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Chapter 1

THE PREVALENCE OF *HEPATOZOON CANIS* IN TURKEY

Burçak ASLAN ÇELİK[1]

1 Dr.Öğr.Üyesi Burçak ASLAN ÇELİK, Siirt Üniversitesi Veteriner Fakültesi Parazitoloji ABD, https://orcid.org/0000-0002-0130-970X

1. Introduction

Hepatozooon species are protozoan parasites classified in the Phylum Apicomplexa, Family Hepatozoidae, and are closely related to *Plasmodium* spp. and pyroplasmas. There are more than 300 species in the *hepatozoa* genus and they can infect a wide variety of domestic and wild carnivores, birds, reptiles and amphibians (Aydin, Sevinc, & Sevinc, 2015; Baneth et al., 2003; Baneth, Samish, & Shkap, 2007; Ivanov & Tsachev, 2008; Little et al., 2009; Smith, 1996).

Canine hepatozoonosis is a protozoal disease that primarily affects dogs, and is caused by *Hepatozoon canis* (Baneth et al., 2007; O'dwyer, Massard, & de Souza, 2001). The disease was first identified in a dog in India and has initially been been named as *Leucocytozoon canis* (Baneth et al., 2007; Bentley, 1905). Although canine hepatozoonosis is common all over the world, the geographical distribution of the disease-causing species differs depending on the type of tick that transmits the parasite (Altay, Aktas, & Dumanlı, 2013). Two hepatozoan species, *Hepatozoon canis* and *Hepatozoon americanum* have been identified in dogs. These common species associated with canine hepatozoonosis are *H. Canis;* in Southern Europe, Middle East, Africa and Far East, and *H. Americanum* in the United States are (Altay et al., 2013; Baneth, 2011; Inokuma, Okuda, Ohno, Shimoda, & Onishi, 2002; Little et al., 2009).

The main vector *of H. canis* is the *Rhipicephalus sanguineus* type ticks, whereas for *H. americanum*, the vector is *Amblyomma maculatum* type ticks (Baneth, 2011; Baneth et al., 2003; Craig, Smallwood, Knauer, & McGrath, 1978; Gavazza, Bizzeti, & Papini, 2003; Little et al., 2009; Voyvoda, Pasa, & Uner, 2004). Studies show that vertical transmission can also occur in offspring born to infected mothers (Murata, Inoue, Tateyama, Taura, & Nakama, 1993).

All hepatozoa species have a basic life cycle. The life cycle is sexual development and sporogony in the hematophagous invertebrate definitive host, and merogony followed by gamontogonia in a vertebrate intermediate host (Baneth, 2011; O'Dwyer, 2011). Many tick-borne bacterial and protozoal diseases are transmitted through the salivary glands of ticks, while hepatozoan transmission takes place by ingestion of ticks containing *Hepatozoon* oocysts while dogs self-groom (Altay et al., 2013; Baneth, 2011; Baneth et al., 2007; O'Dwyer, Saito, Hasegawa, & Kohayagawa, 2004).

The sporozoites are released from the oocysts after dogs ingest the infected ticks. The sporozoites then penetrate the intestinal wall, invade mononuclear cells, and enter the circulation. Through the circulation they reach many organs such as bone marrow, spleen, liver, kidneys, lungs,

intestine and even lymph nodes (Baneth, 2011; O'dwyer et al., 2001).

The clinical manifestations of the disease may vary depending on the agent, diet, and other individual factors. The disease can evolve from an asymptomatic state to a severe, life-threatening and fatal condition (Altay et al., 2019; Baneth et al., 2003; Baneth & Weigler, 1997; Gavazza et al., 2003). *H. canis* is considered to be less virulent than *H. americanum*, and rarely causes clinical symptoms, however a clinical table that may result in death can still occur. Significant symptoms of the disease include fever, lethargy, and weight loss (Aktas et al., 2015; Altay et al., 2013). Fever, generalized pain, muscle atrophy, weakness, depression, reluctance to move, and mucopurulent ocular discharge, thirst, cough, and rarely excessive urination can also be observed during *H. americanum* infections (Altay et al., 2013; Altay et al., 2019).

Microscopic examination of giemsa-stained peripheral blood smears, serological (Baneth, 2011; Elias & Homans, 1988; Paşa, Kiral, Karagenc, Atasoy, & Seyrek, 2009), [Indirect Fluorescent Antibody Technique (IFAT), enzyme immunosorbent assay (ELISA)] (Baneth, 2011; Gonen et al., 2004; Karagenc et al., 2006) and molecular methods [Polymerase Chain Reaction (PCR)] (Aktas, Özübek, & Ipek, 2013; Altay et al., 2019; Aydin et al., 2015; Baneth, 2011; Inokuma et al., 2002; Karagenc et al., 2006; Rubini, dos Santos Paduan, Cavalcante, Ribolla, & O'Dwyer, 2005) are used in the diagnosis of the disease. It is reported that molecular methods are more sensitive and specific than microscopic and serological methods in the diagnosis of hepatozoonosis and other blood parasites (Altay et al., 2019).

5-6 mg/kg imidocarb dipropionate is used intramuscularly every 14 days for the treatment of *H. canis* until no gamonts are found in the blood. In addition, doxycycline 10 mg/kg orally can be used in combination with imidocarb for 21 days. Removal of gamonts from peripheral blood may take up to 8 weeks (Altay et al., 2013; Aytuğ, 2012). Clinical findings may recur if treatment is not performed regularly (Aytuğ, 2012). It was reported in a study that positive results were obtained from the application of toltrazuril, trimethoprim-sulfamethoxazole, and B complex vitamins (Voyvoda et al., 2004). There is no effective compound in the *H.americanum* treatment. Trimethoprim-sulphadiazine 15 mg/kg twice a day, clindamycin 10 mg/kg three times a day, and pyrimethrine 0.25 mg/kg once a day can be used for 14 days (Altay et al., 2013).

Dogs should be checked regularly for ticks and tick control should be performed using appropriate acaricides for the prevention of disease (Altay et al., 2013; Aytuğ, 2012). Dogs should be prevented from eating raw meat of wild animals in areas where the disease is endemic(Altay et al., 2013).

2. Studies on ***Hepatozoon canis*** In Turkey

Studies were conducted in different provinces (Figure 1) with various methods to determine *H.canis* prevalence in animals in Turkey (Table 1). The highest positivity was reported in the Aegean region (33.66%), and the lowest positivity was reported in the Black Sea region (Table 2).

First data on canine hepatozoonosis in Turkey was reported by Tüzdil (1933) in a dog. Aslantaş, Çelebi, and Usluca (2020) reported that no positivity was found in Mersin while in a study conducted by Aktas et al. (2015) a prevalence of 10.8% was reported in the same province. A prevalence of 0% and 2.44% was determined in Adana and Hatay respectively in the studies carried out by Aslantaş et al. (2020).

In a study carried out by Karagenc et al. (2006) using microscopic, PCR and IFAT methods in İzmir, a prevalence of 38.46%, 61.54% and 69.23% was determined, respectively. A prevalence of 26.7% was determined with the PCR method by Aktas et al. (2015) in the same province. In a study conducted in Aydın with microscopic, PCR, and IFAT methods, a prevalence of 3.7%, 14.81% and 20.37% was found, respectively (Karagenc et al., 2006). A case study performed by Voyvoda et al. (2004) using microscopic method was also reported in the same province. In the studies performed by Karagenc et al. (2006) with microscopic, PCR and IFAT methods, prevalences were reported as 4.35%, 8.7%, 26.09% in Manisa, and 5.05%, 24.24% and 37.37% in Muğla, respectively.

In the study performed by Orkun et al. (2018) with microscopic and PCR methods in Ankara, a prevalence of 3.8% and 49.5% was determined respectively. Aktas et al. (2015) reported a prevalence of 4% by PCR method in the same province. In a study conducted in Konya and Karaman provinces, a prevalence of 2% and 4.9% was reported, respectively (Aydin et al., 2015). Prevalence was determined as 5.3% in Kayseri (Düzlü, İnci, Yıldırım, Önder, & Çiloğlu, 2014) and 3.9% in Nevşehir (Aktas et al., 2015).

In a study conducted by Bölükbaş et al. (2016) in Samsun, it was reported that no prevalence was detected by the microscopic method, while a prevalence of 0.5% was determined by PCR method. In Giresun, a prevalence of 18% was reported (Aktas et al., 2015).

In the studies carried out by Aktas et al. (2015)in Sakarya and Kocaeli, the prevalence was reported as 21.5% and 17.4%, respectively.

In Erzurum province, a prevalence of 42.8% was determined by Aktas et al. (2015), while a prevalence of 5.3% was determined by Guven, Avcioglu, Cengiz, and Hayirli (2017). Another study reported 25.3% prevalence in Elazığ province (Aktas et al., 2015).

In a study conducted in Diyarbakır, a prevalence of 15.87% was determined (Aktas et al., 2013). It was reported that no positivity was found in studies conducted in Batman and Gaziantep (Aslantaş et al., 2020).

