid implants are the absence of enough width and height bone between premolar and molar areas of the maxillary jaw. Pterygoid implants use when the sinus wall does not allow placing implant, due to the maxillary sinus hyperpneumatization (33). Pterygoid implants permit the immediate loading thus they use with patients who want immediate prosthesis after surgery (30).

The main contraindication of the pterygoid implants is the existence of an uncontrolled systemic disease such as diabetes mellitus, heart disease, etc, or patients with ongoing maxillary radiation therapy. Patients who have trismus or inadequate mouth opening that does not allow accessing the posterior region of the maxillary jaw create contraindication. Also, an impacted wisdom tooth which prevents access considered as a contraindication. Furthermore, the lack of bone in the pterygoid area is another contraindication (2, 23, 30, 33, 34).

5. Advantages and Disadvantages of the Pterygoid Implants

Pterygoid implants have several advantages and quite a few disadvantages (3, 8, 17, 22, 23, 29, 33) listed in Figure 5 and Figure 6.

Figure 5. *Advantages of the pterygoid implants*

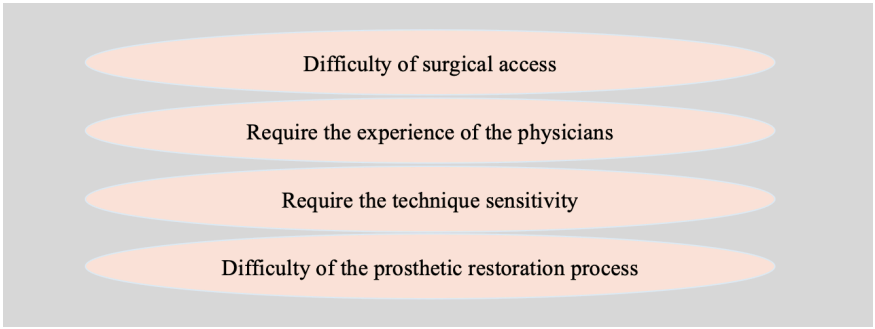

Figure 6. *Disadvantages of the pterygoid implants*

6. Complications

Pterygoid implant surgery widely considered safe and surgical complications reported in the literature are uncommon. The reported complications include hemorrhage, hypoesthesia of the palatine nerve, pain, venous bleeding, trismus, infection, rhinosinusitis, oroantral fistula, misplacement of implant (18, 20). Reychler and Olszewski (35) reported an intracerebral penetration occurs in a zygomatic implant into the pterygoid region. Valero and Valeron (36) reported minor venous bleeding resolved with local hemostatic agents. Krekmanov (37) reported losing implant during placement due to over drilling. Displacement cases also reported to infratemporal fossa by Nocini et al. (38) and pterygoid fossa by Dryer et al. (39).

7. Surgical Procedure

Preoperative Evaluation

A stepwise preoperative evaluation that includes medical/dental history, preoperative clinical/radiological examinations, and pre-treatment photographs have important to perform adequate planning (2). Imaging employing high-quality orthopantomograph considered providing enough data for preoperative planning, however computerized tomography (CT) particularly Cone Beam Computed Tomography (CBCT) enables a more detailed evaluation of the region since the extent of sinus pneumatization and relative bone density of pterygoid plates to the maxillary tuberosity can be evaluated (8, 29). Especially, before the surgical procedure of the implants 20 mm and below in length CBCT is recommended to prevent complications (40).

The insertion of pterygoid implants is suggested being performed specifically to individual anatomy. With the advances in the 3D technologies and virtual planning usage of surgical stents increasingly take place in the preoperative planning of the pterygoid implants (3, 6, 20, 21). The use of

a surgical guide avoids perforations into adjacent anatomical sites (palatal or buccal). Models help to identify the patient-specific anatomy, point of entery, mesiodistal and buccopalatal angulations (6). Guided surgery with stents useful in stabilization of initial drilling and adjust angulation. However, it may also pose limitation due to the thickness of the stent along with the anatomical difficulties of the area, and resulted in complications like implant displacement (8).

Surgical technique

Although previously general anesthesia suggested for implant placement, recently surgery usually performed under local anesthesia (41). Pterygoid implants can be placed to the placed to the tuberosity and the pterygoid process thus the surgical procedure show difference among them (6), (Figure 7).

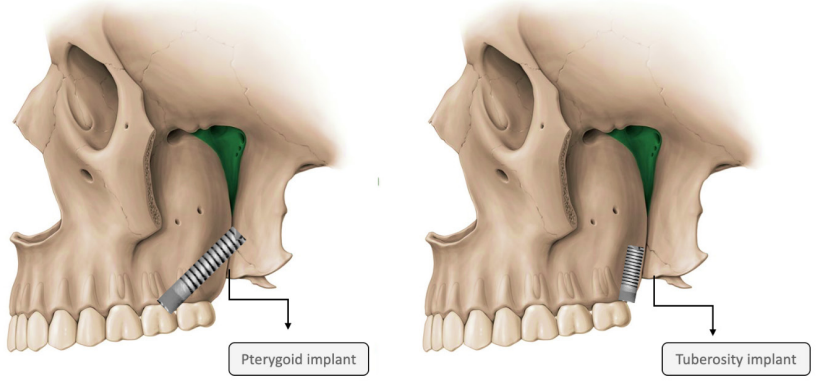

Figure 7. *Placement of pterygoid and tuberosity implant*

The standard surgical technique: After blockage of the maxillary nerve and the palatine nerve with local anesthesia an incision was made on the crest of the tuberosity from the pterygomaxillary fissure to the premolar region, and a relaxing incision is performed. A full-thickness mucoperiosteal flap was raised to uncover the region entirely. The drill entry located 3-4 mm in front of the tuberosity. The drill axis is about 20-30° in the horizontal and 45° in the maxillary plane. Antero-posterior and mesio-distal drilling angles were adjusted to the patient's anatomy. The hamular process is palpated and the drill is directed 5 mm laterally at approximately 45 degrees to the occlusal plane. This is the guide to determine the thickest part of the pterygoid bone. Drilling with a pilot drill performed to the pterygopalatine-tuberosity suture. If the path is correct dense cortical bone of pterygoid plate reached after 10 to 14

mm insertion. After drilling sinus controlled and if the sinus has been perforated, a new enterance located at least 3 mm posterior to the first one must be determined. An implant engaging 3 to 4 mm to the pterygoid process is selected. Usually, a self-tapping, 15- to 20-mm implant placed. Distal angulation of 35° and 55°, adjusted based on the sinus floor and the bone height of the tuberosity. In this technique, the gradual progress from cancellous to cortical bone, without a break should be perceived. In some cases, it is suggested to extract the third molars. The cover screw or the abutment was placed, and the flap was sutured subsequently. In some instances with wide gingiva more than 4 mm, soft tissue plasty can be performed (10, 20, 29).

To increase primary stability, reduce trauma, and the failure of various modifications of the surgical techniques have been reported. Venturelli et al. (42) described a soft technique with a single drill at 600 rpm. Consequently, the implant was inserted manually. This technique was suggested to improve primary implant stability and early prosthodontic rehabilitation. Valeron and Valeron(36) combines drills and straight osteotomes to preserve bone and to reduce complications. Penarrocha et al. (43) aimed to minimize surgical risk, preserve bone, and tactile control and facilitate the formation of implant bed and recommended a combined burs and osteotomes usage during surgery.

Tuberosity implants are placed into the tuberosity, parallel to the sinus posterior wall. Although the surgical approach is similar to pterygoid implants, curved osteotomes preferred and insertion performed with an angle of 10–20 (6, 24).

8. Prosthetic Rehabilitation Supported by Pterygoid Implants

Implant-supported prostheses ensure a reliable option for the rehabilitation of edentulous jaws (44). However, the quality and quantity of bone, amount of resorption of the alveolar ridge effect the location of inserted implants as well as the size and length of implants. Generally, in the maxillary posterior region with D4 bone, some limitations rise and cause difficulty (45). Hyperpneumatization of the maxillary sinus is cause a complexity to insert the implants in the posterior area (33). Sinus lifting and/or bone augmentation may require in the posterior of the maxilla, in order to insert the implant and ensure the suitable osseointegration. In a situation where these techniques cannot be performed, implants may be inserted in the anterior area of the maxillary jaw and prosthetic rehabilitation may be manufactured with posterior cantilevers. However occlusal forces are increased in the molar areas while mastication and prostheses with posterior cantilevers may not overcome the bite forces

and it cause some complications, such as screw and prosthesis fracture, marginal bone loss with several degrees, and finally loss of implant osseointegration. Utilizing the pterygoid implants ensure avoiding the distal cantilevers (23, 33). Furthermore, in certain patients with very wide and high smiles, a shorter restoration would also result in unaesthetic black spaces at the distal extent of the buccal corridors. Increases A-P spread, allowing for significantly longer full arch restorations with more chewing surface and improved aesthetics for certain patients with wide smiles (8).

It is possible to perform prosthetic rehabilitation either immediately after the surgical operation (33) or after the healing period (10, 46). Both constant and provisional prostheses are built in the same way. The process of the prosthetic restoration was schematized in Figure 8.

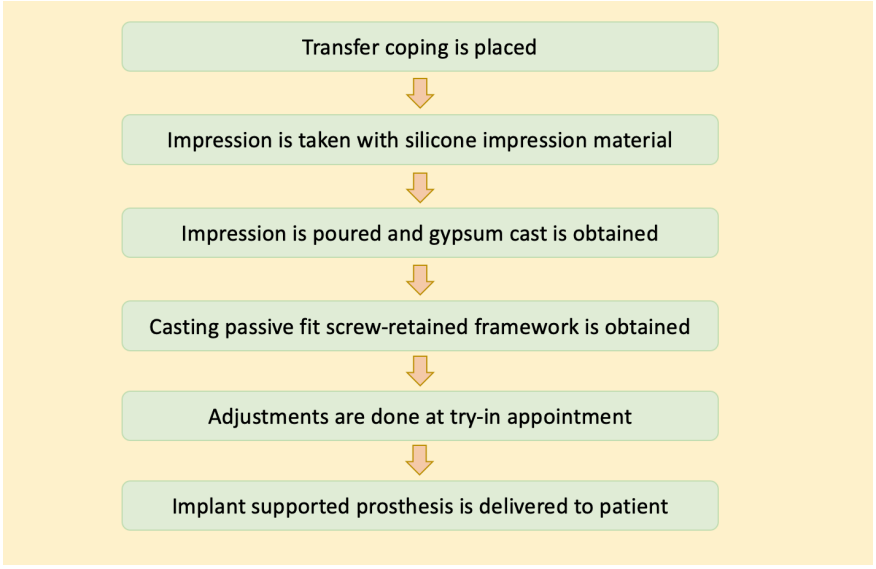

Figure 8. *The process of the prosthetic restoration*

9. Clinical implications of Pterygoid Implants

Tulasne reported an 80 %, success rate in the early usage period of pterygoid implants, and with the advances in biotechnology higher success rates observed regarding pterygoid implants (18). Recently in a systematic review Araujo et al. (5), reported a 10-year survival rate of pterygoid implants 94 % and similar to conventional implants. Regarding different length and angulation of the implants, different success rates exist in the literature. Clinical outcomes of the pterygoid implants in literature, clinical studies that related to pterygoid implants, and their success rates summarized in Table 2.

10. Conclusion

Comparable survival rates and long-term clinical outcomes between pterygoid and conventional implants in maxilla point out these implants as a predictable solution for the rehabilitation of severely atrophic jaws by revealing low rates of complication.

Table 2: *Clinical studies about the pteryoid and tuberosity implants*

Author/Year	Study design	No. of patients	Number/ Type of implants	Width and lenght of implants (mm)	Complication	Success (%)	Follow-up
Graves, 1994 (47)	Clinical case	49	64	NR	NR	89	NR
Balshi et al., 1995 (48)	Prospective study	44	51 PI	3.75-4/ 10-20	NR	86.3	3 years
Valeron et al., 1997 (49)	Prospective study	19	31 TI	NR	NR	93.5	3 years
Balshi et al., 1999 (50)	Prospective study	189	356 PI	3.75-7/ 8.5-20	NR	88.2	4.68 years
Krekmanov, 2000 (37)	Prospective study	22	9 PI	NR	Mobility	NR	1.5 years
Vrielinck et al., 2003 (51)	Prospective study	29	14 PI	3.75/-	NR	71	6-24 months
Balshi and Wolfinger, 2003 (52)	Case report	1	2 TI	NR	NC	NR	3 years
Penarrocha et al.,2004 (53)	Case report	1	2 PI	4.1/ 16	NC	Satisfactory	6 months
Balshi et al., 2005 (54)	Retrospective study	82	164 PI	3.75-4/ 13-18	NR	96.3	2.6 years
Valeron et al., 2007 (55)	Retrospective Study	92	152 PI	NR	NR	94.7	10 years
Ridell et al., 2009 (56)	Retrospective study	21	22 TI	3.75-4/ 13-20	NR	Satisfactory	8 years
Peñarrocha et al., 2009 (57)	Retrospective Study	45	68 PI	4/ 16	NR	97.05	1 year
Sherry et al., 2010 (58)	Case report	1	2 PI	4/ 18	NR	NR	30 months
Park et al., 2010 (59)	Retrospective study	7	7 TI	3.75/ 11.5-15	NC	NR	NR

Peñarrocha et al., 2012 (60)	Retrospective study	18	10 TI	4.2-5.5/ 10-13	NR	NR	1-7 years
Rodriguez et al., 2012 (61)	Retrospective study	392	454 PI	3.75-4.2/ 15-20	Hemorrhage Hypoesthesia	96.5	6 years
Anandakrishna and Rao, 2012 (62)	Case report	2	1 TI	5/ 13	NC	NR	NR
			2 TI	NR			
Nocini et al., 2013 (63)	Case report	1	1 PI	NR	NC	NR	6 months
Bidra et al., 2013 (64)	Case report	1	2 PI	4/ 15	NC	NR	1.5 years
Balshi et al., 2013 (11)	Retrospective Study	NR	67 PI	4/ 7-13	NC	88.06	NR
		NR	925 PI	4/ 15-18	NC	94.16	
Curi et al., 2015 (23)	Retrospective Study	56	66 PI	3.75-4/ 18-20	NC	100	3 years
Cucchi et al., 2017 (65)	Case report	1	2 PI	4.1/ 15	NC	NR	1 years
Ardekian et al., 2018 (46)	Prospective study	20	35 PI	-/ 10-20	NC	91.4	11 months
Holtzclaw and Telles 2018 (8)	Retrospective study	16	25 PI	3.5-4.3/ 11.5-13	NR	100	6-40 months
Jimoh and Diederich, 2018 (10)	Case report	2	3 PI	3.5/ 20	NC	NR	6 months
Dryer et al., 2019 (66)	Case report	1	2 PI	4.3 mm/ -	ID	Pain LMO	1 year
Loewenstein et al., 2020 (67)	Case report	1	2 PI	4/20	NR	NR	1 year
Nag et al., 2019 (30)	Case report	1	2 PI	-/ 22-25	NR	NR	1 year
Osman et al., 2020 (68)	Case report	1	2 PI	3.5/ 29-35	NC	Satisfactory	4 years
Signorini et al., 2020 (33)	Prospective study	15	28 PI	4/ 13-18	Mucositis Complications with the interim prosthesis	100	1 year
Stafenalli et al., 2020 (34)	Case series	14	28 PI	NR	NC	96.4	1 month

NR: Not reported, **NC:** No complication, **PI:** Pterygoid implant, **TI:** Tuberosity implant, **LMO:** Limited mouth opening, **ID:** Implant displacement

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Chapter 26

APPROACH TO BLEACHING APPLICATIONS IN DENTISTRY

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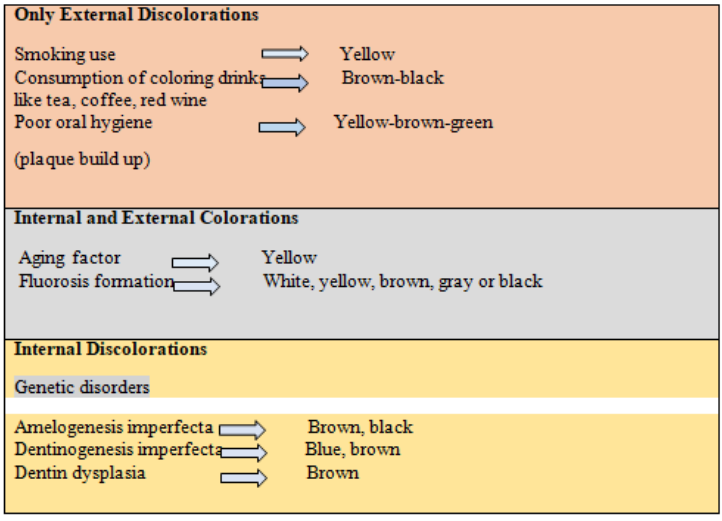
With the increasing interest in aesthetics, the demands for improving the defects in the teeth have also started to come to the fore. The color changes in teeth, which are aesthetically important, are an element that cannot be ignored since they affect the appearance and self-confidence of the person (Kelleher & Roe, 1999). This factor, which causes changes in smile, is provided by tooth bleaching methods, which is the most popular cosmetic treatment method (Nutter et al., 2013; Moreira de Freitas et al., 2016).

The etiology of tooth discoloration depends on many factors. External discoloration, which is generally seen in the interfaces of the teeth and gingival margins, occurs depending on the poor oral hygiene, consumption of colored foods and beverages, smoking and the effects of chlorhexidine (Şeker & Sarı, 2019). These colorations occur only in the enamel. Internal coloration related to enamel and dentin are listed as genetic disorders such as amelogenesis imperfecta, dentinogenesis imperfecta, systemic diseases, drug use that affect tooth development such as tetracycline, pulp necrosis, passage of blood into dentin tubules during pulp extirpation, leaving residual filling material in the pulp chamber, restorative and endodontic materials, body products such as bilirubin and hemoglobin (Abbott,1997; AM Sulieman, 2008). While external discoloration can be partially removed by tooth brushing and mechanical cleaning, this is not the case for internal discoloration (Walsh, 2000; Yap & Wattanapayungkul, 2002).

In the literature review, it was seen that many methods were used for vital tooth bleaching. There are many approaches in different samples depending on the bleaching agents, application times and concentrations of these agents, and whether light activation is used or not (Goldstein & Garber, 1995; Sulieman, 2004).



Discolouration due to drug use during tooth development		
Tetracycline	⇨	Brown, gray and black
Fluorosis	⇨	White, yellow, gray and black
Ciprofloxacin	⇨	Green
Minocyclin	⇨	Brown
Discolouration due to trauma		
Pulpal hemorrhage	⇨	Pink
Enamel hypoplasia	⇨	Green, black
Discoloration due to resorption in the roots		

Table 1. *Types of tooth discoloration and the color changes (Şeker & Sarı, 2019)*

Bleaching

It is aimed to lighten the tooth color by using chemical agents in the tooth bleaching process, which is one of the most desired treatments by the patients and started to be applied at the beginning of the 1800s (Joiner, 2006). Transformation from peroxides in the structure of bleaching agents to nonstable free oxygen radicals is in question in tooth bleaching which takes place in the form of an oxidation-reduction reaction (Albers, 1991).

The best time to apply tooth bleaching procedures in children is after the age of 6. Another important factor in determining the treatment time is root development. It is recommended to start the bleaching process after completing the root formation. Children's teeth colors lighten faster, especially because enamel permeability varies with age (Şeker & Sarı, 2019).

With the increasing popularity of tooth bleaching, which is the main factor for perfect smiles, many different bleaching agents have been introduced to the market. These items are presented in a variety of ways for the individual's own use or for professional application by the physician (Hasson et al., 2006).

The bleaching process has many advantages. This type of treatment is frequently used because of its low cost, low post-treatment side effects, high reliability, and good aesthetics. Considering the indications, this method of treatment is used in bleaching darkened teeth in order to ensure complete aesthetics before composite restoration or laminate veneers, fluorosis and tetracycline coloring, coloring caused by chromogenic foods, traumatic coloring, smooth surface homogeneous coloration, nicotine-induced coloration, porphyria coloration. (Özel et al., 2007; Koruk & Kırzıoğlu, 2010).

Bleaching treatments only affect natural teeth. Since it has no effect on porcelain or restorations, in these cases, the application of these treatments is contraindicated. These treatments are not recommended in patients with hypersensitivity in their teeth, in patients with adaptation problems, in cases of excessive enamel loss, during pregnancy and breastfeeding, in teeth with large pulp, in teeth with open root surfaces, and in cases with advanced discoloration such as fluorosis and tetracycline (Koruk & Kırzıoğlu, 2010).

When the side effects of bleaching treatment are examined, it has been observed that the most common negative condition is hipersensitivity. There are low molecular weight peroxides inside bleaching agents in high concentrations. These peroxides that pass from dentine to pulp cause hypersensitivity in teeth (Council on Dental Therapeutics, 1994). In the plates used for home bleaching, sensitivity may occur in the teeth due to excessive pressure. Apart from these, the duration of application of these agents and the pain threshold of the patient are also effective factors on sensitivity (Cooper et al., 1992). In order to prevent this, potassium nitrate and fluoride were added to the bleaching gels.

Another side effect is gingival irritations that occur in the gingiva. In this case, which occurs as a result of the bleaching agent coming into contact with the gingiva, while the tissue turns white, recovery is observed after a while (Haywood, 2000). In order to prevent this negative situation, the plate should be made compatible and the material should be used in a way that does not overflow. When using bleaching agents, rubberdam or a protective barrier for the gingiva should definitely be used (Majeed et al., 2015). Apart from tooth sensitivity and gingival irritation, serious problems can occur in the temporamandibular joint due to the improper preparation of the plate.

Ingestion of the bleaching agent (hydrogen peroxide) can result in cyanosis, respiratory distress or even death. Important here is the amount and concentration of hydrogen peroxide solution swallowed (Sherman et al., 1994). In order to prevent all these side effects, preparations containing low peroxide concentrations should be used and the time allocated for treatment should be reduced (Küçük & Keçeci, 2019).

Bleaching Agents

Hydrogen peroxide and its derivatives are mostly used in tooth bleaching. Apart from this, agents such as sodium perborate and carbamide peroxide are bleaching agents used in dentistry in combination with hydrogen peroxide or separately. While hydrogen peroxide and carbamide peroxide are generally preferred for extracoronal bleaching, sodium perborate is recommended for intracoronal bleaching (Rotstein et al., 1996).

Hydrogen Peroxide (H2O2)

Hydrogen peroxide, which is an oxidizing agent and is weakly acidic in pure aqueous form, can form highly reactive free radicals such as perhydroxyl (HO2-) and oxygen (O). HO2 is strong, O is a weak free radical. Perhydroxyl anion alone can also be effective in bleaching. The formation of oxygen occurs by ionizing hydrogen peroxide. The bleaching process occurs by penetrating oxygen molecules, which are produced by the breakdown of hydrogen peroxide, into teeth and breaking down pigmented molecules (Gimeno et al., 2008).

Concentrations of hydrogen peroxide in use range from 5% to 35%. 30% solution of hydrogen peroxide called superoxol in water is the most commonly used material in tooth bleaching (Plotino et al., 2008). Its bleaching ability is good because its 25% solution in ether named 'Pyrozen' penetrates well into dentin canals. However, it has disadvantages such as bad smell and if it leaks, it creatures caustic effect (Çalışkan, 2006).

While applying the bleaching treatment, care should be taken to ensure that the teeth are especially dry and clean due to the presence of in the mouth some enzymes that prevent the ionization of hydrogen peroxide (Carlsson, 1987).

Carbamide Peroxide (CH6N2O3)

Carbamide peroxide, an aqueous solution, is used in tooth bleaching treatments. In the case of contact with the tissue, hydrogen peroxide, carbonic acid, urea and ammonium are formed. 3.4% hydrogen peroxide and 10% carbamide peroxide have equivalent bleaching effect (Gökay & Müjdeci, 1998). When the carbamide peroxide is broken down, the ammonia released increases the pH of the environment. Thus, bleaching reactions are also facilitated (Dahl & Pallesen, 2003).

The pH of carbamide peroxide, which is generally found in concentrations of 3% to 20%, varies between 5 and 6.5 (Dahl & Pallesen, 2003). While the rate of carbamide peroxide is 10% in bleaching agents used at home, this rate is 15% and 20% in products applied by a physician (Oktay, 2006). The application time of carbamide peroxide in home bleaching can be 2 weeks or more.

There are carbapol or glycerin in products with carbamide peroxide. Carbapol does not change in the effectiveness of the bleaching agent, but slows the release of hydrogen peroxide. It also extends the shelf life of the material. Glycerin is not preferred because it causes dehydration in the teeth (Çoban, 2000). When evaluated in terms of oxygen release, it has been seen that this release is 2.5 times slower in those containing

carbapol. However, this situation has no negative effect on bleaching (Dahl & Pallesen, 2003).

Sodium Perborate (NaBO3.4H2O)

Another frequently used bleaching agent is sodium perborate. There are 3 forms as monohydrate, tetrahydrate and trihydrate. These water contents in the crystallization of sodium perborate affect the amount of oxygen released. In terms of oxygen concentrations, the most oxygen-containing form is monohydrate (16%), while this ratio is the least in tetrahydrate (10.4%) (Weiger et al., 1994). It is more reliable than hydrogen peroxide. Sodium perborate, which is in powder form and is stable in dry conditions, decomposes into hydrogen peroxide and free oxygen when it encounters hot air, acid and water (Rotstein, & Friedman, 1991).

Unlike carbamide peroxide, sodium perborate generates sufficient free radicals without causing the formation of hydrogen peroxide. This way too, the bleaching process can be performed (Greenwall, 2001).

Vital Tooth Bleaching

There are three main approaches to bleaching vital teeth (Gerlach & Zhou, 2001). These;

- Home bleaching,
- Office bleaching (power bleaching)
- Bleaching done with over-the-counter products without the control of a physician.

Before the bleaching procedure, the teeth to be treated should be evaluated in terms of caries, sensitivity, etc., and treatment should be initiated considering the indications and contraindications.

• Home Bleaching

Home bleaching, first described by Haywood and Heymann, is the process of applying by the patient bleaching agents with low concentration via a carrier plate (Haywood, 1989). There are many advantages in home bleaching. The most important of these are its low cost, self-application and high reliability (Leonard Jr et al., 2003). In this method, the patient is recommended to use at home by putting the determined amounts of bleaching agent on the plate prepared by the dentist. This bleaching method has become the gold standard with the used bleaching agents and concentrations.

Another advantage of home bleaching treatments is that it takes longer time to recycle while obtaining slower results compared to bleaching treatments performed in clinics. While the use of highly concentrated

bleaching agents accelerates the treatment process, on the other hand, it negatively affects the comfort of the patient (Touti et al., 1999).

There are products in different concentrations for this on the market. Home bleaching application using products such as carbamide peroxide and hydrogen peroxide is in the form of use 30 minutes to 2 hours in day for 2-6 weeks (Haywood, 1989). When the clinical studies on this subject are examined, the only approval was given for 10% carbamide peroxide by ADA (American Dental Association) in bleaching treatments in 2006 (American Dental Association, 2006). Another advantage of 10% carbamide peroxide is that it is safe for use in children.

In home bleaching it is extremely important patients to be patient. However, if the patient is incompatible and wants his teeth to be whitened as soon as possible, in such cases applying this form of treatment is a contraindicated situation (Şeker & Sarı, 2019). Sometimes, creation excessive sensitivity of the bleaching agent causes the patients to leave half their treatment. Apart from these situations, treatment should be done separately to the lower and upper jaws in patients with joint problems (Ozduman & Celik, 2017; Heymann et al., 2013).

Also, color change is very important in this treatment. In patients who do not use their apparatus meticulously, the results show a negative appearance lower than expected. In contrast, overuse is sometimes seen in enthusiastic patients who are aware of the importance of treatment. This often results in thermal sensitivity at high rates, up to 67% too.

• Office Bleaching

A lot of research has been done to find a powerful material in these bleaching treatments, also called Office Bleaching. Abbot, who activated superoxol with a light source in 1918, pioneered this form of treatment (Abbot, 1918).

Firstly, tooth bleaching agents (25-40% hydrogen peroxide) are used in high concentration in office bleaching. Here the dentist has full control throughout the procedure and has the ability to stop it when the desired effect is achieved. Under normal circumstances, there may be a significant color change even in one go in office type bleaching, but much more is required to achieve the best results (Tezel et al., 2007; Haywood, 2006).

In this process in which 35% hydrogen peroxide is used, priority should be given to the protection of soft tissues such as tongue, lips and gingiva. For this purpose, rubber dam or gingiva protectors should be used. High concentrations of bleaching agents are applied to the teeth after the soft tissues are well eliminated. Here the dentist has full control (Ozduman

& Celik, 2017; Prathap et al., 2013). The treatment can be finished when the desired color is achieved.

In this treatment, in addition to hydrogen peroxide, the use of carbamide peroxide at home is also recommended (Leonard Jr et al., 2003; Alqahtani, 2014). Using 10-20% carbamide peroxide is equivalent to 3.5-6.5% hydrogen peroxide. In this treatment, which should be supervised by a physician afterwards, it is sufficient to use 10% carbamide peroxide 8 hours a day and 15-20% carbamide peroxide 3-4 hours a day.

In office bleaching, treatment should be completed in four sessions. By activating the used material with heat or light source, it is ensured bleaching would done in a shorter time. Each session should last 15-20 minutes on average and it is recommended not to exceed this period.

The toxic effect of pulp is related to the application time of hydrogen peroxide. For this reason, the application period should not be exceeded as much as possible for less toxicity and coloring.

Heat and light are used together with the bleaching agent in the power bleaching process recently, in which used high concentration hydrogen peroxide, also called the thermophotocatalytic method (Koruk & Kırzıoğlu, 2010). At the end of the procedure, the bleaching agent is removed from the tooth. However, also in many studies, it has been reported that the use of high concentrations of hydrogen peroxide causes tooth sensitivity and irritation in the pulp (Sheets et al., 2002). However, the rapid bleaching causes not only tooth sensitivity, but also side effects such as nausea, gingiva and throat irritation.