Table 1. Distribution of studies carried out in Turkey by province, method and results

Provinces	Examined (n)	Positive		Methods	References
		(n)	(%)		
Mersin	36	0	0	PCR	(Aslantaş et al., 2020)
	74	8	10.8	PCR	(Aktas et al., 2015)
Adana	45	0	0	PCR	(Aslantaş et al., 2020)
Hatay	41	1	2.44	PCR	(Aslantaş et al., 2020)
İzmir	65	25	38.46	Microscopic	(Karagenc et al., 2006)
		40	61.54	PCR	(Karagenc et al., 2006)
		45	69.23	IFAT	(Karagenc et al., 2006)
	60	16	26.7	PCR	(Aktas et al., 2015)
Aydın	1	1	100	Microscopic	(Voyvoda et al., 2004)
	162	6	3.7	Microscopic	(Karagenc et al., 2006)
		24	14.81	PCR	(Karagenc et al., 2006)
		33	20.37	IFAT	(Karagenc et al., 2006)
Manisa	23	1	4.35	Microscopic	(Karagenc et al., 2006)
		2	8.7	PCR	(Karagenc et al., 2006)
		6	26.09	IFAT	(Karagenc et al., 2006)
Muğla	99	5	5.05	Microscopic	(Karagenc et al., 2006)
		24	24.24	PCR	(Karagenc et al., 2006)
		37	37.37	IFAT	(Karagenc et al., 2006)
Ankara	103	4	3.8	Microscopic	(Orkun et al., 2018)
		51	49.5	PCR	(Orkun et al., 2018)
	49	2	4	PCR	(Aktas et al., 2015)
Konya	50	1	2	PCR	(Aydin et al., 2015)
Karaman	171	7	4.9	PCR	(Aydin et al., 2015)
Nevşehir	51	2	3.9	PCR	(Aktas et al., 2015)
Kayseri	400	21	5.3	PCR	(Düzlü et al., 2014)
Giresun	50	9	18	PCR	(Aktas et al., 2015)
Samsun	200	0	0	Microscopic	(Bölükbaş et al., 2016)
		1	0.5	PCR	(Bölükbaş et al., 2016)
Sakarya	65	14	21.5	PCR	(Aktas et al., 2015)
Kocaeli	69	12	17.4	PCR	(Aktas et al., 2015)

Erzurum	133	7	5.3	PCR	(Guven et al., 2017)
	126	54	42.8	PCR	(Aktas et al., 2015)
Elâzığ	150	38	25.3	PCR	(Aktas et al., 2015)
Batman	50	0	0	PCR	(Aslantaş et al., 2020)
Gaziantep	14	0	0	PCR	(Aslantaş et al., 2020)
Diyarbakır	63	10	15.87	PCR	(Aktas et al., 2013)

Table 2. Distribution of studies carried out in Turkey by regions

Regions	Examined (n)	Positive	
		n	%
The Mediterranean region	196	9	4.59
Aegean Region	410	138	33.66
Central Anatolia Region	824	84	10.19
black Sea region	250	10	4.00
Marmara Region	134	26	19.40
Eastern Anatolia Region	409	99	24.21
Southeast Anatolia Region	127	10	7.87
Overall	2350	376	16.00

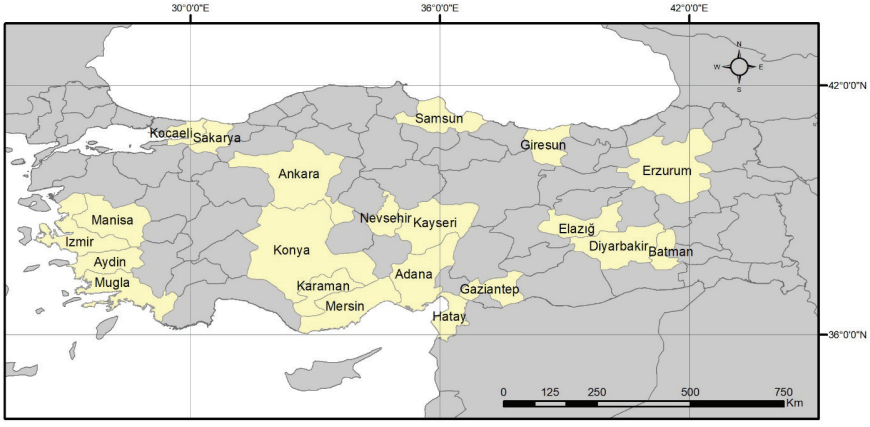

Figure 1. Provinces where H. canis was studied

3. Conclusion

In conclusion; Canine hepatozoonosis positivity has been reported at different rates in studies conducted in different parts of Turkey. It is of great importance to take preventive measures, especially to fight ticks with appropriate acaricides, since there is no vaccine to prevent the disease. Clinicians should be aware of this disease as well as others for accurate differential diagnosis of patients with ticks. It should be remembered that the elimination of gametocytes from the peripheral blood is slow and an 8-week treatment is required to completely cure the disease.

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Chapter 2

REMINERALIZATION AND CARIES PREVENTIVE AGENTS

Hakan DEMİR[1]

1 Prof., Sivas Cumhuriyet University, Faculty of Dentistry, Department of Prosthodontics, hdemir@cumhuriyet.edu.tr, Orcid: 0000-0002-1769-1667

Remineralization and Caries Preventive Agents

Hydroxyapatite is a crystalline mineral, a complex form of calcium phosphate, and is the main inorganic component of bones and teeth (1). In the demineralization process, mineral ions are removed from the hydroxyapatite crystals of hard tissues such as enamel, dentin, cementum and bone. Re-acquisition of these mineral ions by hydroxyapatite crystals is called remineralization. Demineralization is a reversible process; therefore, repair can occur if partially demineralized hydroxyapatite crystals in teeth are exposed to an environment that promotes remineralization (2).

It has been reported that there is a continuous ion exchange in a dynamic balance between the enamel surface and plaque and salivary fluid throughout the day. It is known that remineralization is the natural repair process of our body against caries lesions located under the surface and not yet cavitated (3-4).

Chemical demineralization of teeth is caused by acid attacks in two main ways: dietary acid consumed through food or drink and microbial attack by bacteria in the mouth. The critical pH value at which demineralization takes place in the enamel is 5.2-5.5. During an acidic attack or a typical demineralization process, chemical dissolution of both organic and inorganic matrix components takes place (4-6).

It has been determined that the acid produced by the plaque bacteria is buffered by the saliva and the pH increases and becomes neutral. It has been explained that when the pH of the plaque exceeds the critical pH, the dissolved minerals begin to precipitate and remineralization occurs through saliva with the effect of the minerals dissolving from the enamel (7). In contrast to demineralization, it has been reported that passive transport during remineralization is not by H^+ ion transfer, but by passive transport from saliva and plaque to the lesion body where the concentrations of Ca^{+2} and $PO4^{-3}$ ions are low (4). During the remineralization period, minerals accumulate in the crystal cavities formed during demineralization, and it is known that the lost minerals are compensated. It has been stated that since the repaired crystals may be smaller or larger than the actual crystal size, the permeability of the enamel decreases and it becomes more resistant to acid attacks (8). Saliva, fluoride therapy, dietary control, and probiotic bacteria are defined as preventatives for tooth demineralization (2, 3, 9).

It is known that saliva is a solution that takes part in remineralization. It has been determined that Ca^{+2} and $PO4^{-3}$ in the salivary structure provide remineralization of the crystal structure in the demineralized areas by diffusing in the enamel structure with the help of the F^- ion, which acts as a catalyst. It is known that this new structure contains fluoride hydroxyapatite, that is, fluorapatite. It has been emphasized that this new

structure is more resistant to acid attacks than the first structure (8).

Low carbohydrate consumption, low number of cariogenic bacteria in the plaque, high saliva buffering capacity and flow rate, high concentration of inorganic ions in saliva, low carious lesion depth and inactivity, effective mechanical cleaning and the use of remineralization agents It has been reported to be among the factors that accelerate the process (10).

In this review, researches on Fluoride, Propolis and Chitosan will be discussed when remineralization agents and caries preventive agents are examined.

Remineralization Agents and Caries Preventive Agents; Fluoride, Propolis and Chitosan

Propolis

Propolis is a resinous hive product collected by bees from various plants and processed with bees' enzymes. Its color can vary in shades of green, red and dark brown. It has a characteristic odor and shows sticky properties as it interacts with proteins and fats in the skin (11).

Composition; It varies depending on the plant it collects, the region, the season and the colony. However, it consists of an average of 50% resin, 30% wax, 10% essential and aromatic oils, 5% pollen, 5% other substances and organic residues (11, 12).

Propolis collection is carried out by worker honey bees. Going on a propolis expedition, the bee first pulls the propolis off the plant with its mandibles. It makes pellets by moistening, softening and adding some enzymes in its mouth, and transfers the pellet to the pollen basket on its hind legs using its front legs. While the worker bee, which comes to the hive loaded with propolis, clings tightly to the honeycomb using its feet; Other worker bees in the hive hang with their mandibles and take the propolis from the pollen basket of the carrier bee. They use propolis in the hive by accumulating propolis behind the bottom board, frame edges and entrance hole (13).

Bees add propolis to thin parts of honeycombs or to cavities where they live. Propolis is used to close holes and tears, to repair honeycombs, to thicken weak parts of honeycombs, to make air holes in the hive or to help hive defense. They also use propolis in the hive by mixing it with beeswax for polishing and sterilizing larval nests. Bees benefit from both the mechanical and biological activity of propolis. Depending on the source of the resin, the color of propolis can vary from yellowish green to red and dark brown, or it can be transparent. Its physical structure is hard, brittle in cold, sticky in heat. (13)

Propolis is slightly soluble in water and hydrocarbon solvents, and highly soluble in alcohols. Apart from ethanol, it can also be dissolved in solvents such as ether, glycol, methanol, oil. When solvents other than ethanol are used, it has been observed that the substances to be isolated from propolis differ and many components cannot be isolated. Therefore, the most commonly used solvent for propolis is 96% ethanol. Propolis extraction methods can change the effect of propolis. Because its solubility in different solvents and its extracted parts will differ according to the solvent. The most commonly used solvents in biological studies are mixtures of ethanol, water and methanol at different concentrations (14, 15).

The chemical content of propolis is very complex, more than 300 chemicals have been identified so far, and this content varies depending on the plant and flora from which the propolis is taken. Due to this change in propolis content, problems are experienced in its medical use and standardization (13, 15).