Lasers and high intensity light sources are also used to accelerate bleaching. The use of lasers in bleaching first started with argon and CO2 lasers in 1996 with the approval of the FDA (Food and Drug Administration, 1988). In vitro studies constitute the majority of research on this subject (Burrows, 2009). While these applications are not recommended by some researchers due to their negative results in the clinic, some researchers have advocated the necessity of using these materials in office bleaching. Dostalova et al. also found in their studies on this subject that the use of 38% hydrogen peroxide shortens the duration of the laser action (Dostalova et al., 2004).

Bleaching done in the clinic is generally preferred in impatient patients to achieve quick results or who want to carry out the treatment process under the control of a physician. Sensitivity may occur in the teeth during treatment sessions. However, as a result, it has been stated in studies that there is no pulpal effect at the end of the treatment.

The Counter-Top-Products Applied Without Physician Control

They are low cost bleaching products that are provided by the patients themselves without being recommended by the physician. These products, which are generally sold in pharmacies and markets, are toothpaste, dental floss, varnish, mouthwashes and whitening tapes (Ozduman & Celik, 2017). The popularity of these counter top bleaching products has grown enormously in recent years. Generally, there are low concentration hydrogen peroxide in their contents.

There are many bleaching agents that provide whitening in toothpastes. Especially hydrogen and carbamide peroxide content is very effective in bleaching. Abrasives in the structure of pastes remove external discolorations thanks to their abrasive effects. However, they cause more sensitivity in teeth than other products. Similarly, bleach-effective mouthwashes with hydrogen peroxide are used 2 times a day for 60 seconds (Carey, 2014). Whitening bands that cause 1-2 tons of opening, like mouthwashes should be used 1-2 times a day for 5-60 minutes (Şeker & Sarı, 2019).

Also known as the growing sector of dentistry, the reliability of these counter top products is questionable. The reason for this is that the production of all these products are not made by the Food and Drug Administration.

Devital Bleaching

Also known as intracoronal bleaching, this bleaching type was first introduced by Spasser in 1961 (Spasser, 1961). By applying to devital teeth, it allows the elimination of aesthetic concerns. For this purpose, a careful examination should be done before treatment. Impermeability of root canal treatments of teeth should be good, periodontal tissues should be healthy (Baratieri et al., 1995).

After a properly completed root canal treatment, an cavity should be opened 2 mm below the enamel-cement joint. Afterwards, the coronal canal filling should be closed with glass ionomer cement and impermeability should be ensured (Koruk & Kırzıoğlu, 2010). The cavity should be temporarily closed by placing a small cotton pellet after the bleaching agent applied. At regular intervals, the patient should be invited to treatment sessions.

Devital bleaching agents:

- Hydrogen peroxide
- Sodium perborate
- Carbamide peroxide

Before starting the devital bleaching process, the walls of the pulp chamber should be cleaned with the help of a bur and the colored dentin should be removed. Thus, a cleaner treatment is started (Plotino et al., 2008).

Devital bleaching techniques used today:

- Walking bleach
- Modified walking bleach
- Ultraviolet photooxidation technique
- Thermocatalytic technique
- Planned endodontic treatment and bleaching (Alqahtani, 2014)

In the walking bleach technique, which is one of the most used bleaching techniques with its simplicity, reliability, cheapness and effect, by mixing water and sodium perborate, placed in the pulp chamber (Kaneko et al., 2000). In this technique, the bleaching agent should be changed more frequently. The reason for this is that the bleaching effect of the mixture with distilled water can take a short time (Çalışkan, 2006). Sometimes a combination of sodium perborate and hydrogen peroxide is used. A better bleaching is provided in this mixture. It can give results in as little as 1 week (Rotstein, 2001). These procedures are continued until the physician's desired result is achieved. If the results are not satisfactory at the end of all sessions, supportive treatment is started with office bleaching (Setien et al., 2008).

The number of sessions has been reduced in the modified walking bleach technique (Caughman et al., 1999). Generally, 10% carbamide peroxide is used in this method. This agent is regularly applied to the teeth, but in this method, the pulp chamber should not be closed during the treatment, but should be left open. The disadvantage of this method is that if the cement covering the pulp is broken, in this instance periapical tissues become contaminated and endodontic treatment fails. Apart from these, the bleaching agent can be swallowed by the patient and staining may occur in the intracoronal dentin (AM Sulieman, 2008). As a result of a study on tooth staining and bleaching, there wasn’t seen no difference between walking and modified walking techniques (Ozduman & Celik, 2017).

In the ultraviolet photooxidant technique, which is not used today, 2 minutes of ultraviolet light is applied after the bleaching agent is placed. As a result, oxidation occurs.

Hydrogen peroxide is preferably used in the thermocatalytic technique and the application of heat is maintained with special lamps and electrical

devices. The heat application process is done 3-4 times after the bleaching agent is renewed in each session (Plotino et al., 2008). The most important complication that may occur is external root resorption.

In cases where bleaching treatments are insufficient in vital teeth in the planned endodontic treatment and bleaching technique (tetracycline coloring), endodontic treatment procedures are applied to the teeth. For this reason, patients should be given detailed information about the treatment to be performed and the procedure should not be started without patient approval. The most important thing in this technique is the need for good patient compliance (AM Sulieman, 2008).

Low concentration bleaching agents should be used to prevent the risk of external root resorption in teeth with devital bleaching (Leith et al., 2009).

Tooth bleaching applications constitute an important place in eliminating the aesthetic complaints of patients. In achieving aesthetic success, patient-physician harmony and a treatment procedure in accordance with the rules should be provided. For these reasons, taking into account the patient's expectation, a treatment protocol should be followed with a correct approach. As a result, it has been stated that this treatment method is the most conservative method in providing aesthetics by changing the tooth color.

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Chapter 27

A NEW APPORACH RAPID GLUCOSE6-PHOSPHATE DEHYDOGENASE ENZYME DETERMINATION BY BIOSENSOR

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INTRODUCTION

Erythrocytes need energy to maintain their normal life. Since there are no mitochondria in erythrocytes, the required energy is obtained through the Embden Meyerhof pathway. They derive from anaerobic glycolysis. Energy for erythrocytes to survive In addition to meeting their needs, hemoglobin and proteins in the cell oxidant they need protection from the effects. The glucose-6-phosphate dehydrogenase enzyme (G6PD), located in the pentosmonophosphate pathway in erythrocytes, functions to protect the cell from oxidant damage(Luzzatto, Ally, & Notaro, 2020).

Erythrocytes play a role in vital functions such as the supply of oxygen (O_2) necessary for the life of the organism, removal of CO_2, and adjustment of blood pH. During the reactions due to the high concentration of O_2 it carries, 2 superoxide radicals and metabolites that cause oxidative damage such as hydrogen peroxide are formed. These reactive derivatives are harmful to the cell. Reduced glutathione (GSH) is used by erythrocytes to protect against oxidative agents such as certain drugs, broad beans and infections. Nicotinamide Adenine Dinucleotide Phosphate (NADPH), the co-factor of the GSH pathway, is obtained from the pentose phosphate pathway. Glucose-6-phosphate dehydrogenase (G6PD) enzyme is an important enzyme in the first step of this pathway(Beutler et al., 1974). Any loss of function in this enzyme causes the cessation of NADPH formation, leaving erythrocytes vulnerable to oxidative damage. People with G6PD deficiency may be asymptomatic or present with neonatal jaundice, chronic nonspherocytic anemia, and acute hemolytic anemia (Pinna et al., 2019).

In the case of G6PD enzyme deficiency, in the first days after birth, bilirubin cannot be metabolized as rapidly as required due to the deficiency in liver functions and enzyme deficiency and neonatal jaundice occurs(La Vieille et al., 2019)

G6PD enzyme deficiency, one of the most common enzymopathies in the world, is an inherited disease that is estimated to be carried by 400 million people. More than 400 variants and approximately 170 mutations of the enzyme have been identified. The frequency of G6PD deficiency in the world varies according to geographic region and ethnicity. By the World Health Organization list, which was published in 1989 in Turkey, G6PD deficiency is most commonly seen in the ranking of Italy, Greece, located along the West African and Southeast Asian countries. 400 in the world than the specified variant and the most common in the Mediterranean region of Turkey G6PD deficiency G6PD deficiency is more Mediterranean type(Parsanathan & Jain 2020).

Glucose-6-phosphate dehydrogenase (EC:1.1.1.49, G6PD; D-glucose-6-phosphate: NADP+ 1-oxidoreductase) catalyzes the first step of the pentose phosphate pathway. It is result glucose can not go outside from cytoplazmic membrane. Pathway involves the transformation of glucose 6-phosphate to 6-phosphogluconolactone concomitant with conversion of NADP to NADPH. The NADPH produced protects erythrocytes from oxidative damage (Sobngw et al., 2005). G6PD deficiency is a common enzymopathy affecting more than 500 million people worldwide. G6PD deficiency may result in hemolytic anemia due to drug toxicities, infections during the neonatal period, consumption of beans and stress conditions(Oppenheim et al., 1993).

Measurement of G6P levels is important for mediterranean pepople, because G6PD mutation is an epidemic disease of mediterranean countries. Fast and practic determination of G6PD activity can help early treatment at childhood(Castro et al., 2006). Biosensors are practic, cheap and mobile useable equipment. We aimed to design a new biosensor for rapid determining of G6PD enzyme activity and G6P levels.

Biosensors

Biosensors basically; It is based on the principle of the formation of a signal proportional to the amount of analyte on the transducer (transducer) surface as a result of the interaction of the substance to be analyzed with the biocomponent (biological recognition zone) on the biosensor surface and the transmission of this signal to the measuring device(Kökbaş, Kayrin & Tuli, 2013). Enzymes, microorganisms, plant and animal tissues, receptors, antibodies and nucleic acids can be used as biocomponents in biosensors. In accordance with the molecule to be analyzed, a biocomponent and a suitable transducer that converts the electrochemical, optical or gravimetric signal generated as a result of the transformation of the analyte into an electrical signal should be selected. Transducer and biocomponent can be connected to each other by a suitable physical or chemical method(Kökbaş et al., 2020).

Biosensors are classified according to measurement principles and transducer type as follows:

a. Electrochemical based (Amperometry, Potentiometry)

b. Optical based (Photometry, Fluorometry, Bioluminescence)

c. Piezoelectric based (Quartz crystal microbalance, Microcentilevers)

D. Calorimetry based (Thermistors)

Enzyme-based biosensors

Considering the historical background of biosensor technology, it

is seen that the first studies in this field started with enzyme sensors. Glucose oxidase enzyme electrodes for glucose determination, reported by Clark and Lyons in 1962 and Updike and Hick in 1967, are the first examples in this regard. The first examples in biosensor technology emerged as amperometric and potentiometric based enzyme electrodes. The most important reason for this situation was that the knowledge and technological know-how at that time did not reach a sufficient level for these studies. When the biological materials used in biosensor technology are listed according to their increasing complexity characteristics; They can be classified as ionophores, antibodies, enzymes, liposomes, biomembrane fragments (eg receptor), cell organelles (eg mitochondria) tissue, or all cells and organs (eg sight and smell). In the most general sense, enzyme sensors, like other biosensors, consist of bioactive layer, transmitter and measurement system. The only difference from other biosensors is that there are enzymes in the bioactive layer as biomolecules. On the other hand, as in other biosensors, membranes on the inner and outer surfaces of the bioactive layer, signal amplifiers between the transmitter and the measurement setup, microprocessors or recorders or computer systems associated with the measurement setup are elements added according to their requirements(Kökbaş et al., 2019).

Tissue-Based Biosensors

It is known that some animal and plant tissues and organelles are rich in enzymes. Instead of isolated preparations of these enzymes, these tissue pieces, where they are concentrated directly, are also used as biocomponents. It is advantageous to use tissue containing these enzymes and use tissue fragments as biocomponents, if the enzyme of interest is not commercially available, rather than using different enzymes together in the multi-step conversion of the target analyte, especially since it saves the difficulty of lengthy and costly enzyme purification. Due to the complexity of the tissues, the response characteristics for each new type of electrode are different, so optimization is required for each biosensor (Coulet & Blum, 2019).

In this type of biosensors, enzymes are very advantageous in terms of catalytic stability since they will be found in their natural environment. However, the response time is prolonged due to the increase in the diffusion barrier that the substrate must overcome to reach the enzyme. In order to reduce this disadvantage partially, the tissues are used by homogenization(Grupta et al., 2019). In addition, since tissues contain many enzymes, substances and conditions that will inhibit other enzyme systems can be used when preparing the biosensor to prevent errors that may occur when the analyte is transformed with other enzymes other than the desired enzyme(Kökbaş et al., 2019).

DNA Based Biosensors

Biosensor systems using single-stranded DNA oligomers as biocomponents. The emergence of DNA structure, hybridization, amplification and development of recombinant DNA technologies pioneered the development of DNA sensors. Generally, DNA sensors are based on the principle of hybridization of a single-stranded DNA oligomer (DNA probe) fixed to the transducer surface and a single-stranded DNA segment that symbolizes a certain disease, an inherited character or pathogenicity of a bacterium or virus. Thanks to the double stranded DNA formed by this hybridization, an electrochemical or optical signal is formed and the signal is made readable with an optical, piezoelectric, electrochemical transducer. The DNA probes used are usually 20-30 bases short single stranded DNA(Nguyen et al., 2019).

Antibody / Antigen (Immuno Sensor) Based Biosensors

Biosensors using antibodies as biocomponents are called antibody-based biosensors or immunosensors. Substances with protein structure produced by immune system cells against foreign organisms (viruses, bacteria and protozoa) or their protein products entering the body are called antibodies. Since there is a specific interaction between antibodies and antigens, highly specific and sensitive analyzes can be performed with immunosensors. Immunosensors can be developed for the determination of hormones, drugs, viruses, bacteria and other molecules, pesticides, biomedical substances that are environmental contaminants by matching antibodies with appropriate transducers (Metkar & Girigoswami, 2019).

Microbial Biosensors

A microbial biosensor is formed by immobilizing and immobilizing living or non-viable microbial cells with a transducer. The microorganisms used in the construction of biosensors have a number of advantages. Microorganisms can metabolize a wide range of chemical compounds. Microorganisms have a great capacity to comply with adverse conditions and can form new molecules over time by developing their abilities. Microorganisms are also an economical source of intracellular enzymes for genetic modifications caused by mutation or recombinant DNA technology (Killard, 2020).

One of the main limiting factors of using cells compared to enzyme-based sensors is that diffusion of substrates and products across the cell membrane provides a slower response. One way to overcome this problem is to increase cell permeability. The permeability of cells can be increased by physical (freeze-thaw), chemical (organic solvents / detergents) and enzymatic (lysozyme, papain) processes. The most used method in

this regard is chemical methods using organic solvents such as toluene, chloroform, ethanol and butanol, and detergents such as N-acetyl-N, N, N-trimethyl ammonium bromide (CTAB), Na-deoxycholate and digitonin. Such chemical processes cause the separation of some lipids in the cell membrane, facilitating the diffusion of small molecular weight substrates and products across the membrane by keeping macromolecules such as enzymes in the cell. Although the permeation process causes the death of cells, it enables intracellular enzymes to be used economically. This method is used in simple biosensor constructions that do not require cofactor regeneration or metabolic respiration. Compared to pure enzymes, the low specific activity of cell-based biosensors is another factor limiting cell use, so the use of enzymatic biosensors is analytically better (Christopher et al., 2020).

Immobilization Methods in Biosensors

After selecting the appropriate biocomponent and transducer for the sample targeted to be analyzed, these two elements should be connected to each other, that is, fixed on the biocomponent transducer surface (Snoek et al., 2020).

The basic methods used for fixing:

a. Adsorption (non-covalent bonding)

b. Covalent Bonding

c. Arrest

d. Cross Linking

Since the life of the biosensor depends on how long the biocomponent can be retained on the transducer surface by fixing, it should prevent the biocomponent from leaving the surface for a long time. In fixing enzymes, care should be taken not to damage the enzyme active center during fixation or to prevent a decrease in enzyme activity due to steric hindrance (Chesterfield et al., 2020).

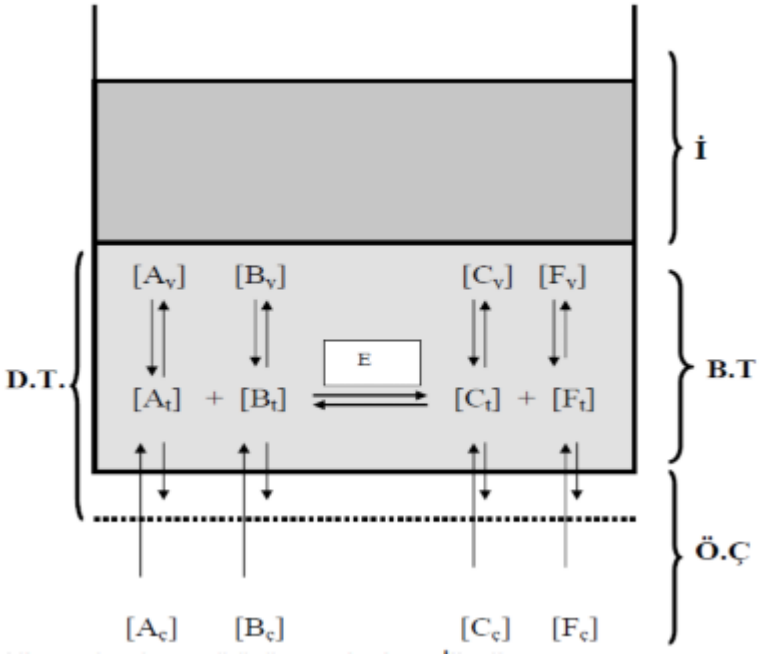

Figure 1. *General Operating Principle of Enzyme Biosensor3. (A: Substrate, B: Cosubstrate or Coenzyme, C and F: Products, ç: Concentrations in measurement solution, t: bioactive layer, and y: electrode surface. DT: Diffusion layer, Ö.Ç .: Measurement solution, BT: Bioactive layer, I: Transmissive)*

Electrochemistry Based Enzyme Biosensors

Electrochemical based transducers are used in most enzyme based biosensors. Since these transducers can be measured according to the principles of amperometry and / or potentiometry, enzymes of the oxidoreductase (electron releasing enzymes) class are generally used. An enzyme biosensor is shown schematically in Figure 1. As can be seen from the figure, substrate A is converted to C and F by the enzyme fixed on the electrode surface with the help of the B coenzyme. After this transformation, the differentiation in substance concentrations on the electrode surface is reflected as a signal by the transmitter(Bollella, & Katz, 2020).

Features Required in Qualified Biosensors

a. Sensitivity: It means that the device responds exactly to the change in the analyte.

b. Optional: Indicates analyte specificity of the instrument only. The instrument does not show any interest in other reagents and does not give erroneous results.

c. Measuring range: It is the range of analyte concentration that the device can measure.

D. Measurement time: Indicates the measuring speed of a type of device.

to. Consistency: Refers to the consistency in the results of the device.

f. Measurement limit: Refers to the lowest analyte concentration that the device can measure hindrance (Vokhmyanina et al., 2020).

g. Life: The service life of the device without any noticeable decrease in its performance.

Materials and Methods

Chemicals

All chemicals used in biosensor establishement were purchased from Randox Laborotaries Ltd., UK and Sigma Chemical Co., USA. All solutions were prepared freshly just before experiment.

Apparatus

To perform the electrochmeical measurements, corundum ceramic based screen printed gold electrode (BVT Technologies, CZ) combined with the reference Ag/AgCl electrode, and the auxiliary AuPd (98/2%) electrode was used with. PalmSens potentiostat (Holland) to measure the electrochemical potential. In the experiments, automatic pipets (Gilson, France), magnetic stirrer (Germany) and a thermostat (Nuve, Turkey) were used. Ultra-pure mili-q water in the preparation of solutions was obtained water purification system (Mili-Q and Milipore RIOS-DI 3 UV, USA).

Preparation of biosensor

Firstly, BSA-Gelatin mixture coated onto gold electrode, than G6PD enzyme is crosslinked by gluteraldehyde. The immobilized was used to regenerate NADPH from the NADP+. Optimization studies show that assay buffer is 0.5 mM of NADP+ and 1 mM glucose-6-phosphates in 50 mM potassium phosphate buffer, pH 7.4. We obtained NADPH peak at 50 mV of differansiel pulse voltammetry.

Measurements

All the measurements were performed by using a thermostatic reaction cells contained varying amount of glucose 6 phosphate dehidrogenase enzyme concentrations in the solution of phosphate buffer (50 mM, pH 7.0) and the mediator (50 mM Ferrociyanide), at 35 °C.

CONCLUSIONS

For the investigation of the electrochemical characteristics of the biosensor cyclic voltammetry used. Cyclic voltammogram was used for determination potantial point of study. (Fig 2)

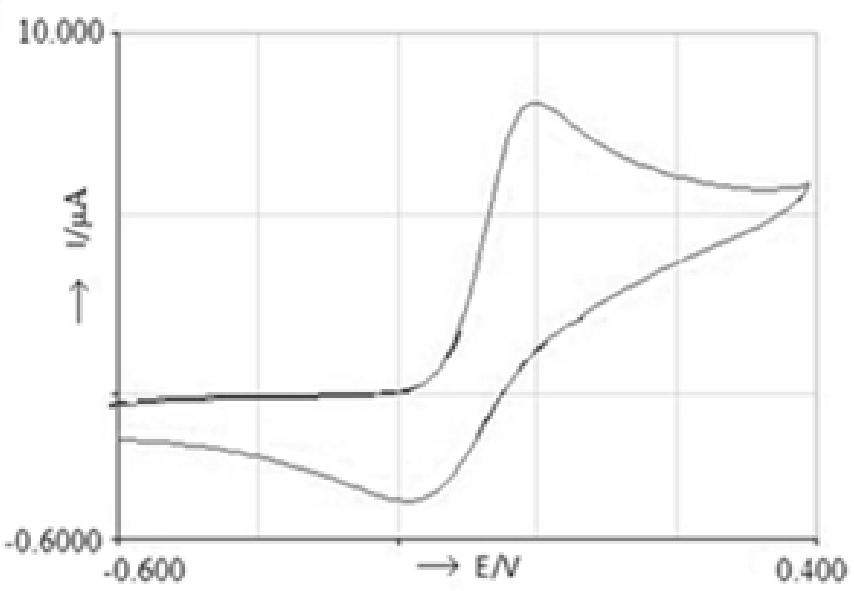

Fig. 2. Cyclic voltammogram of G6PD biosensor.

In this study, a portable equipment developed for G6PD activity determination. This equipment is both faster and easy useable for clinical analyses. It have more adventages such as accuracy, precision, less cost.

The characterization studies showed that this equipment can perfectly mesause G6P levels at 20- 55 C^{o} with one drop of whoole blood. This measurement is take just 1 minute. (Fig. 3.)

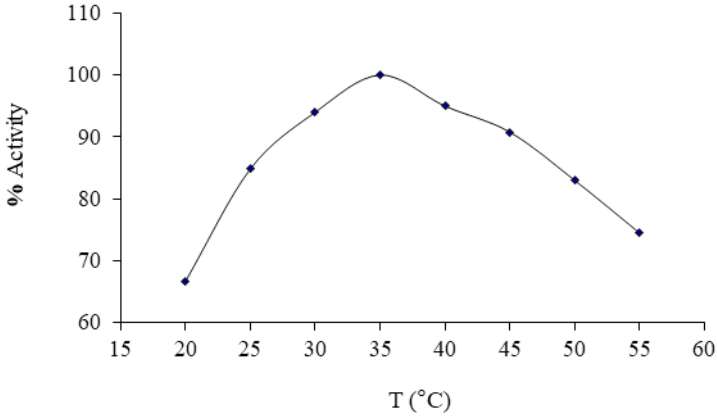

Fig. 3. *Temperature- Enzyme activity diagram of biosensor.*

For detection the effect of the pH value on the biosensor response, different buffer systems were investigated. For this purpose, 50 mM concentration of acetate (pH 5.0-5.5), phosphate (pH 6.0-6.5-7.0-7.5) and Tris-HCl (8.0-8.5) buffers were used. The optimum pH values were from 6.0 to 7.5. (Fig. 4.)

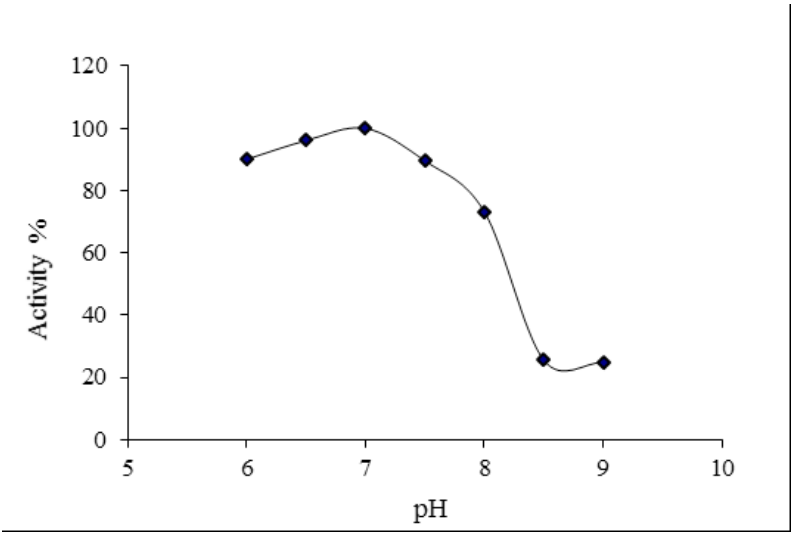

Fig. 4. *Diagram of the effect of the pH value on the biosensor response.*

A vast number of methods of immobilization are currently available; however, an economical and small process of immobilization is still necessity. It was determined that the method is sensitive, economic, practical and less time-consuming. The current advancement in microprocessing and microelectronic devices has created a promising future for the application of G6PD as biosensors.

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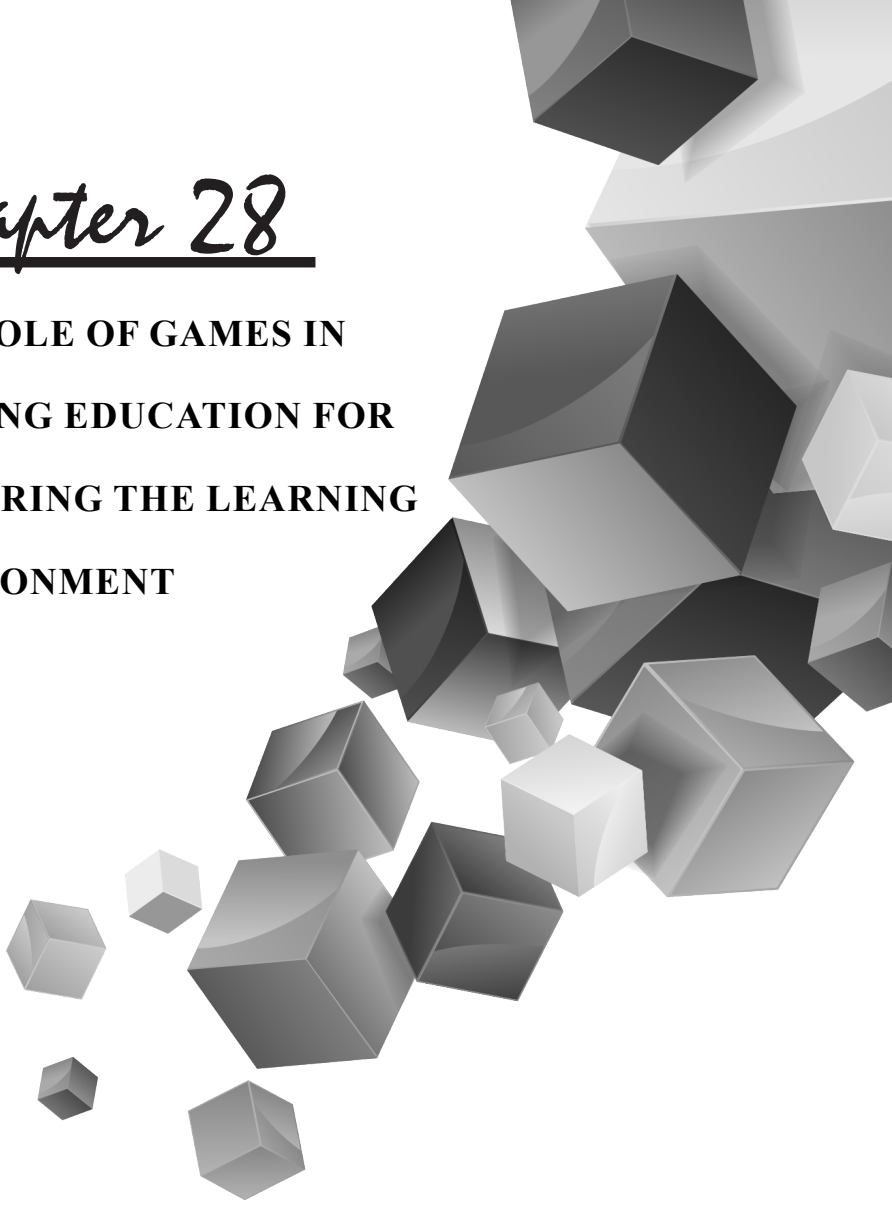
Chapter 28

THE ROLE OF GAMES IN NURSING EDUCATION FOR PREPARING THE LEARNING ENVIRONMENT



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INTRODUCTION

Contributing to the obtaining and assessment of learned information by reviewing and consolidating, the game is an interactive process. Games are used not only for teaching, but also are enjoyed by the participants (Bayer-Hummel, 2010). The use of games in the course ensures that all students are "winners". Because they all have the equal opportunity to participate in experiential learning.

Game also adds versatility throughout the classroom, increasing attention and speeding up the teaching experience. Game helps the individual to have an idea about the behavior and self-awareness through peer interaction and feedback (Allery, 2004; Bayer-Hummel, 2010).

In the literature, it is argued that game-based learning can increase students' problem-solving, targeted behavior development, participation and motivation, as well as helping to develop social networking, strategic thinking, group decision-making and higher cognitive skills (Brom, Šisler, & Slavik, 2010; Freitas & De Freitas, 2013). There is a need for a systematic process in establishing a balance between a strong pedagogically based education and entertainment in the realization of the lessons by using game (Thompson & Tanimoto, 2013).

For all these reasons, this article aims to examine and provide information about the use of games as a new teaching strategy in the education of nursing students.