Propolis contains more than 300 compounds covering various chemical structures such as polyphenols (flavonoid aglycones, phenolic acids) and their esters, phenolic aldehydes, alcohols and ketones, secuiterpene quinones, coumarins, steroids, amino acids and inorganic compounds. The part containing resin and balsam contains substances such as terpenes, polysaccharides, caffeic acid. Beeswax, on the other hand, contains fatty acids, B vitamins, and vitamins C and E. Propolis; It has been determined that it contains B1, B2, B6, C, E vitamins and minerals, silver, cesium, mercury, lanthanum, antimony, copper, Mn^{+3}, Fe^{+3}, aluminum, Ca^{+2}, vanadium (12, 14).

Flavonoids (polyphenolic content), the most important part of the organic part, are the most studied substances and are responsible for a significant part of the biological activity of propolis. This part contains substances such as pinosembrin, pinostrobin, queracetin. Propolis also contains different mineral and oligo elements. Flavones and flavonoids are substances that give propolis antifungal, antiviral and antibacterial properties. The main chemical compounds it contains are; flavonoids, cinnamic acid and its derivatives are benzoic acid, synaptic and isopherulic acids, various aldehydes, ketones and trace elements, cleredon, diterpenes, sesquiterpenes and tripenes (14-17).

It has been determined that the main components of propolis are flavonoids. The flavonoid structure of propolis may also show some differences depending on the plant from which it is collected. The ratio of flavonoids from all components in propolis is over 25%. Flavonoids are polyphenolic compounds. Due to their free radical scavenging properties,

they are antioxidants and inhibit lipid peroxidation. Alternatively, it is stated that they can be antioxidants because they form metal chelates (13-17).

It has been stated that the biological activity of propolis is formed by the synergistic effect of phenolic and other compounds in its resin (12). At the same time, it has been reported that mixtures of pinosembrin, galangin, and caffeic acid phenyl ester inhibit bacterial RNA polymerase and exert antimicrobial effects (16).

The pharmacological value of propolis, the preparation of its medicinal preparations and its antibacterial and antiviral value are due to the secondary metabolites it contains. Among these metabolites; phenolic acids (caffeic acid and cinnamic acid) and their esters, ketones, phenolicaldehydes, flavones and flavonoids (pinosembrin, pinobanksin, acacetin, chrysin, rutin, catechin, naringenin, galangin, luteolin, campherolricetin, quinomenoids, and terpenestin), terrypenes, aromatic acid and its esters, amino acids, alcohols, aldehydes, aliphatic acid and its esters and some hydrocarbons can be counted (16, 17).

Use of Propolis in Dentistry

Propolis is a highly effective antimicrobial agent against oral microorganisms. Sonmez et al. (18) stated that propolis solutions prepared in appropriate proportions were highly effective against P. gingivalis, P. intermedia, C. rectus, F. Nucleatum, C. Parapsilosis, C. Krusei and C. Albicans and were not cytotoxic against gingival fibroblasts.

S. mutans is the most important bacteria in caries formation. These bacteria produce organic acids that demineralize the enamel and synthesize glucans that provide adhesion of other cariogenic bacteria to the tooth surface. Therefore, bacterial control is important in preventing caries formation. In studies, it has been determined that propolis is very effective in destroying or reducing the effectiveness of bacteria that are effective in the formation of dental caries. (19, 20). Park et al. In their study examining the effect of propolis on the proliferation and enzyme activities of S. mutans, they showed that propolis highly inhibited bacterial growth and glucosyltransferase synthesis (21).

Propolis has been used successfully in the treatment of inflammatory lesions of gingival-periodontal tissues and buccal mucosa (such as gingivitis, periodontitis, aphthous ulcers, stomatitis, glossitis) without being exposed to the side effects of other drugs (19, 22).

Al-Shaher et al. reported that aqueous solutions of propolis prepared at 4mg/ml or lower concentrations showed minimal toxicity against periodontal ligament cells and pulp fibroblasts, whereas calcium

hydroxide at the same concentrations was highly toxic (23). In one study, it was stated that propolis stimulated the healing process and reduced tissue inflammation in the treatment of direct pulp capping (24). Silva et al. reported that propolis can be easily used as an intracanal drug in endodontic treatments (25).

Propolis has started to be used for prophylactic purposes for caries and periodontal diseases by adding it to toothpastes, mouthwashes, dental floss and gums. It has been reported that toothpastes with propolis are very good plaque cleaner, prevent plaque formation and have anti-inflammatory effects (26). While the topical application of propolis accelerates epithelial healing after tooth extraction, it has not been found to have an effect on socket wound healing (27).

It has been reported that the teeth can be stored in propolis solutions in order to maintain the vitality of the periodontal ligament cells until the treatment procedures are started in cases of avulsion, and that the best alternative to storage media such as milk and saliva is propolis solutions (28).

In the study of Dualibe et al. (2007), in their study with propolis-containing mouthwash, it was reported that the amount of S. mutans was 49% lower in saliva samples collected before and after mouthwash use (29). Netto et al. (2013), in a study in which propolis-containing mouthwashes were compared with chlorhexidine-containing mouthwashes, it was found that propolis-containing mouthwashes were more effective than chlorhexidine in reducing the number of S. mutans and Lactobacillus (30).

Chitosan

Chitosan has been defined as a natural biopolymer with a chemical structure closest to cellulose, which is common in nature. It is stated that it is obtained from the cell walls of arthropods, crustaceans, fungi and yeasts. It is known that chitin is a natural cationic polysaccharide found in the shells of crustaceans, fungal cell walls, and insect cuticle, similar to chitosan. It has been reported that it has some biological activities such as antimicrobial and hemostatic activity (31).

It has been reported that many years ago, Koreans ground the shells and bones of sea creatures into powder and applied them to bone and skin wounds. It has been emphasized that chitosan is a useful derivative of chitin. (32).

Antimicrobial activity of chitosan on certain fungi and bacteria has been described, depending on the type, degree of polymerization, physical and chemical properties of chitosan. It has been determined that chitosan with short chain length has reduced antimicrobial activity, and partial

depolymerization of chitosan increases its antibacterial properties. It is stated that the degree of deacetylation affects the activity of chitosan. It was emphasized that chitin constitutes the majority of the dry weight in the exoskeleton of crustaceans. It has been reported that chitosan is found in nature in lesser amounts than chitin (32, 33).

It is emphasized that the main interest in chitosan derivatives stems from their cationic structure in acidic solutions. It is known that this structure allows chitosan to be used in other water-based treatments. It has been determined that chitin can dissolve in the soil without harming the environment and is biocompatible. It has been reported that chitin has biological properties such as cell viability and antitumor activity (33-35).

Chitosan; It has been stated that it has properties such as promoting wound healing, absorbing heavy metals and absorbing dangerous oral microorganisms. It has been determined that the effect of chitin on wound healing is accelerating. Chitin derivatives. It has been stated that the glycosaminoglycan components of the scar tissue have a role in the restructuring of the newly formed collagen in the granulation tissue of the healing wound. It has been reported that another mechanism by which chitin derivatives affect wound healing is related to macrophages. Along with these, chitosan and its derivatives; It has been stated that it has healing-accelerating effects during tissue regeneration and is also bactericidal (33, 34).

Use of Chitin and Chitosan Derivatives in Dentistry

It has been stated that chitosan is used in the prevention of dental caries due to its bacteriostatic and bactericidal properties. At the same time, it has been stated that chitosan can buffer the effects of organic acids that lower the pH values in the mouth (36).

The role of S. mutans, which is the most important etiological factor in dental caries, in the early stage of dental caries has been defined and attributed to its ability to colonize the tooth surface. It has been reported that the basis of the proposed approach for the prophylaxis of caries at an early stage is the inhibition of HAP binding and colonization of S. mutans. It has been emphasized that chitosan, which is an N-deacetylation derivative of chitin, is important because it protects against the harmful effects of organic acids, stimulates the regulated regeneration of oral soft tissues, and shows bactericidal properties against certain pathogens (32, 37).

It has been disclosed that chitosan-modified chitosans have a negative logarithm of the acidity constant Ka (pKa) of 6.3. It has been reported that this pH value is suitable for buffering at high oral pH value and is sufficient

to protect against the destructive effect of organic acids. It has also been shown to have a bactericidal effect against many pathogens, including S. mutans. Chelation of basic metal ions and formation of polyelectrolyte complexes with bacterial surface components, enzyme inactivation have been reported to be among the mechanisms of its bactericidal effect (35-37).

Antibacterial compounds; It has been reported that host sub-minimal inhibitory concentration (sub-MIC) impairs the production and function of bacterial adhesins, as well as inhibits the attachment and colonization of bacteria to host tissues. Therefore, considering that chitosan sub-MICs can be obtained in the oral cavity when mouth rinses and toothpastes containing these polymers are used, it has been discussed whether the binding of S. mutans to HAP can be reduced by chitosan sublethal concentrations (36, 37).

Ong et al. (2017) evaluated the role of chitosan-propolis nanoparticles for their antimicrobial and anti-biofilm properties against E. faecalis. With this formulation, E. faecalis inhibited biofilm formation and it was concluded that it reduced the number of bacteria in the biofilm. They reported that the formulation not only reduced bacterial numbers, but also physically disrupted the biofilm structure, as observed by scanning electron microscopy. It was determined that exposure of biofilms to formulation containing chitosan-propolis nanoparticles changed the expression of biofilm-related genes in E. faecalis. As a result, it is thought that chitosan-propolis nanoformulation can be considered as a potential anti-biofilm agent in resisting infections involving biofilm formation such as chronic wounds and surgical site infections (38).

Fluoride

Fluoride is widely used in the prevention of dental caries. The effect of fluorine on preventing tooth decay occurs in different ways. Fluor prevents demineralization of enamel as a result of acid attacks and provides remineralization. In addition, it inhibits bacterial metabolism, prevents acid production and increases plaque pH (7, 39). Fluor ions replace the hydroxyl (OH^-) group in apatite crystals, forming fluorapatite crystals with lower solubility and more resistance to caries. In topical fluorine applications, CaF_2, a loosely bound fluorine compound, is formed on the enamel surface. CaF_2, which accumulates in tissues such as plaque and saliva, helps remineralization by providing fluorine ions in case of enamel demineralization (40, 41).