BACKGROUND

Many educators experience that it is difficult to keep high students' motivation, attendance, and concentration in the classroom or learning environment during the lesson. Lack of motivation in students can cause that learning objectives are not fully achieved and a negative educational atmosphere emerges in the classroom (Liu, Bridgeman, & Adler, 2012). In crowded classrooms, a high level of interaction between students cannot be achieved.

In educational researches conducted in higher education, the more active the learning activities and the students participate in these activities, the more education is emphasized (Wang & Tahir, 2020).

Although nowadays traditional teaching methods are used in classrooms and clinical settings, new methods should be implemented as teaching strategies for nursing students due to the rapidly increasing use of technology and social media (Boctor, 2013; Ferguson, Davidson, Scott, Jackson, & Hickman, 2015; Kinder & Kurz, 2018; Sharma, 2017). In fact, this situation offers a chance to innovate nursing education, by starting to

use games which range from paper and pencil to high-tech games (Boctor, 2013; McEnroe-Petitte & Farris, 2020). Game use in education is cost-effective and user-friendly (McEnroe-Petitte & Farris, 2020).

The right balance between education and entertainment is required to optimize game-based learning. With the use of high-level graphics and animation in a not well-designed game, students can distract players by earning points by competing, rather than learning, which can prevent reaching the real goal (Admiraal, Huizenga, Akkerman, & Dam, 2011). Games can also be useful to provide a deeper understanding of certain basic principles, especially when dealing with complex and versatile issues that are difficult to understand by just giving information (Brom et al., 2010).

The idea of digital game-based learning has existed for more than thirty years; however, it has become even more popular with the development of information technology and the internet. It has been suggested that even most full-fledged video games can effectively support classical curriculum education (Brom et al., 2010). Likewise, well-designed video games are argued to be effective in learning environments because they motivate their learners unknowingly (J. P. Gee, 2003).

Why Games should be used as a Teaching Strategy?

Today, it is very difficult for nursing educators to attract the attention of new-generation students in classes or online environments due to technological stimulus which starts when they are born (Brown, 2018; McEnroe-Petitte & Farris, 2020). Innovative and active teaching methods like games including technology in educational environments attracts the attention of new-generation nursing students (McEnroe-Petitte & Farris, 2020).

Nursing educators use games to motivate students and reinforce learning. By using games, a competitive learning environment is created among the students, thereby increasing the motivation of the students (Gallegos, Tesar, Connor, & Martz, 2017). In a research performed with nursing students, it was determined that students wanted to receive feedback immediately in their learning processes, and they valued learning by seeing and experiencing (Robb, 2014). According to Dale (1969), “People remember 10% of what they read, 20% of what they hear, 30% of what they see, 50% of what they hear and see, 70% of what they say, 90% of what they do” (Kennedy, 2006). In previous researches, it was reported that students have 3 different learning styles, which are auditory, visual, and kinesthetic (Lujan & Dicarlo, 2006; Meehan-Andrews, 2009; Pourhosein Gilakjani, 2012). Teachers have difficulty in using different

teaching strategies considering that their students have different learning styles (Boctor, 2013). Students are different from each other, and have different learning styles. Multiple approaches can be adapted to different learning styles of students in education. Many learning styles can benefit from games by the use of audio and visual stimuli, while promoting group discussion and participation (Boctor, 2013). It has been shown that games make learning more enjoyable in education (Charlier & De Fraine, 2013), and strengthen knowledge acquisition when the information learned is evaluated one month after the game is played (Brom, Preuss, & Klement, 2011).

The use of games in nursing education increases critical thinking, and requires students to analyze the contribution of others while reaching a common decision while encouraging students to work in teams to make a decision (Pront, Müller, Koschade, & Hutton, 2018). Games are an effective way to consolidate previously learned knowledge, and help students review it. Games have been found to increase the protection of information (Skirton & Blakely, 2009).

Is the Game Effective in Learning Process?

Game-based teaching and learning is an educational approach towards students involving being participant and create a team and work together to solve problems. Studies have shown that teaching activities which include involving games promote active learning, and increase learning motivation of students and outcomes (Admiraal et al., 2011; Virvou, Katsionis, & Manos, 2005; Walsh et al., 2018; Yang, 2019). Games used as a teaching strategy in the classroom or clinical environment increase motivation of students and encourage effective learning, and cause positive changes in learning process (McEnroe-Petitte & Farris, 2020; Sharma, 2017; Xu, 2016). It enhances excitement by learning, having fun while solving problems, and giving a chance to nursing students to observe their own learning abilities, and most importantly, improve their critical thinking skills (McEnroe-Petitte & Farris, 2020; Verkuyl, Atack, Mastrilli, & Romaniuk, 2016). Game methods used as a teaching strategy have been found to be useful in learning for a range of conditions like heart and pulmonary diseases, home-care and leadership in the field of health (Brown, 2018; Fernandes, Martins, Gomes, Gomes, & Gonçalves, 2016; McEnroe-Petitte & Farris, 2020).

Game enables more interaction in the classroom, improves the active learning process that ensures the efficient storage of information, and causes the development of problem-based learning and critical thinking skills of nursing students (Johnsen, Fossum, Vivekananda-Schmidt, Fruhling, & Slettebø, 2018; McEnroe-Petitte & Farris, 2020). During a

game, students have to cooperate and make a team work while trying to solve a problem (Brown, 2018; Johnsen et al., 2018; McEnroe-Petitte & Farris, 2020). It has been found to be beneficial for students who have different learning styles (Boctor, 2013; McEnroe-Petitte & Farris, 2020; Murad, 2017; Sharma, 2017).

Games have a huge impact on students' learning experiences, helping students learn deeply instead of superficial learning (Baid & Lambert, 2010). For example, playing a game for teaching a theory also helps students develop their discussion, critical thinking, clinical reasoning, problem-solving and prioritization skills (Baid & Lambert, 2010). Game not only teaches the desired content, but also provides indirect unexpected benefits, such as communication, collaboration and leadership among students (Uhles, Weimer-Elder, & Lee, 2008).

Many reasons are considered why games and game strategies are advantageous in education. The most important reason is that games improve experiential learning (Graham & Richardson, 2008; Murad, 2017; Strickland & Kaylor, 2016). The other reasons are that games increase nursing students' participation and motivation (Fernandes et al., 2016; Graham & Richardson, 2008), and allow participation of students with different strengths and weaknesses (Kinder & Kurz, 2018). Also, they provide a safe learning environment and content on risk management to students (Azriel, Erthal, & Starr, 2005; Brown, 2018). Games also support traditional methods (Azriel et al., 2005), promoting teamwork (Azriel et al., 2005; Brown, 2018). Koivisto et al. (2018) stated that games can be repeated as simulations, offering the same contents to all nursing students, and allow them to progress at their own pace depending on the games (Koivisto et al., 2017).

Games have their advantages as well as their disadvantages. Games may require additional space in large classes (Brown, 2018; Graham & Richardson, 2008; Strickland & Kaylor, 2016), and can cause stress, being shy or unwilling to respond to questions for students (Boctor, 2013; Graham & Richardson, 2008). Games can be noisy, confusing, and destructive in the classroom environment.

The size and density of the educational content to be handled may be affected by the additional expense needs for the materials to be used in the game, time-consuming game preparations of the trainer (Kinder & Kurz, 2018; Strickland & Kaylor, 2016), and the existence of possible time constraints (Boctor, 2013).

Which Games can be Played as a Teaching Strategy?

Games and game strategies may be employed in classrooms, online education, nursing professional skills laboratories and clinical settings.

Online formative assessment tools such as Kahoot and mini quizzes, puzzles, role plays, card games, PICO, ESCAPE, Jeopardy, Jenga, augmented and virtual reality are used as teaching strategies (Brown, 2018; Johnsen et al., 2018; McEnroe-Petitte & Farris, 2020; Verkuyl et al., 2016; Xu, 2016).

Pico

PICO was developed to identify population (P), intervention (I), comparison intervention (C), and outcome (O) as clinical scenarios focusing on interventions, therapies, or evidence-based practices. Teachers should prepare PICO questions according to scenarios before the game (Milner & Cosme, 2017).

The aim of the game is to be the first player or group by finding three PICO questions and their answers correctly. In the game, there are scenario cards about different issues related to medical stories of the patients and focusing on comparing an intervention to traditional or evidence-based care. The scenario cards could change according to teacher's preparation or editing the game. Each eighty cards are prepared according to P, I, C, or O words by matching the scenario. Students choose three scenario cards and hide them from their partners. The dealer (teacher) gives five cards to each player; and other cards are placed facing down on the table where all students can reach. The top card is faced up. When it is a student's turn, he or she must take the top card from the pile or put it into another student's hand by hiding from others or can take one card from the discard pile. The discard pile is fanned out, and students may take from the entire pile, not just the face up card, but if he or she draws a card, the other student must replace that card with the same type (e.g., P card for P card). If the player takes a card from the stockpile, the other student can discard any type of card. Players say "PICO" when they finish all cards according to the scenario question, and the dealer/ teacher checks them according to the PICO answer key card; if all the cards are correct, it is placed in front of the player and the game continues. Diversity of the game includes playing in teams, and the team who finishes completing three PICO cards according to the scenario questions first wins, or the number of completed PICO questions to win may be increased (Milner & Cosme, 2017).

For example, if you prepare the game depending on one scenario, then you could wait answers from the student groups for the most three important health problems, related to nursing diagnosis, interventions and evaluations by giving them a certain amount of time. The group that complete the game first picks up their cards by saying PICO, and after the confirmation of the correctness of the steps by the responsible instructor, the 1st group members are given an award, which could be adding points to

their exam grades or evaluation methods. The important point is discussing the answers of all groups to correct their wrong knowledge about the issue.

Jenga

Jenga is used to learn especially about security and performance improvement in leadership and risk management. This game has been used in a case study in which a student group had to define patient risk areas before any dangerous condition for the patient. The patient's portrait was depicted as Jenga (brand) blocks. According to the instructions developed for the original game, nursing students answered the questions in Jenga game. Whether the answers are true or false, a student will remove one block from the tower. The group whose tower does not collapse wins the game. Debriefing sessions, which focused on the strengths and weaknesses of the activities in the scenarios, were held with the end of the game. When learning was evaluated, it was expressed as encouraging and entertaining learning, and helped to increase the real life situations of nursing students (Brown, 2018)

Online Formative Assessment Tools

Education platforms that provide online formative assessment such as Kahoot!, Quizizz, and Socrative are defined as Student Response System (SRS). These tools are used to create short answers, simple true /false and more complex questions. Questions can be adjusted to the speed of the nursing student or trainer, accessible to the internet, and can be used on laptops, tablets or smartphones, which is cost-effective compared to older click devices that are expensive for institutions and students (Chaiyo & Nokham, 2017; Dellos, 2015; Ismail & Mohammad, 2017; Nawalaniec, 2015; Orhan Göksün & Gürsoy, 2019).

Student Response Systems (SRS) were developed as a solution in the 1960s to make crowded classes more interactive during the lesson (Judson & Sawada, 2002) and were started to be used in classrooms in the early 1970s (Bessler & Nisbet, 1971). SRSs have a positive effect on classrooms, students teacher perceptions, and learning performances (Caldwell, 2007). New methods of increasing classroom interaction have been sought for reasons such as increased internet network and computer use in lessons and students' bringing their own digital devices to the school (Chaiyo & Nokham, 2017). One of them is Kahoot! It allows to integrate the technical infrastructure of schools with SRS, students' own digital devices, social networks and games in one learning platform (Wang & Tahir, 2020). Kahoot! is a Game-Based Student Response System (GSRS) where the class is temporarily converted into a game show, instructor is the game show host, and students are competitors (Dellos, 2015). Kahoot's goal is to

increase participation, motivation, enjoyment and concentration to improve learning performance and class dynamics (Baker, D'Mello, Rodrigo, & Graesser, 2010). Kahoot was launched in September 2013. The basic requirements for using Kahoot are that trainers create contents for lesson topics. These online game-based exams are perceived as competitions while playing. In the game, a competitive learning environment is created by making a separate score for each correct answer according to the speed of the students. The winner of the contest is determined by announcing the top three at the end of the game. While entering the game, student use a nickname they want, and complete the game in a comfortable learning environment without being embarrassed by their answers as their personal identity is anonymous (Wang, 2015).

Quizizz is a very similar platform compared to Kahoot. Teacher creates an exam to start. A game code is given as in Kahoot. Students join the game from browsers on their mobile phones, tablets, or computers by entering join.quizzizz.com by using their names. Quizizz has some differences from Kahoot. While the multiple choice questions are shown on the large screen in Kahoot!, there are figures related to the answers that the students want to choose on their devices. There is no need for a projector in Quizizz. Students participate in the game by seeing the questions and answers on their devices. While students in Kahoot see the same questions at the same time, each student sees different questions in the Quizizz program, and answers the questions at their own learning speed. In other words, questions reach randomly to each student, preventing cheating among students. The most suitable program for individualized teaching is Quizizz. In Kahoot, after each question, the game can be stopped by the teacher, and the discussion can be performed over the answers, while in Quizizz, the teacher can give information about the general situation of the students after the game (Chaiyo & Nokham, 2017). In the studies comparing Kahoot and Quizizz platforms, it was determined that Kahoot is more fun, and students' post test scores are higher (Chaiyo & Nokham, 2017; Orhan Göksün & Gürsoy, 2019).

Socrative is another SRS platform which has a similar infrastructure with Kahoot and Quizizz. It can be used to make formative or summative assessments by teachers to determine the status of students before the lessons (Nawalaniec, 2015). Socrative ensures real-time formative evaluation for collecting data from students, and offers Space Race in which student teams answer questions to move their rockets quickly on the screen (Coca Mendez & Slisko, 2013; Nawalaniec, 2015). In the Space Race game, students are divided into groups and compete to give the most correct answers. Wrong answers cause the rocket to stay in place, while correct answers advance the rocket (Nawalaniec, 2015). The difference of

the Socrative platform from Kahoot and Quizizz platforms is that students compete in groups and increase cooperation within the group (Nawalaniec, 2015).

No matter which SRS platform is used in the classroom, the teacher should explain the accuracy rates of the answers given by students after the game providing feedback to students about their wrong answers. Thus, when the game is used as a competition in the learning process, the motivation of the students will be increased. It will also allow students' meaningful learning with teachers' immediate feedback.

Escape Room

Escape room is one of the popular collaborative live-action games (Morrell & Ball, 2018). Today, the escape room game has been implemented to class learning activities. During the game, nursing students can be active, and try to find clues about the problem prepared by the trainer according to a scenario. In this game, puzzles, crosswords, hangman, and medical equipment are used to find the clues related to the problem they have to solve according to their theoretical and practical knowledge. Nursing students need skills such as finding objects, checking vitals, wearing surgical gloves, controlling urine levels, removing stitches, and staples in a locked room to find the key (Morrell & Ball, 2018). To move on to the next stage of the game, it is necessary to find the correct answers or to complete the skill successfully. Also, after all the tasks are completed, students must find the keys to escape from the room. Nursing students said that the escape room was fun, and helped them learn the topic, and demanded that more games be used as a teaching strategy in nursing (Gómez-Urquiza et al., 2018). Escape room game brings students together in problem-solving, critical thinking, and teamwork (Morrell & Ball, 2018).

Before the game, there is a theoretical planning and appropriate environment need. The instructor should organize places, and hide all the necessary elements (puzzle, medical instruments, key of the room, etc.), and test the game in a locked classroom or skill laboratory. A scenario may be needed, which could help students to understand what the instructor expects them to make in this room. The students should be divided into teams before the game and separated from the experienced ones after the game to prevent bias. When the game starts, student groups are called to enter the room in order, and in a certain amount of time to play the game, and to relocate all tools in their original positions to provide the same environment for all groups. As the event begins, the teacher shows an instructional video which has team instructions and the goal of the game. During the game, while each group is in the classroom with a scary-sounding music, monitors or cameras could be used to assess their

performance and to ensure that the techniques required were being correctly carried out. The instructor does not give any assistance before and during the game. But if the game environment is a simulation laboratory, teacher could only speak with students to provide one-two clues and, if necessary, to indicate that a technique is not being correctly performed. The teacher should be the same for all the groups, who is deeply knowledgeable about the processes and the objects' locations. At the end of the game, there will be a debriefing session with students. Also, pre and post quizzes can be implemented to see the improvement in students' learning before and after the game (Gómez-Urquiza et al., 2018).

Jeopardy

Jeopardy is a television game and used as a tool to improve learning. By using five categories, Jeopardy can be customized to cover broad management topics (e.g. organizational behavior, human resource management, strategic management), or specific sections in a textbook. Jeopardy can be applied in two or more rounds. Students can split into two (or more) teams who will work together to provide the correct "question" to compete for points (Azriel et al., 2005).

The game is executed using a Jeopardy template power point program that is available online, and is similar to the television version (Boctor, 2013). Jeopardy "answers" could be selected from a textbook and course materials. Teams work in turn to select categories and values until the board is cleared. If one group cannot provide the correct answer, the other group will have a chance to respond and earn points. At the end of the two rounds, the trainer asks a final Jeopardy question to groups, then the game points are calculated, and the winning team is announced. Jeopardy has been found to increase student engagement, improve teamwork, and facilitate the overall learning environment (Azriel et al., 2005).

"Jeopardy" game is designed as follows for nursing students. Care management in the NCLEX-RN exam is structured according to risk reduction, safety and infection control, physiological adaptation, and basic care. Sound, theme music, picture, and applause effects have been added to the game. In the game, students see the question categories on the power point and the money numbers that they will earn. Students are also free to choose the category and amount of money as a team. There are questions worth $ 100, $ 200, $ 300, $ 400, $ 500 in each category. The question arises when the amount is clicked by the teacher in accordance with the category determined by the students. The students work as a team to give the correct answer to the question they choose. If the team cannot give the correct answer within the specified time, the right to reply passes to the other group. While the teams score points for each correct answer they

give, the points are deleted for their wrong answers. After all questions are answered, a final round is held, where students as a team can bet as much as they want on a question, as in the last Jeopardy round on television. Also, the discussion environment is provided with the teacher through the answers that the teams know correctly in the game. Questions in the game can also be chosen from multiple choice (Boctor, 2013). In the researches, it was determined that "Jeopardy" game increased the participation, pleasure and motivation of nursing students (Baid & Lambert, 2010; Bayer-Hummel, 2010; Boctor, 2013).

Bingo

Bingo is a team-based game used for increasing the motivation of students, and evaluating the learning outcomes. The game takes approximately 40-50 minutes. Before starting, students form groups of three or four. The instructor prepares the playing cards and the test questions. Empty cards are given to each group. Numbers are written on the empty cards as many as the number of test questions. The same amounts of question cards are distributed to all student groups. Each group is given 20 minutes to discuss and decide their answers to multiple choice questions.

The group then replies to questions by using blue ink to write their answers into the squares they previously assigned on the board. Students are reminded not to review the answers after they are written on the relevant square. If the group answered the question correctly, the choosing and answering right for the next question is given. Groups use "O" for correct answers and "X" for wrong answers in each frame for question. The aim is to combine five squares on a line with correct answers marked "O", whether vertical, horizontal, or diagonal. A completed "Bingo!" becomes the winner and finishes the game (Hsieh, 2016).

Augmented and Virtual Reality

Augmented reality and virtual reality (VR) offer a more immersive student experience than ever before, where new learning paths are needed (Emre, Selçuk, Budak, Bütün, & Şimşek, 2019; Holopainen et al., 2020; Klopfer & Squire, 2008; Radianti, Majchrzak, Fromm, & Wohlgenannt, 2020). VR is a technology that allows the individual to swift from a physical environment to a virtual environment by stimulating different sensory organs (Emre et al., 2019; Guttentag, 2010; Schwienhorst, 2002). The transition to virtual environment is defined as "immersion, the feeling of being in a virtual environment by cutting the individual's connection to the physical environment with VR devices with certain limitations" (Emre et al., 2019).

Different technologies such as VR glasses (head mounted display - HMD) and virtual caves (Cave automatic virtual environment - CAVE) are used to provide a VR experience (Emre et al., 2019; Guttentag, 2010; Schwienhorst, 2002). In VR, avatars are used, which are digital assets that represent the user in their digital environment. The user typically exists in the virtual environment from the "eyes" of the avatar which is the first person perspective or from the back of the avatar which is the third person perspective (Howard & Gutworth, 2020). For example, "Second life" computer-aided VR game and "Ghosthands" have been used as a learning platform in nursing education (Ferguson et al., 2015).

When interacting with VR by stimulating multiple sensory organs, it is emphasized that the learner facilitates immersion within the environment, and has a positive effect on recalling information from memory (Krokos, Plaisant, & Varshney, 2018). It allows educators to measure students' competence by allowing VR skills and content to be repeated through the application (Ericsson, 2006), and provides learning outcomes (Samosorn, Gilbert, Bauman, Khine, & McGonigle, 2020). The use of simulation is guided and presented through a standard scenario built into VR technology, enabling students to interact and experience contents in the same way by feeling and seeing at the same time (Squire, 2006). VR gives students a chance to experience things that cannot be experienced in the real world (Keys, Luctkar-Flude, Tyerman, Sears, & Woo, 2020). In addition, VR potentially increases didactic learning, enabling student to gain rich visual and kinesthetic experiences (Samosorn et al., 2020).

One of the VR games is Virtual Simulation Games (VSG). Also known as educational proposed game, VSG is a new and innovative teaching method that requires more research (Keys et al., 2020). VSG provides students to implement theoretical concepts in a safe, simulated clinical setting with educational purposes, while also promoting self-learning and providing error learning (Keys et al., 2020).

In a study, it was reported that participants responded to an increasing education call in the field of resuscitation science by creating an innovative VSG that demonstrated optimal nursing care for patients with secondary cardiac arrest due to ventricular fibrillation (Keys et al., 2020). VSG is defined as "An interactive computer application with a challenging purpose, with or without an important hardware component, playing and engaging, including some scoring mechanisms, and providing skills, knowledge and / or attitudes that are actually useful to the user" (Tan et al., 2017). There are many benefits to using VSG as an educational tool for nursing practice and as a cost-effective method for learning. Since these games can be easily developed by nurse educators, they can be used to prepare students for situations in which contextual representation in clinical settings is not

easy (Verkuyl, Romaniuk, Atack, & Mastrilli, 2017). With VSG, students are often expected to act as team leaders and encourage critical thinking when there are time and resource constraints (Keys et al., 2020)

A VSG was performed to intervene in a patient who had a heart attack. To create content firstly for this VSG, they developed learning outcomes that formed the basis of decision points. In order to reach these learning goals, GoPro technology was used for the video parts of the game. As a patient with cardiac arrest, a high quality dummy, a defibrillator, and a video with standard simulated drugs were prepared and used (Keys et al., 2020).

To improve the airway placement skills of the nursing students, VR technology game was used consisting of six scenarios. In this game, total teaching period is 20 minutes and it involves attempting an apnea patient. During the game, each scenario was defined as a learning module in which the students realized the airway application skill with the right technique by receiving instructions in the form of voice guidance. It completes the learning process in a virtual environment by using handheld controllers to follow both audio and visual cues irrigated for each learning module with voice guidance. Tactile feedback is provided through the hand controls to provide additional positive support for the completion of the correct hand-eye task. As a result of the study, it was determined that the knowledge and skill levels of students regarding airway application have increased (Samosorn et al., 2020).

SOLUTIONS AND RECOMMENDATIONS

How should Games be Prepared in the Learning Environment?

In order to use the game effectively, and ensure that the game reaches the educational objectives in the learning environment, strict rules or learning objectives must be determined by the trainer, and these rules must be followed during the game (Brown, 2018; Gómez-Urquiza et al., 2018; McEnroe-Petitte & Farris, 2020; Xu, 2016).

Games must be active, creative, include emotions, be enjoyable, engaging, support interaction, focus on experiential knowledge and skills, encourage participation and ultimately create a sense of general competition for being effective (Brown, 2018; Gómez-Urquiza et al., 2018; McEnroe-Petitte & Farris, 2020; Xu, 2016). Games must provide instant feedback (Fernandes et al., 2016). Thus, it enables nursing students to evaluate what they know or do not by providing formative assessment (Brown, 2018; Gómez-Urquiza et al., 2018; McEnroe-Petitte & Farris, 2020; Xu, 2016).

Gee (2008) reported that a successful game-based teaching approach includes the following features:

Step 1: Identification, where participants create a sense of identity in the game

Step 2: Interaction

Step 3: Risk taking, that is, compared to real life, failing a game has no serious consequences, so participants are given the freedom to take risks

Step 4: Autonomy, that is, participants have control over the game

Step 5: Well-organized problems, that is, the game is designed to include issues that are relevant and allow participants to grow and develop gradually

Step 6: List is challenging to design the game to contain problems that challenge students' current professional knowledge

Step 7: Students acquire the necessary information to develop their critical thinking skills instantly

Step 8: Built-in and meaningful learning, that is, students can learn new concepts through game scenarios

Step 9: Pretty annoying

Step 10: Discovery, the understanding and rethinking that the in-depth game forces players to expand contextual knowledge to carry out comprehensive and in-depth thinking

Step 11: Opportunities and environment for teamwork and

Step 12: Game-based teaching should also be a kind of problem-based learning that improves students' problem-solving skills (J. Gee, 2008; Yang, 2019).

It has been determined that games have a high potential to increase learning when used in education. Studies have found a positive correlation between game activities and learning (Marklund & Taylor, 2016).

When integrating game-based learning into the curriculum, attention should be paid to the presence of devices to be used, the duration and learning objectives of the lesson, and the number of students (Marklund & Taylor, 2016). In addition, a balanced integrity must be ensured between the design of the game, its contents, and assessment of learning objectives (Papadakis, 2018).

The curriculum plays an important role in planning the game and choosing the game type. Concern about whether physical infrastructure facilities will be sufficient to ensure the integration of the game into the classroom and the educators' perception of competence in using this

equipment are effective. In eliminating these concerns, it is necessary to identify the infrastructure facilities of the institutions and the aspects that need to be improved, to provide the missing resources and to support/ encourage the trainers to use the games in the classroom. During the play of the games in the classroom, teachers should make the necessary preparations in advance, and act as the game manager. Also, it is imperative for the teacher to master the students and to discuss the learning objectives. Before the game, it should be remembered that students will play with a collaborative approach, which may cause problems. Students should be explained how to play the game, and if necessary, pre-application should be done (Marklund & Taylor, 2016). Game-based learning in the classroom encourages students to understand the topic in context (Papadakis, 2018).

Classroom roles of teachers change when the games are integrated into learning environments (Becker, 2016; Hangh, 2013). A teacher using a game assumes the role of evaluator, guide, instructor, observer, actor, playmaker, referee, role actor and counselor in the classroom (Becker, 2016; Papadakis, 2018). While in the role of the evaluator, the teacher evaluates students' learning experiences and outcomes. In the role of a guide, the teacher provides students with a guiding scaffolding to achieve their learning goals. In the role of the instructor, the teacher includes the role of traditional teacher, who starts the lesson. The observer role is a passive role, which is like watching students in a distance. The actor role is a role that should be taken before the lesson begins. The playmaker role helps the operational use of the game. In the role of the counselor, it facilitates a kind of competition by focusing on the players playing together or by helping the students get into the game. When the teacher gets the personality of a character or the person who fits the narrative in the game, s/he performs the role of an actor/actress. When acting as a consultant for the players on issues related to the subject discussed in the game, the teacher fulfills the counselor role (Becker, 2016; Papadakis, 2018).

FUTURE RESEARCH DIRECTIONS

Game using in education started by Dewey at the beginning of the 20th century. Moreover, today, limited number of videogames are using in nursing education in literature. The most important benefit of these games is providing clinical decision-making ability of students. Possibility that using and changing environments/ scenarios and time pressure of the videogames could prepare nursing students to make time-efficient, informed clinical reasoning and problem-solving decisions around tailored patient care. In addition to all, this games providing financial and logistical benefits for students and educators, which are needed in nursing education in these covid-19 pandemic days (Pront et al., 2018). For future studies,

which videogames could increase decision-making, problem-solving and fast reaction time abilities of nursing students by using features such as multisensory stimuli, time limitations, hologram like real time applications, feedback, and repeated exposure could be recommended.

CONCLUSION

Nursing educators should improve the traditional and one-sided (students only become a listener) education model by using active education strategies, and utilizing games to motivate students in lessons and reinforce learning. During lectures, many learning styles can be used with the help of visual and auditory stimuli of game method. Especially, using games in large groups encourages students' participation in group discussions. Today, games make learning in education more fun for students who love the use of technology. It makes it easier for students to keep in mind the information they have learned, that is, to encode the information into their consciousness.

Using games in nursing education provides skills of critical and analytical thinking, problem solving and teamwork which are aimed to be reached in nursing program outputs. Nowadays, game using has become more important for nursing students who experience emergency remote teaching and have problems in practicing in hospitals due to Covid-19 pandemia. Due to this process, it has become more important that nursing students bring the information to the application level in the classroom or online learning environment. The games and game strategies can be employed in classrooms, online education, nursing professional skills laboratories and clinical settings. For all these reasons, it is essential to use games in the education of nursing students.

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Chapter 29

BOTOX APPLICATIONS IN DENTISTRY

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Botulinum toxin

Botulinum toxin (botox), a highly neurotoxic protein, is produced by clostridium botulinum which is a gram-positive anaerobic bacteria. Nowadays, botox is used for cosmetic purposes such as removing lines and wrinkles. However, contrary to popular belief, botox has been used for therapeutic purposes for a long time. Botox is an effective method applied in cases such as chronic muscle spasm, cervical tone disorders, strabismus, laryngeal dystonia, migraine, smooth muscle sphincter spasms, myofascial pain and movement disorders. Botox is used for medical purposes as well as therapeutically in dentistry (Kattimani et al., 2019).

Clostridium botulinum produces botulinum neurotoxin, which causes poisoning called botulismus (Ngeow & Nair, 2010). There are immunologically different but structurally similar neurotoxins in serotype A, B, C, D, E, F, and G produced by Clostridium botulinum. Among these neurotoxins A, B, E and rarely F type botulinum cause poisoning in humans (A. B. Scott & Suzuki, 1988).