It is known that the acid that emerges during the activities of cariogenic bacteria lowers the oral pH below the critical level of 5.5, and then the demineralization process begins. Initially, it was stated that this process is

reversible. If there is an F- ion in the plaque fluid, it has been reported that this ion participates in the crystal structure of the enamel and prevents the dissolution of Ca_2^+ and $PO4^{-3}$ from the tooth enamel. It is emphasized that when the pH rises above the critical level, F^- ions join the HAP structure and form a more durable crystal, fluoroapatite. In this way, it was stated that remineralization took place. At the same time, it is stated that F^- ions contribute to the prevention of demineralization indirectly by disrupting the microbial cell physiology (42).

High to moderate evidence has been reported that fluoride technologies and fissure sealants are primarily effective in the prevention of dental caries. In terms of secondary caries formation, the evidence has been shown to be weaker. It has been reported that the role of fluoride in caries prevention is undisputed today. It has been announced that fluoride is the only compound accepted as a caries preventative by the Food and Drug Administration (FDA) (43).

Fluoride Application Methods

Systemic Fluoride Applications

It is known that systemic fluoride applications have been used around the world for many years. Although systemic fluoride applications have decreased with the widespread use of fluoride toothpastes in recent years, the use of systemic fluoride supplements is still recommended by the European Academy of Pediatric Dentistry (EAPD) (2009). Accordingly, it has been emphasized that the use of systemic fluoride supplements should be considered if the daily intake of fluoride with drinking water is less than one unit per 0.6 million (ppm) (44).

Fluoridation of Drinking Water

Fluoridation of drinking water; It is expressed as bringing fluoride, which is naturally present in waters, to an optimal level for dental health. Fluoridation of water has been reported as an effective method for caries prevention (44).

Fluoridation of Milk

The EAPD shows that fluoridated milk has caries-reducing activity, according to evidence-based systematic reviews on milk fluoridation in 2009. It has been stated that the optimal fluoride concentration in milk should be 2.5-5.0 ppm (44).

When the effectiveness of different concentrations of fluoridated milk on enamel remineralization was investigated, it was found that milk containing 1.0 ppm fluoride was clearly effective in remineralization. It was found that the remineralization efficiency increased up to 5.0 ppm (45).

Fluoridation of Salt

It has been reported that fluoridated salt is used in more than 30 countries in the world and such use of systemic fluoride is recommended by the World Health Organization (WHO). It is stated that the salt is fluoridated to contain 250 milligrams (mg) of fluoride per kilogram. Although fluoridation of salt has a caries-preventing effect, it should be noted that children in the younger age group may not be able to benefit from this effect sufficiently because they consume less salt (44).

Fluoride Tablets and Drops

The American Academy of Pediatric Dentistry (AAPD) (2012) stated that the use of fluoride tablets should be considered for children older than 6 months whose daily fluoride intake dose is below 0.6 ppm. The daily fluoride intake dose of the child should be determined by taking into account the drinking water and diet; Accordingly, it was emphasized that it is necessary to calculate the required fluoride tablet dose. It has been stated that it is recommended that the child chew or suck the tablets in order to obtain a topical effect in addition to the systemic effect (46). In 2009, EAPD stated that if the daily amount of fluoride taken is between 0.3 and 0.6 mg, there will be no need for additional fluoride tablet/drop support for the use of fluoride toothpaste in the 2-3 age group. It has been reported that 0.25 mg fluoride support can be used in higher age groups (44).

Topical Fluoride Applications

In 2012, AAPD stated that before deciding on the frequency of professional topical fluoride application, it is necessary to determine which risk group the patient is in. Every 6 months for patients in the intermediate risk group; it has been reported that topical fluoride application is required every 3-6 months for patients in the high-risk group (46).

Fluoride Varnishes, Gels and Mouthwashes

The effectiveness of fluoride gels and mouthwashes on primary teeth is not certain, but they have a caries preventive effect on permanent teeth; It has been emphasized that gels and mouthwashes should not be used in children younger than 6 years of age due to the risk of swallowing. Fluoride varnishes, on the other hand, have caries-preventing effects on primary and permanent teeth; It has been reported that it can also be used in children younger than 6 years old (44).

Fluoride Toothpastes

EAPD stated in 2009 that one of the main reasons for the significant decrease in dental caries rates in recent years is the use of fluoride toothpastes. Use of fluoride toothpaste; It is an ideal public health method

in terms of ease of use, prevalence, cheapness and traditionality. One of the harms of using fluoride toothpaste is the risk of swallowing by young children. Children under 3 years of age should be careful in this respect. Parents should be informed that only a pea-sized amount of paste should be used and the child should be accompanied while brushing teeth until at least seven years of age. In order to prevent a possible risk, the use of paste containing less fluoride in children may be considered, but it has been reported that the paste must contain at least 500 ppm fluoride in order to have anti-caries effectiveness (44).

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Chapter 3

DIURETICS: DOPING AGENTS[1]

Anıl KARAAĞAÇ
Durişehvar ÖZER ÜNAL

1 Anıl Karaağaç, Durişehvar Özer Ünal (Orcid No: 0000-0003-0754-1240)

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Doping is defined as "the conscious or unconscious use of substances or methods prohibited by the World Anti-Doping Agency (WADA) by the athlete in order to increase the physical and mental performance of the athlete during the competition". Doping is against the ethics of sports, as it lays the groundwork for unfair competition, impairs the health of athletes for a short and long term, and even causes possible death risks (1).

According to WADA, committing one or more of the following rule violations is considered doping:

• Detection of a prohibited substance or its metabolites or markers in the sample taken from the athlete

• Use or attempted use of a prohibited substance or a prohibited method by athletes

• Avoiding, refusing to give an example or not giving an example

• A combination of three missed test and/or reporting misconduct actions by an athlete in a registered control pool within a twelve-month period.

• Violating or attempting to circumvent any part of doping control, cheating or attempting to cheat

• Possession of a prohibited substance or a prohibited method

• Engaging in or attempting to illegally trade any prohibited substance or prohibited method

• Administering or attempting to administer any prohibited substance or prohibited method to any athlete in a competition, or administering or attempting to administer any prohibited substance or prohibited method outside the competition to any athlete during a non-competition period.

• Conspiracy or attempted complicity by an athlete or other person

• Prohibited cooperation by an athlete or other person

• Actions taken by an athlete or other person with the aim of discouraging or harming a person who wants to inform the authorities (2).

Why Doping Is Needed?

Physician Robert Goldman posed a question to top athletes in 1984: Would you use a drug for sporting success (for example, winning an Olympic medal) that would not be detected in doping tests but would cause your death within five years? More than half of the athletes said "yes". More recently, Julian Woolf, who works in the field of kinesiology, which studies human movement, at the University of Windsor in Canada, argues that this picture has changed. The study, in which Woolf, together with

Jason Mazanov and James Connor, asked 212 athletes, shows that this rate has decreased (3).

There are multiple reasons for the need for doping, they vary from athlete to athlete and from situation to situation. E.g;

• Some athletes resort to doping because they feel pressured to do their best and can't see any other way.

• Athletes resorting to doping to accelerate the healing process of injury and long-term physical restraints.

• Not knowing exactly which substances and methods are on WADA's banned list and this ignorance, for example; may result in a positive doping test through malnutrition or food supplements

• Some athletes do not accept their natural limits and resort to doping to improve their performance.

• Seeing doping as the only solution after a failure (4)

• Doping for the purpose of masking other doping agents (5)

History of Doping

The word doping derives from an alcoholic beverage called "dope", which the Bantous natives of South Africa believed to increase stamina. Later, the term was given to a drink developed by the Boers, the whites of Dutch origin, and brought to England. It was recorded in the English literature as "Doping" in 1889 (6).

The history of doping use goes back to before Christ. B.C. In the 7th century, it was reported that the Romans gave their horses a drink called hydromel, a mixture of honey and water, in chariot races, to make the horses run faster. B.C. It is known that in the 3rd century, Greek athletes used special diets and stimulants to increase their performance. While doping use of canal swimmers was reported in Amsterdam in 1865, it was reported that large amounts of drugs were used in the bicycle races held in 1869, and the death of cyclist Arthur Linton in 1879 as a result of drug use during the competition drew attention to performance-enhancing drugs. In the early 1900s, the use of alcohol, strychnine mixtures, and the use of caffeine and cocaine before competitions increased. Upon the increase in the use of such substances, the International Amateur Athletic Federation banned the use of doping substances in the field of sports in 1928, and it was the first federation to put a ban in this field. The introduction of amphetamine in the 1920s and the introduction of synthetic hormones in the 1930s led to the widespread use of these substances in sports in the 1940s and 1950s. Anabolic-androgenic drugs were widely used in 1950-1970. The death of Danish cyclist Knud Enemark Jensen as a result of a

heart attack during the 1960 Rome Olympic Games gave rise to the idea of doing a doping test for athletes. In 1962, the International Olympic Committee (IOC) started to take measures against doping in sports. In 1963, it was decided to establish a commission to fight doping in the Council of Europe. This commission organized the first doping control for cyclists at the 1964 Tokyo Olympics. The first official doping control was carried out at the 1968 Mexico Olympics. In 1986, it was decided to carry out doping controls outside the competition. In 1988, diuretics were included in the list of prohibited drugs. Until 1999, anti-doping work was organized by the IOC Health Commission. In order for anti-doping organizations to continue their work in a more organized and widespread manner, under the leadership of the IOC, the first "World Doping Conference" was convened in February 1999 and the independent "World Anti-Doping Agency" was established in order to prevent doping practices that seriously threaten the health of athletes. (November 10, 1999 - Lausanne) (6).

WADA has provided for the implementation of out-of-competition doping testing rather than competition. Prohibited substances and methods on the doping list are determined by WADA and published in lists each year (6).