The German Physicist Justinus Kerner (1786-1862) first used botulinum toxin for therapeutic purposes. This neurotoxin was started to be used clinically in the late 1970s to reduce pain and excessive muscle contractions (Dastoor, Misch, & Wang, 2007). Alan Scott made a breakthrough in ophthalmology in 1981 by treating strabismus with botulinum toxin (Alan B Scott, 1980). In the following years, this neurotoxic protein began to be used cosmetically to remove deep facial lines (Münchau & Bhatia, 2000).

Mechanism of action

When botulinum toxin is injected into the muscles, it prevents excessive and unwanted contractions of the muscles and acts by creating paralysis. This effect varies depending on the dose given, the structure of the muscle and the response of the host. All serotypes with similar structures and molecular weights act with the same mechanism but produce different paralysis effects. Type A is the most widely used type in the market and used in clinics for treatment and cosmetic purposes (Duruel, Ataman-Duruel, Berker, & Tözüm, 2019).

Figure 1 shows the summary of mechanism of botox. Botulinum toxin acts by blocking acetylcholine release. Acetylcholine is a neurotransmitter chemical agent that provides transmission in sympathetic and cholinergic nerve endings. Botulinum toxin causes chemical degeneration in the muscles, causing a partial decrease in muscle activity and even paralysis. Botulinum toxin begins to show its effect in 2-3 days following the injection

and provides its effect fully on the 15th day and the effect continues for 3-6 months (Omer Majid, 2009).

Botulinum toxin consists of 3 basic protein complexes containing high molecular weight proteins. The lethal dose for type A toxin is the fetal dose for 50% of the human population (individuals with an average weight of 70 kg); it is 70 µg orally, 0.7-0.9 µg by inhalation and 0.09-0.15 µg intravenously. The lethal dose of botox injection to be applied to the muscle in studies is approximately 2500-3000 U (35-40U / kg in individuals with an average weight of 70 kg) (A. B. Scott & Suzuki, 1988).

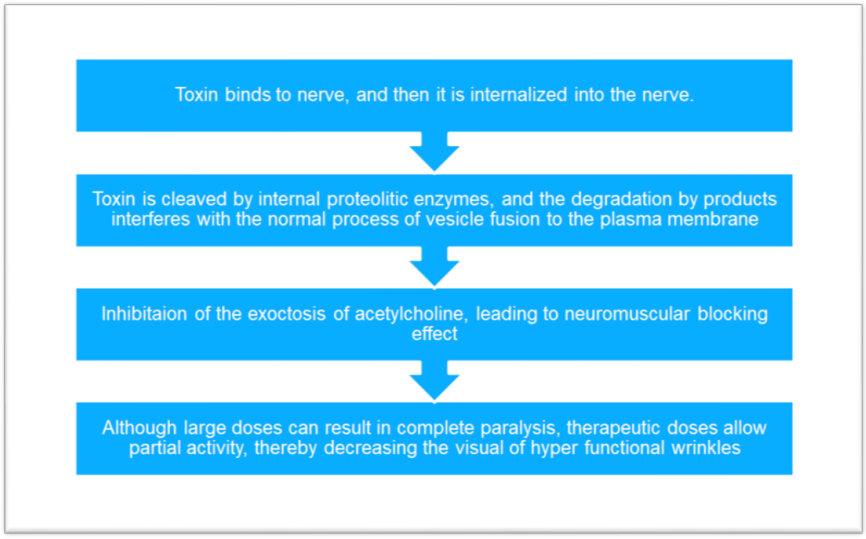

Figure 1: *Flow Chart (Mechanism of botulinum toxin action) (Azam, Manchanda, Thotapalli, & Kotha, 2015)*

Preparation and contents

Botulinum toxin has different types of preparations. Botox's trade names and countries of origin are:

- Botox® (Botox-A, Allergan, USA)
- Dysport (Botox-A, Ipsen Ltd., Germany)
- Xeomin (Botox-A, Merz Pharmaceuticals, Germany)
- Myobloc (Botox-B, San Francisco, USA)

Preparations of botulinum toxin are also known in the market under a different name.

Botox onabotulinum toxin A, Dysport abobotulinum toxin A, Xeomin incobotulinum toxin A and Myobloc are also called rimabotulinum toxin B (Chen, Kuziemko, & Stevens, 1998; Dressler & Saberi, 2005).

The treatment dose of botox depends on the brand of toxin used. The dose given for any type of toxin is only valid for the specific preparation and cannot be added or transferred to the doses of the other product unless it is the serotype of the same toxin. The dosage of the toxin should be very well adjusted because different preparations have varying effects on different parts of the body (Figure 2 and 3) (OW Majid, 2010).

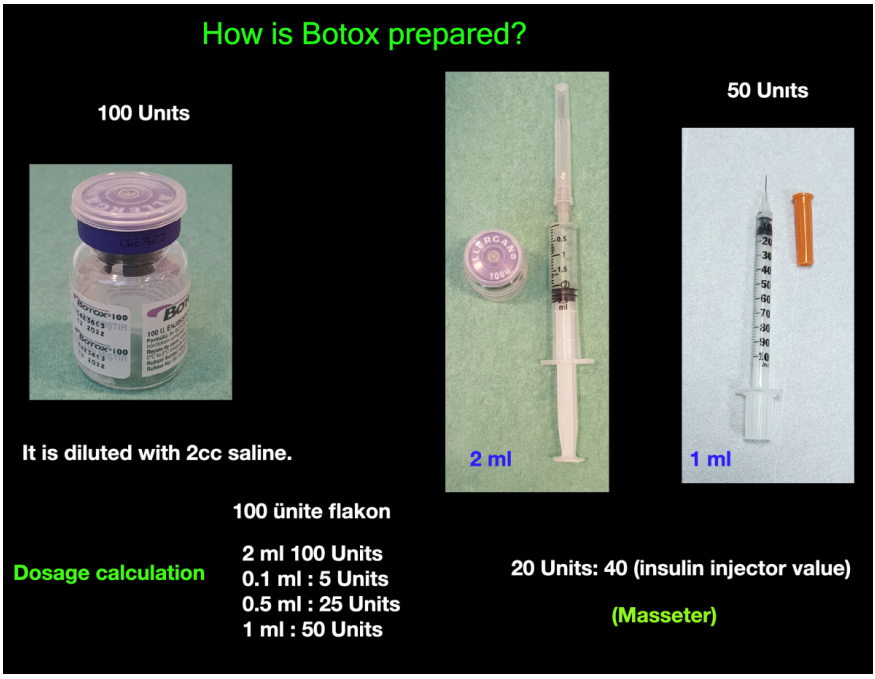

Figure 2: *Preparation of botox*

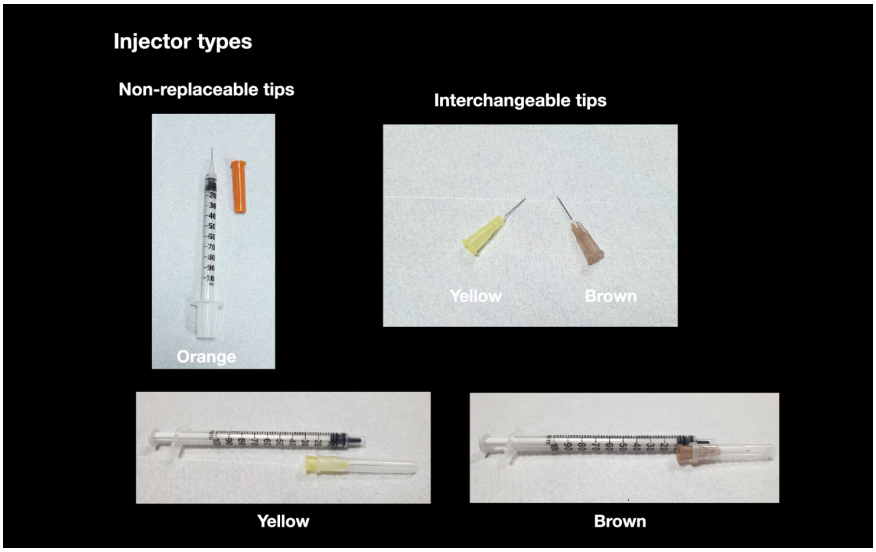

Figure 3: *Injector types used in botox*

Botox® is purified Botox-A isolated from the fermentation of Closterium botulinum. It was first used in the treatment of strabismus in 1968. This is a vacuum dried powder diluted with saline solution. The Allergan company bought purified Botox-A in 1991 and launched it under the trade name Botox®. Each vial of Botox® contains 5 ng (100 U) of air dried toxin (Rowe & Noonan, 2017).

Dysport contains 12.5 ng (500 U) air-dried toxin, 125 μg albumin and 2.5 mg lactose. Since Botox® and Disport are obtained from different bacterial species, their doses are not the same (Odergren et al., 1998). As a result, 1U Botox® is 2.5 times more toxic than 1U Dysport (Figure 4).

Name of the product	Botox	Dysport
Name of the preparation	Onabotulinumtoxin A	Abobotulinumtoxin A
Manufacturer	Allergan ABD	İpsen Biopharma Germany
Serotype	Tip A	Tip A
Active Ingredient	Clostridium botulinum toxin Tip A	Clostridium botulinum toxin Tip A
Unit in Each Vial	50/100 allergan units	300/500 Speywood units

Figure 4: *Details about botox and dysport*

Xeomin is freeze-dried purified botox-a which does not contain other complex proteins (hemagglutinin and non-hemagglutinin). It is less immunogenic than other botox-a products. It is also the only form that can be stored at room temperature, while other botox forms can be stored in the cupboard (Hellman & Torres-Russotto, 2015).

Myobloc is more effective in treating movement disorders than cosmetic use. In 2001, it was approved by the FDA for use only in the treatment of cervical dystonia and hemifacial spasms.

It can be used as an alternative in cases with droopy eyelids, for some wrinkles and in patients with resistance to botox-a products for cosmetic neuroblock (Hellman & Torres-Russotto, 2015; OW Majid, 2010).

Storage Conditions

Botulinum toxins can be stored for 3 years in a refrigerator at 2-8°C. Its reconstituted form can maintain its clinical efficacy for 6 weeks at 4°C. Vials should be stored in accordance with the manufacturer's recommendations and should not be exposed to direct sunlight or heat that would denature the protein in the toxin (Alshadwi, Nadershah, & Osborn, 2015).

Directly exposed to sunlight, the toxin is inactivated within 1-3 hours, 30 minutes at 80°C and 10 minutes at 100°C. In addition, the concentration

of the toxin can deteriorate in a much shorter time as the temperature increases depending on the pH of the environment (Lam, 2003).

Indications in dentistry

The general usage of Botox can be seen in the Table 1. The indications of botox in dentistry can be summarized as follows:

1. Bruxism
2. Extreme Gingival Appearance (Gummy smile)
3. Masseter hypertrophy
4. Trigeminal neuralgia
5. Temporomandibular joint disorder
6. Mandibular spasm
7. Oromandibular dystonia
8. Implant and prosthesis
9. In orthodontics
10. In maxillofacial surgery
11. Sialore

Table 1 *Botulinum toxin's uses according to the disorders and their subtypes (Azam et al., 2015)*

Disorder	Subtype
Cosmetic use	i. Hyperkinetic facial lines ii. Hypertrophic platysma muscle bands
Focal dystonia • Involuntary, sustained or spasmodic patterned muscle activity	i. Blepharospasm ii. Cervical dystonia iii. Laryngeal dystonia iv. Limb dystonia v. Orolingual dystonia vi. Oromandibular dystonia vii. Truncal dystonia
Non-dystonic disorders of involuntary muscle activity	i. Hemifacial spasm ii. Hereditary muscle cramps iii. Myoclonus iv. Myokymia and synkinesis v. Tics vi. Tremor
Smooth muscle hyperactive disorders	i. Achalasia cardia ii. Chronic anal fissures iii. Detrusor sphincter dyssynergia

Spasticity • Velocity-dependent increase in muscle tone	i. Cerebral palsy ii. Multiple sclerosis iii. Spinal cord injury iv. Stroke v. Traumatic brain injury
Strabismus and nystagmus • Disorder of conjugate eye movement and rapid involuntary rhythmic eye movement Disorders of localized muscle spasm and pain	i. Cervicogenic headache ii. Chronic low back pain iii. Myofascial pain syndrome iv. Migraine headache v. Temporomandibular joint disorders associated with increased muscle activity vi. Tension headache
Sweating disorders	i. Axillary and palmar hyperhidrosis ii. Frey syndrome

1) Bruxism

Bruxism is a repetitive jaw-muscle activity characterized by clenching and grinding caused by abnormal mandibular movement. Bruxism, a parafunctional habit, results in overloading of stomatognathic structures (Beddis, Pemberton, & Davies, 2018).

The prevalence of bruxism varies from 4% to 96% from society to society (Dylina, 2001; Seligman, Pullinger, & Solberg, 1988). The incidence of bruxism does not vary according to gender (Dharmadhikari et al., 2015). However, it is more common in young individuals compared to the elderly (Manfredini, Restrepo, Diaz-Serrano, Winocur, & Lobbezoo, 2013).

The etiology of bruxism is not completely clear. However, it is thought that various factors such as psychosocial, psychological / biological, and exogenous factors may cause bruxism (Pierce, Chrisman, Bennett, & Close, 1995).

Bruxism is divided into two classes as primary and secondary. Primary bruxism is the type of bruxism in which no socio-psychological or medical problems can be detected. Secondary bruxism is the type of bruxism associated with socio-psychological or medical problems (Castrillon & Exposto, 2018; Saito et al., 2013). Also, in another classification, Bruxism is classified under 2 groups as sleep and daytime bruxism (Klasser, Rei, & Lavigne, 2015; Melo et al., 2019).

The fact that it shows a non-specific pathology makes the diagnosis of bruxism difficult. Creaking sounds due to clenching during sleep are a sign of bruxism. However, conditions such as abrasion, headache, jaw locking or temporarily occurring pain and fatigue in the jaw muscles

that occur as a result of teeth grinding allow the diagnosis to be finalized (Beddis et al., 2018).

Treatment of bruxism

In bruxism, the clinician should apply treatments to prevent tooth erosion, reduce / eliminate pain, relax the jaw joint / muscles and improve the quality of sleep. An effective method for the treatment of bruxism has not yet been found (Klasser et al., 2015). Due to the multifactorial etiology of bruxism, a single treatment method cannot be recommended for all patients. Instead, etiologies should be determined for each patient and treatment options should be recommended accordingly (Melo et al., 2019). It can be used as dental, hypnosis, pharmacological, physical therapy applications, behavioral therapy and botox therapy in bruxism (Figure 5 and 6).