Prohibited Substances and Methods in Doping

Prohibited Substances and Methods at all times

Prohibited Substances

• S0: Unapproved substances

• S1: Anabolic substances

• S2: Peptide hormones, growth factors, related substances and mimetics

• S3: Beta-2 agonists

• S4: Hormone and metabolic modulators

• S5: Diuretics and masking agents

Prohibited Methods

• M1: Administration of blood and blood products

• M2: Chemical and physical intervention

• M3: Gene and cell doping

Substances and Methods Prohibited for Use in Competition

• S6: Stimulants

• S7: Narcotics

• S8: Cannabinoids

• S9: Glucocorticoids

Substances Prohibited for Use in Certain Special Sports

• P1: Beta blockers (7)

• Diuretics

Diuretics are therapeutic agents that increase urinary sodium and water excretion to adjust the volume and composition of body fluids or to remove excess fluid from tissues. Although the main pathways of diuretics to increase urine volume are to increase the excretion of salt and water, their effects are not limited to sodium and chloride; they may also affect the renal absorption and excretion of other cations (K+, H+, Ca+2 and Mg+2), anions (HCO3− and H2PO4−), and uric acid. There are two main reasons why diuretics are used by athletes. First, they can provide rapid weight loss due to increasing water excretion from the body and are used to meet the required weight categories in some sports (such as boxing, wrestling). The second is that they are used as masking agents by reducing the concentration of other doping agents in the urine because they increase the volume of urine (8). The nephron is the unit that produces urine in the kidneys. Nephron (Fig. 2.1); It consists of the glomerulus, proximal tubule, loop of Henle, distal tubule, and urine collecting duct (9). Duretics are divided into 4 according to their sites of action (Figure 2.1):

1. Those acting primarily on the proximal tubule

• Carbonic anhydrase inhibitors (acetazolamide, diclofenamide, methazolamide)

2. Those acting primarily on the loop of Henle

• Furosemide, bumetanide, ethacrynic acid, torsemide, azosemide, pyretanide and trypamide

• Osmotic diuretics (glycerine, isosorbide, mannitol and urea)

3. Those acting primarily on the distal tubule

• Thiazide and thiazide diuretics

• Chlortalidone, indapamide, metolazone and kinetazone

4. Those acting primarily on the urine collection duct

• Renal epithelial Na+ channels inhibitors (amiloride, triamterene)

• Aldosterone antagonists (spironolactone, canrenone, potassium canrenoate and eplerenone) (8)

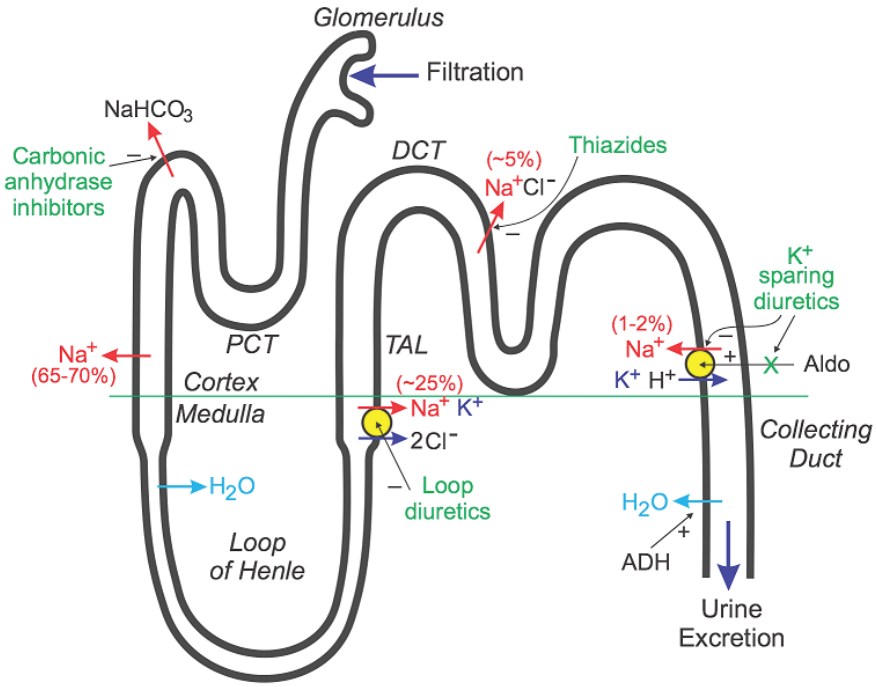
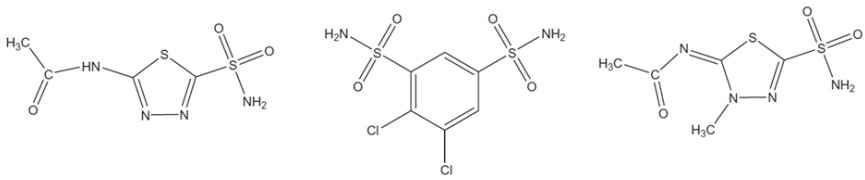


Figure 2.1: Sites of action and mechanisms of diuretics (10)

Carbonic Anhydrase Inhibitors

Carbonic anhydrase (CA) inhibitors (Figure 2.2) block the Na+/H+ exchange as a result of inhibition of the CA enzyme in the proximal tubule and act by increasing sodium excretion (Figure 2.1). They show weak natriuretic and diuretic effects. There are 3 main CA inhibitors with a diuretic effect, and they are acetazolamide (the prototype of the class, a sulfonamide without antibacterial activity), diclofenamide, and methazolamide. All show 100% oral bioavailability with a half-life of 6-14 hours. Acetazolamide and diclofenamide are excreted by the kidneys in an unmetabolized form, while methazolamide is extensively metabolized. The main therapeutic indication for CA inhibitors is open-angle glaucoma. Acetazolamide is used in altitude sickness and in the treatment of edema. CA is present in many extrarenal tissues, including the eye, gastric mucosa, pancreas, central nervous system, and erythrocytes. CA inhibitors have no diuretic use due to the distribution of carbonic anhydrase in the body and the weak diuretic effect of CA inhibitors. They are used to reduce aqueous humor secretion in glaucoma. Most side effects, contraindications, and drug interactions are the result of urinary alkalinization or metabolic acidosis. Infrequent side effects are similar to those of sulfonamides. The side effects of CA inhibitors include the passage of ammonia formed in the kidney from the urine into the systemic circulation, formation of kidney

stones, worsening of metabolic or respiratory acidosis, and decreased urinary excretion rate of weak organic bases (8). Acetazolamide constitutes 2% of the positive diuretic tests (13 positive tests) in 2019 (11).



Acetazolamide *Diclofenamide* *Methazolamide*

Figure 2.2: Chemical structures of carbonic anhydrase inhibitors (8)

Na+/K+/2Cl– Cotransport Inhibitors (Loop Diuretics)

Na+/K+/2Cl– cotransport inhibitors (loop diuretics) (Figure 2.3) are a class of highly potent and short-acting diuretics that bind to the Cl– binding site in the transmembrane domain of the Na+/K+/2Cl– cotransport in the ascending limb of the loop of Henle (Figure 2.1). Blocking this cotransporter causes a significant decrease in the kidney's ability to concentrate urine, resulting in a significant increase in urinary excretion of Na+ and Cl-. Apart from this, there is a significant increase in Ca+2, Mg+2 and K+ excretion. While uric acid excretion increases with acute administration, this situation is reversed with chronic administration. Loop diuretics are furosemide, bumetanide, ethacrynic acid, torsemide, azosemide, pyretanide, and trypamide (8).

Loop diuretics are at least 90% bound to plasma proteins. They are rapidly and extensively absorbed from the gastrointestinal tract (65-90%), but have a very short half-life (less than 1 hour for bumetanide and pyretanide and a maximum of 3.5 hours for torsemide). They undergo partial metabolism (hepatic glucuronidation for bumetanide and torsemide, renal glucuronidation for others) and are excreted by the kidneys in an intact form. Due to their sulfonamide-based nature, some loop diuretics have weak CA-inhibitory activity, which enhances their diuretic effect. The main indication for loop diuretics is the treatment of acute pulmonary edema.

It is also indicated in the treatment of chronic congestive heart failure, treatment of hypertension, edema of cirrhosis and edema of nephrotic syndrome. All of its side effects are associated with fluid and electrolyte imbalance (hyponatremia, extracellular fluid volume reduction, hypochloremic alkalosis, hypokalemia, hypomagnesemia, hyperuricemia, and hyperglycemia). They also increase plasma levels of low-density lipoprotein (LDL) and triglycerides, while lowering high-density

lipoprotein (HDL) plasma levels. Ethacrynic acid can cause ototoxicity. This class of diuretics has many drug-drug interactions, including aminoglycosides, anticoagulants, digitalis glycosides, lithium, propanolol, sulfonylureas, cisplatin, probenecid, and amphotericin B (8). Furosemide is contraindicated in people with gout and diabetes (9). Furosemide accounts for 29% (199 positive tests) of positive diuretic tests in 2019 (11).

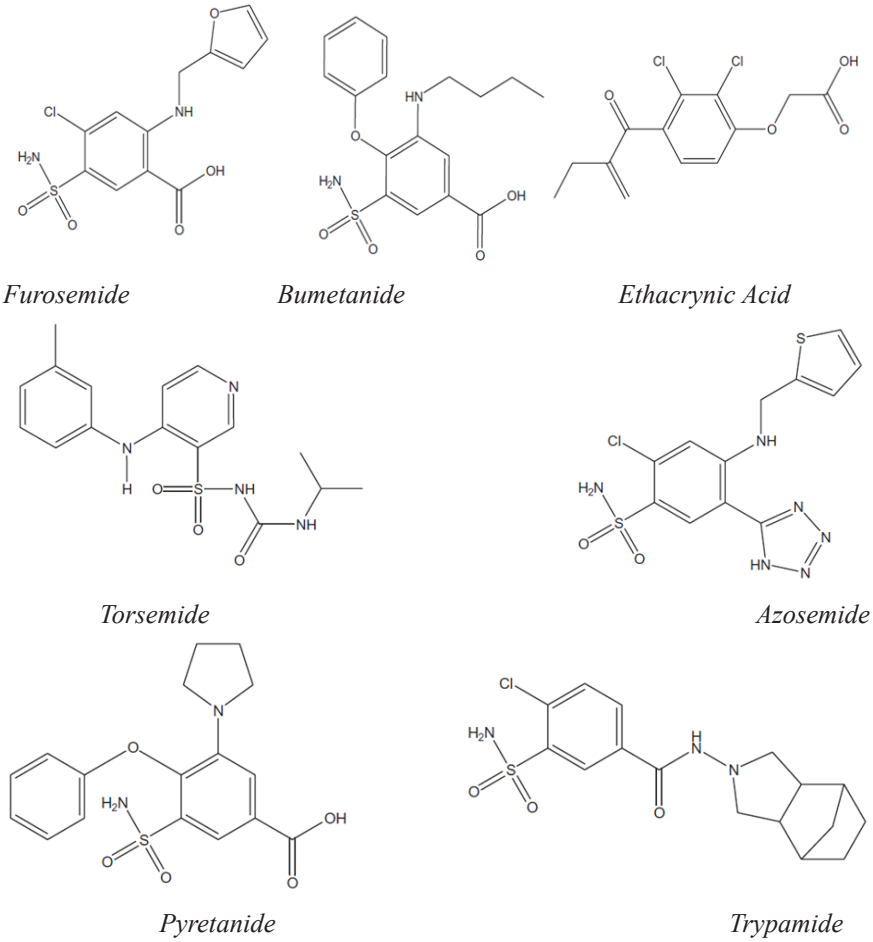


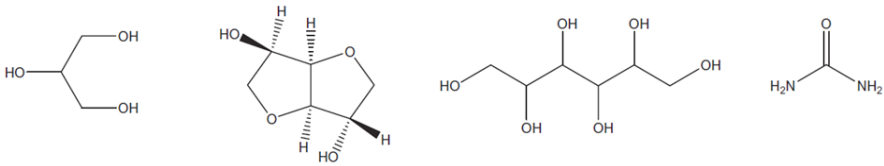
Figure 2.3: Chemical structures of Na+/K+/2Cl– cotransport inhibitors (8)

Osmotic Diuretics

Osmotic diuretics (Figure 2.4) are a class of low molecular weight compounds that cannot be metabolized. Only glycerin, isosorbide, mannitol and urea are included in this class. They significantly increase the osmolality of plasma and tubular fluid, resulting in reduced water reabsorption in the distal tubule and urinary collecting duct. This increases urine osmolality. Osmotic diuretics act in both the proximal tubule and

loop of Henle, with the loop of Henle being the primary site of action (Fig. 2.1). Osmotic diuretics draw water from the cell, expand the extracellular fluid volume, reduce blood viscosity, and inhibit renin release. This causes an increase in the urinary excretion of all electrolytes (Na+, K+, Ca+2, Mg+2, Cl–, HCO3−, PO43−). Its uses are limited to well-defined clinical situations, for example, mannitol is used to reduce cerebral edema before and after neurosurgery and as a kidney protectant in acute tubular necrosis (8).

Osmotic diuretics are used to control intraocular pressure during acute glaucoma attacks and in ocular surgery, as they provide water excretion from the eye and brain. Osmotic diuretics can cause dehydration, hyponatremia (headache, nausea and vomiting) due to electrolyte loss and water loss. Hyperglycemia may occur as a result of glycerin metabolism (8).



Glycerin *İsosorbide* *Mannitol* *Urea*

Figure 2.4: Chemical structures of osmotic diuretics (8)

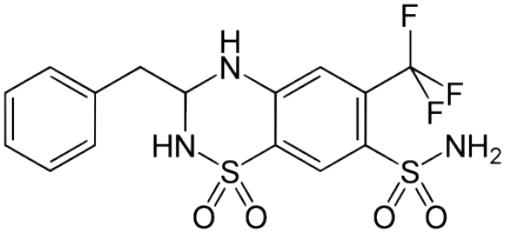
Na+/ Cl– Cotransport Inhibitors (Thiazide and Thiazide Diuretics)

Na+/Cl- cotransport inhibitors (Figure 2.5) have an optimal diuretic effect in the distal tubule and less diuretic effect in the proximal tubule. Some thiazide diuretics are also weak CA inhibitors. They reduce Na+ absorption. Some of the drugs in this class are: bendroflumetazide, chlorothiazide, hydrochlorothiazide, hydroflumethiazide, methyslotiazide, polythiazide, trichlormethiazide, chlorthalidone, indapamide, metolazone and kinetazone. In general, all show good bioavailability after oral administration (100% for bendroflumetazide and polythiazide, at least 50% for hydroflumethiazide and others). They are partially metabolized and partially excreted by the kidneys as intact drugs. Binding to plasma proteins varies within the class. They show a wide half-life range of 1.5 hours for chlorothiazide and 50 hours for chlorthalidone. While this class of diuretics is expected to greatly increase the excretion of Na+ and Cl-, this effect is moderate because approximately 90% of filtered sodium is reabsorbed before reaching the distal tubule (Fig. 2.1). As with loop diuretics, Na+/ Cl- cotransport inhibitors affect K+ and uric acid excretion by the same mechanisms; K+ excretion increases markedly after administration and uric acid excretion increases after acute administration but decreases with

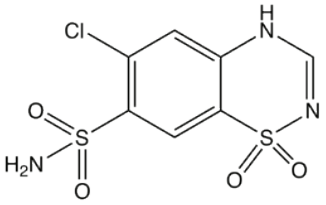
chronic administration. Unlike loop diuretics, they reduce Ca+2 excretion. Thiazide diuretics are the most widely used diuretics. They are used as first-line therapy for hypertension, alone or in combination with other antihypertensive drugs. They are also used in the treatment of edema due to heart, liver and kidney diseases. Thiazide diuretics are frequently used because of their low cost, high tolerance, good compliance (once daily administration), few contraindications, and proven benefits in reducing cardiovascular morbidity and mortality (8).

Most of its side effects are due to abnormalities in fluid and electrolyte balance and include: extracellular fluid volume reduction, hypotension, hypokalemia, hyponatremia, hypochloremia, metabolic alkalosis, hypomagnesemia, hypercalcemia, hyperuricemia, and hyperglycemia. They also increase plasma levels of LDL, total cholesterol and glycerides (8).

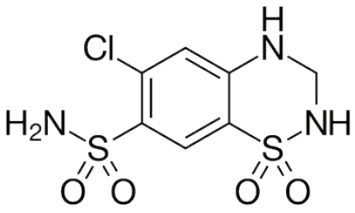
While drug-drug interactions of this group cause a decrease in the effect of anticoagulants, uricosuric agents, sulfonylureas and insulin; anesthetics increase their effects due to the synergy between them with diazoxide, digitalis glycosides, lithium, vitamin D and loop diuretics (8). Hydrochlorothiazide constitutes 21% (141 positive tests) of positive diuretic tests in 2019 (11).



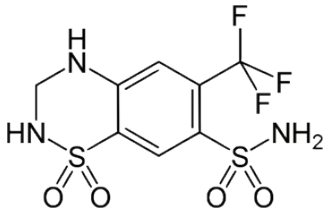
Bendroflumetazide



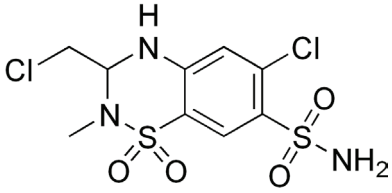
Chlorothiazide



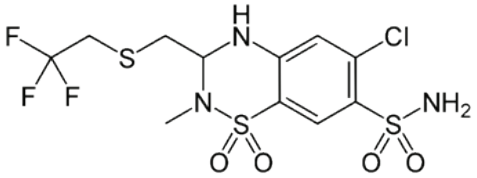
Hydrochlorothiazide



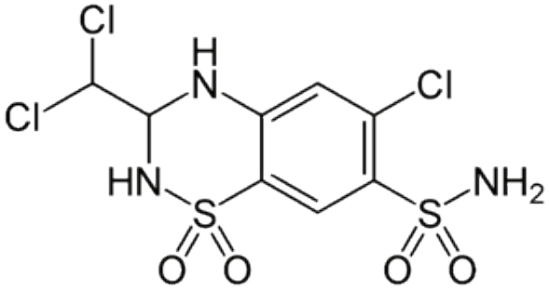
Hydroflumethiazide



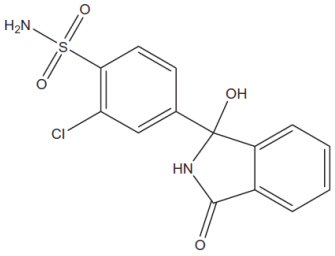
Methyslotiazide



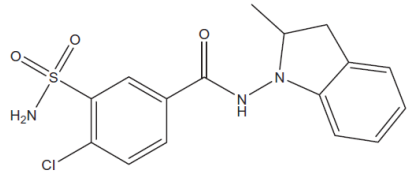
Polythiazide



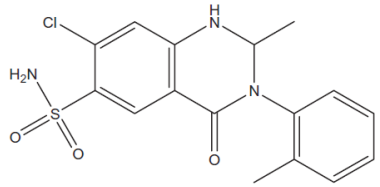
Trichlormethiazide



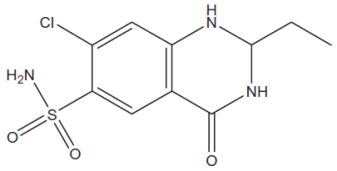
Chlorthalidone



Indapamide



Metolazone

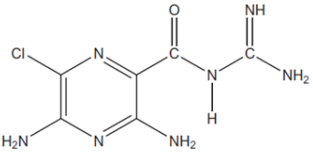


Kinetazone

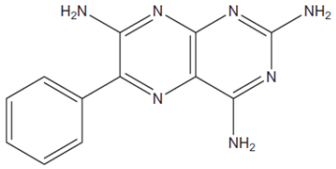
Figure 2.5: Chemical structures of thiazide and thiazide diuretics (12) and chlorthalidone, indapamide, metolazone and kinetazone (8)