Figure 5: *A representative image of intraoral finding in bruxism (abrasions on tooth structures).*

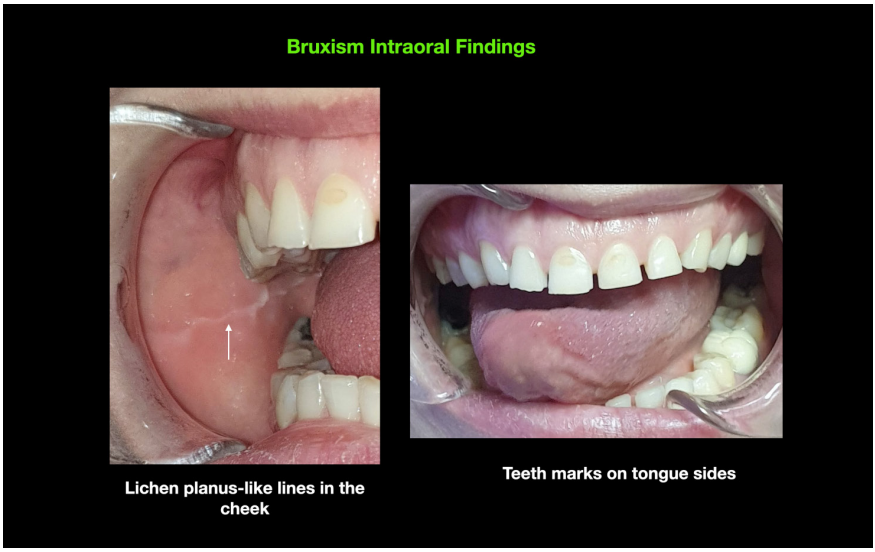

Figure 6: *A representative image of intraoral finding in bruxism (Lichen planus-like lines).*

Dental Treatment

Early contact and occlusion disorders in the teeth should be eliminated in bruxism cases. The effectiveness of oral splints, which are commonly used in dental clinics, has not yet been proven (Hobo, 1996; Raphael, Marbach, Klausner, Teaford, & Fischoff, 2003). Although bruxism is tried to be treated with orthodontic devices, this effect is temporary (Harada, Ichiki, Tsukiyama, & Koyano, 2006; Wali, 2004).

Pharmacological treatment

Pharmacological treatment of bruxism can be used for relief in the short term. There is no evidence that drug therapy cures bruxism. However, it has been shown in a study that clonazepam reduces bruxism by 40% (Saletu, Parapatics, Anderer, Matejka, & Saletu, 2010).

Physical Therapy Applications

Physical therapy methods for bruxism are acupuncture, transcutaneous electrical nerve stimulation, ultrasound, massage, cold and hot applications, injection and exercise. These methods relax contracted muscles and reduce pain (Melo et al., 2019).

Behavioral Therapy

Behavioral treatment of bruxism is also important. Removing bad habits such as smoking and alcohol, and behavioral therapies such as biofeedback can be used to control bruxism (Ommerborn et al., 2007).

Hypnosis

In bruxism, the patient can be controlled and relaxed with hypnotherapy (Ommerborn et al., 2007).

Botox Treatment

Botulinum toxin, which is widely used in facial aesthetics, is also used frequently in temporomandibular joint disorders. Although the use of botulinum toxin in bruxism is popular, further studies are needed on this subject.

A previous report investigated jaw motor episodes after botox application in patients with or without orofacial pain and not responding to oral splint therapy. The authors found that botox is a good treatment strategy for patients with sleeping bruxism. Bruxism can reduce the intensity rather than the generation of the contraction in jaw-closing muscles (Shim et al., 2014).

Ondo et al (Ondo et al., 2018) tested the safety and efficacy of onabotulinum toxin-A injections into the masseter and temporalis muscles in patients with symptomatic sleep bruxism. The authors found that onabotulinum toxin-A effectively and safely improved sleep bruxism.

Shim et al (Shim & Lee, 2020) conducted a randomized controlled study to examine the effects of botulinum toxin type A in sleep bruxism patients. In that study thirty patients with sleeping bruxism were randomly assigned into placebo and botulinum toxin type A. According to the findings, a single botulinum toxin type injection cannot reduce the genesis of sleeping bruxism. The authors stated that botox is an effective management option for reducing the intense of the masseter muscle.

A recent systematic review was conducted to assess the effects of botulinum toxin injections in the management of bruxism. In this study, 3 randomized controlled and 2 uncontrolled clinical studies were included among 904 articles. According to the findings, botulinum toxin type A was found to be a treatment option in sleep bruxism, minimizing signs and symptoms, and decreasing muscle contraction intensities (De la Torre Canales, Câmara-Souza, do Amaral, Garcia, & Manfredini, 2017).

2) Benign Masseter Hypertrophy

Benign masseter hypertrophy is a condition characterized by unilateral or bilateral enlargement of the masseter muscle. Although the etiology of benign masseter hypertrophy is not clear in many cases, it is defined as bilateral swelling of the masseter muscle caused by factors such as bruxism, temporomandibular disorders and malocclusion (Moore & Wood, 1994).

3) Extreme Gingival Appearance (Gummy smile)

Currently, the demand for cosmetic procedures has increased exponentially. Nowadays, cosmetic treatment requests have become very popular. Smiling and communication are of great importance in socializing. Aesthetics are also demanded in the treatments (Bashetty, Nadig, & Kapoor, 2009).

Learning the harmony between lips, teeth and gums will result in a beautiful smile (Bashetty et al., 2009). Incompatibility between these textures may result in an anesthetic smile. For an ideal harmony, it is considered normal that the gums appear 1-3 mm. However, if this gingival appearance exceeds 3 mm, an unacceptable picture emerges in terms of aesthetics (Bashetty et al., 2009).

Gummy smile can be defined as the appearance of more than 3 mm gums while smiling. Gummy smile is frequently seen in women. This is due to the lower laughing line in men compared to women (Sucupira & Abramovitz, 2012).

Causes of excessive gum appearance while smiling; Vertical overgrowth of the premaxilla, delayed passive eruption, overgrowing of the gums, short upper lip and excessive function of the upper lip levator muscles. Orthognathic surgery, crown lengthening (gingivectomy/ gingivoplasty), myectomy and botox applications to reduce excessive gingiva application. Botox applications are the most conservative, fast and reliable method among the treatments in appropriate cases. Botulinum toxin is indicated in the gummy smile caused by an overactive muscle. In addition to being a more conservative approach, the safety of the botox makes it the first-choice treatment for rapid effect. Therefore, botox is considered as an alternative to surgical treatments in appropriate cases (Al-Fouzan et al., 2017).

Smile mainly occurs during contraction of the specific facial muscles such as risorius, orbicularis oris, zygomaticus minor / major, levator labi, alequa nasi. If the muscles are too activated to elevate the upper lip, it causes the gum to appear excessively. Botox injection is performed to the

yonsei point which is the center of these three levator muscles. After the application, with the effect of the toxin, the gum problem disappears as a result of the weakening of these muscles (Al-Fouzan et al., 2017).

Mazzuco and Hexsel divided the gummy smile into 4 subgroups as anterior, posterior, mixed and asymmetrical in order to facilitate botulinum toxin injections and determine the active muscle groups according to the regions with high gingival visibility (Figure 8) (Mazzuco & Hexsel, 2010).

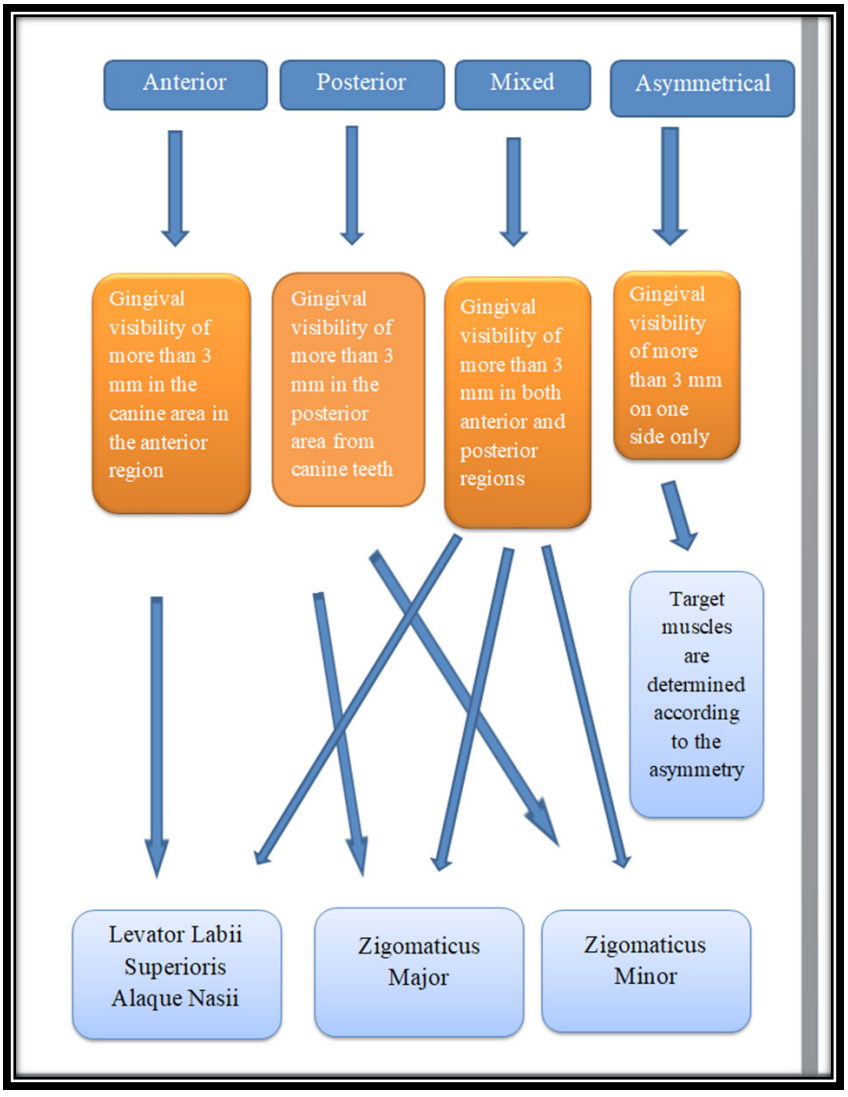

Figure 8: *Gummy Smile Classification*

4)Trigeminal Neuralgia

Trigeminal neuralgia is a condition that reduces the quality of life of patients. Drugs are considered first in its treatment. However, surgical applications are performed in cases that do not respond to drug therapy. Botox is a new hope in patients with trigeminal neuralgia. Botox has been shown by studies to be a safe and effective treatment in trigeminal neuralgia patients. However, further studies are needed on the subject (Castillo-Álvarez, Hernando de la Bárcena, & Marzo-Sola, 2017; Morra et al., 2016).

5) Temporomandibular Joint Disorders

Temporomandibular joint disorders usually include symptoms such as pain, limited mandibular movement, or joint sounds. Botulinum toxin can be used for the treatment of temporomandibular joint disorders' symptoms. Dosage and number of injections are different in studies. However, injection methods are relatively similar. Injection of botulinum toxin into the lateral pterygoid has been shown to be effective in reducing click noise, reducing other muscle disorders related to temporomandibular joint disorders such as pain, hyperactivity and dysfunction (Ataran et al., 2017; Machado et al., 2020).

6) Mandibular Spasm

If the muscles covering the mandible remain spasm, the mouth opening is restricted. This type of muscle spasm restricts the oral hygiene and eating required to maintain oral hygiene. Hypertrophy of the muscular system or the treatment of spastic muscles can be provided with botulinum toxin. The aim is to solve the spasm by injecting the problematic muscle (Alshadwi et al., 2015; SOĞANCI & YAĞCI, 2016).

7) Oromandibular Dystonia

Oromandibular dystonia is a movement disorder characterized by dysfunction of chewing and lower facial muscles and involuntary contractions. It manifests itself with difficulty speaking, swallowing and eating. Although it is a neurological disorder, it is examined under temporomandibular disorders since it concerns the masseter region. Botulinum toxin is made into type A masseter and / or submental. At the same time, temporal and lateral pterygoid muscles may be affected (Alshadwi et al., 2015; SOĞANCI & YAĞCI, 2016).

8) Dental implants and prosthesis

Implant health can be affected by excessive contraction of the masticatory muscles. By applying botulinum toxin to certain parts of the chewing muscles, paralysis occurs in the muscles. Thus, the negative effect of chewing muscles on the implant can be eliminated. The same is true for prostheses (Alshadwi et al., 2015; SOĞANCI & YAĞCI, 2016).

9)In orthodontics

Relapse after orthodontic treatment may develop as a result of strong muscle activity in some cases. This situation is eliminated with Botox. Botox application can be applied to the tongue and other muscles for orthodontic purposes (Alshadwi et al., 2015; SOĞANCI & YAĞCI, 2016).

10)In Maxillofacial surgery

After jaw fractures or repositioning of the jaw by maxillofacial surgical methods, fixation may be impaired as a result of strong muscle activities. Strong muscle activities can be controlled with botox applications (Alshadwi et al., 2015; SOĞANCI & YAĞCI, 2016).

11)Sialorrhea

Sialorrhea is an excessive secretion of saliva and its treatment is usually carried out by pharmacotherapy or behavioral treatment methods. Saliva flow can be reduced as a result of inactivation of the cholinergic receptor as a result of botox application to the parotid or maxillary salivary glands (Alshadwi et al., 2015; SOĞANCI & YAĞCI, 2016).

Contraindications

1. Those with neuromuscular disorders (e.g. myasthenia gravis, Eaton-Lambert syndrome)

2. Those who are allergic to Botox-A or Botox-B components (e.g. albumin, lactose, mineral salt, sodium succinate) (Nayyar, Kumar, Nayyar, & Singh, 2014).

3. If certain drugs are taken that may adversely affect neuromuscular stimulation transmission and increase the effect of botox (e.g. aminoglycoside, penicillamine, quinine and calcium blockers) (Nayyar et al., 2014).

4. Pregnant and breastfeeding patients (Botox is group C in pregnancy category) (Tan, Kim, Koren, & Bozzo, 2013).

5. People working in front of the camera where mimics are important and high expectation (Srivastava, Kharbanda, Pal, & Shah, 2015).

6. If there is an infection in the area to be injected.

Cosmetic use of Botox

Botulinum toxin injection for the treatment of wrinkles on the face is one of the most common aesthetic procedures in the United States. Cosmetically approved by the US Food and Drug Administration, the treatment of frown lines and crow's feet and horizontal forehead lines has predictable results and few side effects. Wrinkles on this face are caused by dermal atrophy and repetitive contraction of the underlying facial muscles (Sundaram et al., 2016; Varlamov, Hinojosa-Amaya, Stack, & Fleseriu, 2019).

When very small amounts of botulinum toxin are injected into overactive muscles, it provides localized muscle relaxation that flatten the skin and reduces wrinkles. It takes about two weeks for the effects of botulinum toxin to be fully seen and this effect lasts for 3-6 months. Dynamic wrinkles seen during muscle function give more dramatic results than static wrinkles that can be seen at rest (Sundaram et al., 2016; Varlamov et al., 2019).

Side effects and complications

The local effects of botulinum toxin are usually temporary. The most common side effect is allergies. Pain, edema, erythema, ecchymosis, infection, dry mouth, facial nerve palsy and short-term nerve insensitivity are other side effects. Transient systemic side effects such as fatigue, nausea and itching are rarely seen regardless of dose. In addition, swallowing difficulty may be seen due to weakness in the muscles (Schantz & Johnson, 1992).

Conclusion

Nowadays, botox is also applied in the field of aesthetic dentistry. In addition to aesthetic expectations, botox, which is also made for treatment purposes, can also be used for problems in the temporomandibular region, bruxism or visible gum treatment. Legal permissions for dentists to apply botox for treatment in the perioral regions. During the application, the important thing is to provide the treatment by injecting into the muscle at the appropriate dose. Otherwise, undesirable results of Botulinum toxin injection may occur. As the injection skill improves, the frequency of side effects decreases and botox applications become safer.

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