Renal Epithelial Na+ Channels Inhibitors

Renal epithelial Na+ channels inhibitors (Figure 2.6) act by inhibiting Na+ reabsorption, K+ and H+ secretion in the distal tubule and urine collection duct (Figure 2.1). There are two drugs in this class with clinical use: amiloride and triamterene. Both drugs by themselves cause a moderate diuretic effect and a small increase in Na+ and Cl- excretion. They are often used in combination with other diuretics to reduce the caliuretic effects of other diuretics and to maintain potassium levels in patients at risk for hypokalemia. Its combination with thiazide group or loop diuretics enhances the diuretic and antihypertensive effect. This group varies widely in low oral bioavailability and half-life (more than 20 hours for amiloride and less than 5 hours for triamterene). The elimination pathway is predominantly renal for intact amiloride, while triamterene is extensively metabolized to active 4-hydroxytriamterene sulfate and excreted in the urine. The most common side effects of this group are nausea, vomiting, diarrhea, headache, leg cramps and dizziness. The most dangerous side effect is hyperkalemia. Triamterene may decrease glucose tolerance and cause photosensitivity (8). Of the positive diuretic tests in 2019, 4% (25 positive tests) were triamterene and 2% (14 positive tests) were amiloride (11).



Amiloride



Triamterene

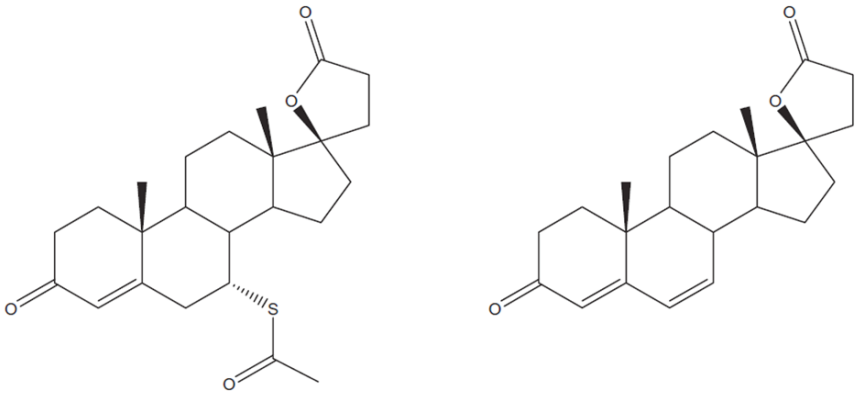
Figure 2.6: Chemical structures of renal epithelial Na+ channels inhibitors (8)

Aldosterone Antagonists (Mineralcorticoid Receptor Antagonists)

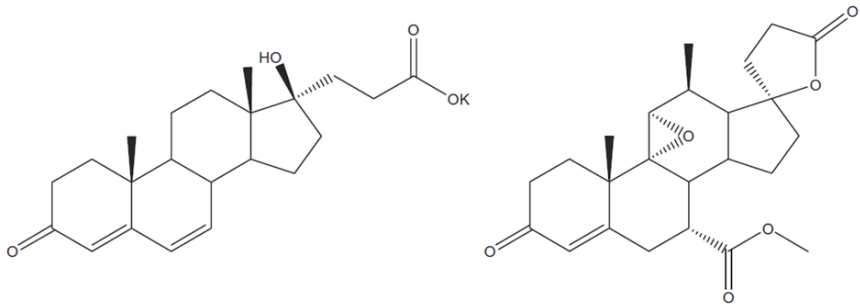
Aldesterone antagonists (Fig. 2.7) are competitive inhibitors of aldosterone (Fig. 2.1), which inhibit mineral corticoid receptors (MR)

located in the epithelial cells of the distal tubule and in the urinary collecting duct. The MR-antagonist complex inhibits the synthesis of aldosterone-induced proteins. Compounds belonging to this class are spironolactone, canrenone, potassium canrenoate, and eplerenone. The oral bioavailability of spironolactone, the prototype molecule of the class, is around 65%, it is extensively metabolized, enters the enterohepatic circulation, is highly bound to plasma proteins, and has a short half-life (about 1.6 hours) (8).

Canrenone is an active metabolite of spironolactone with a half-life of approximately 16.5 hours. Eplerenone has good oral bioavailability and is extensively metabolized. Aldosterone antagonists affect urinary excretion similarly to renal epithelial Na+ channel inhibitors (Fig. 2.1). This group of diuretics is very useful as an alternative to potassium replacement therapy. They are generally used in situations where aldosterone concentrations are high. They are used together with thiazide, loop or K+-sparing diuretics in the treatment of edema and hypertension. Spironolactone is useful in the treatment of primary hyperaldosteronism and refractory edema due to secondary aldosteronism. The most common side effect of MR antagonists is hyperkalemia. Spironolactone also has affinity for progesterone and androgen receptors due to its molecular structure, and may cause side effects such as gynecomastia, impotence and menstrual irregularities. Eplerenone, on the other hand, has very low affinity for progesterone and androgen receptors (<1% and <0.1%, respectively) than spironolactone due to its 9,11-epoxide group. In the case of chronic use of spironolactone, malignant tumors (especially breast cancer) can be induced. As drug-drug interactions, salicylates reduce tubular secretion of canrenone and reduce the diuretic efficacy of spironolactone. Spironolactone, on the other hand, changes the clearance of digitalis glycosides (8). Of the positive diuretic tests in 2019, 10% (69 positive tests) are canrenone and 3% (18 positive tests) are spironolactone (11).



Spironolactone *Canrenone*



Potassium Canrenoate Eplerenone

Figure 2.7: Chemical structures of aldesterone antagonists (8)

Doping and Diuretics

Diuretics are often used illegally in sports. Diuretics are prohibited in all sports, both out-of-competition and in-competition because they can provide rapid weight loss and can be used as masking agents (to hide the detection of other prohibited substances). Diuretics are often prescribed to treat hypertension and other cardiovascular disorders. According to WADA's international standard for therapeutic use exemptions (ISTUE), an athlete can obtain a therapeutic use exemption (TUE) approval by documenting the conditions that require the use of diuretics therapeutically (13). TUE is defined as the authorization to use substances or methods on the list of prohibited substances or methods for therapeutic purposes, when approved by a TUE committee based on a documented medical file prior to use of the substance in sports (8). Permissible therapeutic situations for diuretics are: Use of spironolactone for arterial hypertension and other cardiovascular disorders, polycystic ovarian syndrome, after kidney transplant due to end-stage renal disease (14), and use of spironolactone to reduce testosterone levels in trans female athletes (15). An athlete taking TUE for a diuretic; TUE should also be obtained for threshold substances such as formeterol, salbutamol, norpseudoephedrine, ephedrine, methylephedrine, and pseudoephedrine. If TUE is not obtained and detected for these substances, it is considered as an outlier analytical finding (7).

Although there is little evidence that diuretics improve athletic performance, their abuse is common among athletes seeking rapid weight loss (8). They are used to temporarily reduce body weight in sports with weight categories such as boxing, wrestling, taekwondo and judo, and around 20% of athletes use diuretics for this purpose. It is also used in

gymnastics, a sport without weight categories. Beta-blockers are in category P1 and are prohibited in some specific sports. It has been observed that the use of diuretics is high in shooting, which is one of these sports. Since it is known that diuretics do not make a positive contribution to the hit rate in this sport, it is thought that the reason for their use is to mask the use of beta-blockers (16). Skiers and mountaineers legitimately use acetazolamide to prevent altitude sickness. The diuretics most abused by athletes (furosemide and hydrochlorothiazide) have a short half-life and are therefore undetectable in the urine if samples are not collected within 24-48 hours after administration (8). Table 2.1 presents the statistics of positive diuretic findings from 2015 to the present.

Tablo 2.1: Statistics of positive diuretic findings analyzed by World Anti-Doping Agency (WADA) laboratories (17, 18, 19, 20, 11)

Year	Percentage of diuretics among positive findings	Total number of positive diuretic findings	Most common diuretic (number)	Second most common diuretic (number)
2015	12%	428	Furosemide (153)	Hydrochlorothiazide (125)
2016	12%	499	Hydrochlorothiazide (158)	Furosemide (131)
2017	15%	614	Furosemide (215)	Hydrochlorothiazide (143)
2018	14%	589	Furosemide (172)	Hydrochlorothiazide (127)
2019	16%	677	Furosemide (199)	Hydrochlorothiazide (141)

To evaluate the importance of diuretic use in weight loss, Caldwell et al. (21) compared the different effects of acute dehydration due to exercise, sauna and diuretic on weight change. Results showed a reduction of 2.3 ± 0.8 kg after exercise, 3.5 ± 0.8 kg after sauna, and 3.1 ± 0.8 kg after furosemide administration (8).

Diuretics affect potassium homeostasis during muscle exercise; decrease in intracellular potassium and the resting membrane potential of the cell. Except for potassium-sparing agents (renal epithelial Na+ channels inhibitors and aldosterone antagonists), all diuretics increase kaliuresis by accelerating the consumption of intracellular potassium. The resulting hypokalemia can lead to muscle cramps and cardiac arrhythmias due to electrolyte losses. Excessive use of potassium-sparing diuretics such as spironolactone, triamterene and amiloride can lead to hyperkalemia and

consequently expose athletes to arrhythmias. Most diuretics interfere with uric acid metabolism, which can cause an attack of gout, which can be very painful. Thiazide diuretics are derivatives of sulfonamides and may cause photosensitivity if exercised outdoors at noon. Diuretics are used to reduce weight and dilute urine, but as a result of dehydration, they greatly impair aerobic capacity and muscle strength and reduce metabolic efficiency. This results in a detrimental effect on general sport and exercise ability, and especially on athletic performance (8).

Analysis of Diuretics

Gas chromatography-mass spectrometry (GC-MS), high-performance liquid chromatography-mass spectrometry (HPLC-MS) and high-performance liquid chromatography-mass spectrometry/mass spectrometry (HPLC-MS/MS) are used in the analysis of diuretics for doping (8). WADA has determined the minimum required performance level (MRPL) required for prohibited substances in order to give accurate results in analyzes performed with different devices in different laboratories in the world and to equalize analytical performance between laboratories. The MRPL is the smallest concentration of a prohibited substance that analytical methods used in laboratories must be able to identify and detect with precision. For diuretics, this value is 200 ng/ml. Among all the methods described in the literature, mass spectrometry (MS) is the gold standard for analyzing doping agents in biological samples. Usually, MS is used as a detection method following a separation step such as liquid chromatography (LC) or gas chromatography (GC). These methods are very selective, sensitive and have low detection limits. However, direct sample examination is not possible; MS-based platforms require extensive sample preparation and cleaning steps, including analyte derivatization, extraction, and preconcentration. As noted in numerous studies, these preliminary analytical steps are often the most laborious, time consuming, generate large volumes of waste, and have the greatest source of error in chemical analysis (22).

Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS requires a series of pre-treatments to make samples suitable for analysis. Basically, the critical steps are the extraction of diuretics from the biological matrix and chemical derivatization to increase the volatility and thermal stability of the target compounds. Different methods have been developed for the detection of diuretics in urine using liquid/liquid (L/L) and solid phase extraction (SPE) procedures. With SPE, diuretics can be recovered with higher efficiency. While the use of disposable cartridges increases the overall cost of the pretreatment procedure, it is important to select effective SPE cartridges for analyzes from biological

material. L/L extraction is usually performed in more than one step. Derivatization is required prior to analysis as most diuretics are not sufficiently volatile, lipophilic, or thermally stable for these GC-MS systems. The most common derivatization procedures are silylation and methylation. Methylation is generally preferred as it allows for more stable derivatives in sufficient yield to obtain most diuretics. Methylation can be carried out statically (mixture of methyl iodide and acetone under thermal heating) or dynamically by extractive methylation or by methylation on the column (flash methylation). The best stationary phase for the analysis of diuretic compounds is phenylmethylsilicon, which allows all diuretics to separate within a reasonable time (<15 minutes). Much shorter times can be achieved with the latest generation of analytical columns based on fast electronics and fast GC systems in which mass spectrometric detection is successfully combined. Fast GC systems provide a 10-fold reduction in overall chromatographic analysis time. Electron pulsed ionization and MS detection are the most widely used methods. Mass spectra of methyl derivatives of diuretics have been defined by different researchers (8).

Liquid Chromatography-Mass Spectrometry (LC-MS)

LC-MS is an analytical method that combines the physical separation capabilities of high performance liquid chromatography (HPLC) with the mass analysis capabilities of mass spectrometry (MS). This system enabled the capabilities of both techniques to increase synergistically. LC separates multicomponent mixtures, while MS provides the structural identity of individual components with high molecular specificity and detection sensitivity. The LC-MS system includes an interface that efficiently transfers the separated components from the LC column to the MS ion source; because LC and MS devices are basically incompatible. While the mobile phase in an LC system is a pressurized liquid, MS analyzers generally operate under high vacuum. Therefore, it is not possible to directly pump the eluate from the LC column to the MS source. One of the most widely used LC-MS interfaces is electrospray ionization (ESI). Mass analyzers commonly used in LC-MS systems; quadrupole time-of-flight (TOF), hybrid quadrupole TOF (QTOF) and ion traps (23).

Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LC-MS/MS)

LC-MS/MS is an analytical method that synergistically combines the capabilities of the two systems, similar to LC-MS systems. Analytes separated in the LC unit are ionized and sent to the MS/MS unit. The first spectrometer (MS1) separates these ions according to their mass/charge (m/z) ratio. Ions with a certain m/z ratio from MS1 are selected and split into smaller fragmentation ions by various reactions, for example

induced dissociation, ion-molecule reaction or photodecomposition. These fragmentation ions are then introduced into the second mass spectrometer (MS2), separated and detected according to their m/z ratio. The fragmentation step makes it possible to detect and separate ions with very similar m/z ratios in normal mass spectrometers (24). While there may be many molecules with the same mass/charge ratio, the rate of molecules with the same fragmentation ions is 1/10000 in nature. Thanks to LC-MS/MS, it is possible to obtain results with high sensitivity and accuracy in the analysis of biological samples such as blood, urine and tissue (25).

Electrochemical Paper-Based Analytical Instruments (ePADs)

ePADs are an alternative to traditional portable analytical techniques due to their low cost, easy surface modification with different materials, and high sensitivity (26).

Paper Spray-Mass Spectrometry (PS-MS)

Ambient ionization techniques are analytical methods that allow MS analysis to be performed with minimal or no sample preparation, simplifying the analytical procedure and reducing equipment requirements and analysis time. As an ambient ionization technique, paper spray (PS) ionization combines sample collection, separation and ionization in a single step. In PS-MS, the untreated sample (e.g. raw urine) is deposited on a paper substrate cut into a triangular tip. A suitable solvent is added to the paper triangle containing the sample, followed by application of a high voltage direct current (~2-5 kV) to the wet paper, resulting in the formation of gas phase ions via an electrospray-like mechanism. PS offers several advantages:

(i) the liquid is transported by capillary action, so no external pumping is required;

(ii) nebulizer gas is also not needed, reducing instrumentation requirements;

(iii) low volume of sample and solvent is required;

(iv) paper is inexpensive and can be safely incinerated, reducing the risk of biosecurity hazards to the analyst (22).

Methods traditionally used for the detection of diuretics are dilute and draw (DnS) and extraction combined with liquid chromatography-high resolution mass spectrometry (LC-HRMS) or LC three quadrupole mass spectrometry. While DnS has the advantages of saving time and effort, problems such as matrix effect are encountered in this method. This makes it difficult to detect the presence of a prohibited substance according to existing regulations. While the matrix problem can be avoided by

extraction, this causes a waste of time. Görgens et al developed a two-dimensional LC-HRMS screening method incorporating diuretics. Thanks to online cleaning, they have minimized the matrix effect. Turbulent flow can be used to achieve a deeper clean using online SPE. The turbulent flow technique was developed by Quinn and Takarewski. A high flow rate (1-5 ml/min) is applied to load the samples into the capture column, which causes turbulent flow, calculated by the Reynolds number. Since diffusion of large molecules is slower than diffusion of smaller molecules, large barrier molecules (protein fragments) do not have time to enter the pores of the SPE column during this turbulent flow and are excreted during the capture step. Small molecules have time to diffuse into the pores and can be trapped in the conventional way. Wilde et al. developed a turbulent flow SPE (two-dimensional liquid chromatography, 2D-LC-MS/MS) method to enhance sample purification as a method for detecting diuretics in urine. The method has been validated against current guidelines. In the first step of the analysis, the sample is loaded onto the SPE column at high flow rate. Unwanted compounds flow to waste. Then the flow rate decreases and the valve positions change. The organic solvent in the loop, which was filled during the previous analysis and connected to the first valve, transfers the retained compounds from the SPE column to the analytical column, where the second dimension in the 2D-LC application begins. While chromatographic separation is running on the analytical column, the second valve is reversed so that the loop can recharge with organic solvent at a high flow rate without affecting the separation on the analytical column. After chromatographic separation, the valves are put into transfer mode to flush the entire system with organic solvent. In the final step, the valves rebalance and prepare the columns for the next analysis. A solvent with elucidation (methanol) is used to separate the compounds in the SPE column. Detection of diuretics was performed by ESI in selective reaction mode (SRM). A comparison was made between 2D and 1D analysis to see the benefit of applying 2D to reduce the matrix effect. For most compounds, the matrix effect was found to be smaller in the sample with the turbulent flow online SPE method. This can be explained by the loss of matrix in the first part of the 2D application. The combination of SPE and turbulent flow ensures that unwanted matrix compounds are kept out. The validation results show that 50 diuretics and masking agents can be detected in the urine, with LODs ranging from 1 to 20 ng/ml, values that are at least ten times better than MRPL. As a result, the turbulent flow online SPE LC-MS/MS method was developed and validated for the detection of 50 masking agents. The developed method has shown results to reduce the workload, time and matrix effect. This method can be used to confirm the presence of a masking agent in the urine, as the urine samples used meet WADA's identification criteria and several urines previously stated to be

positive can also be confirmed. The comparison between turbulent flow and conventional flow rate shows the benefits of turbulent flow. Reduced reagent use and reduced sample preparation time saves money and time with this method (29).

Doping has been a problem since people started playing sports competitively. Their use dating back to before Christ still continues today. The IOC began taking anti-doping measures in the 1960s. The establishment of WADA in 1999 took this challenge to the next level. Thanks to WADA, doping tests not only in competition but also out of competition have started to be done widely. Prohibited substances and methods on the doping list are updated by WADA every year. Diuretics are a class of drugs that differ in their structures, physicochemical properties, sites of action and mechanisms. It has many therapeutic uses, especially in hypertension. Although diuretics are known to negatively affect athlete performance, athletes continue to use this drug class as a doping agent. There are two main reasons why athletes use diuretics. The first is to meet the weight categories required in some sports as they can provide rapid weight loss, and the second is to be used as a masking agent by reducing the concentration of other doping agents in the urine. When diuretics were included in the list of prohibited substances in 1988, the first methods for their detection relied on a screening method based on HPLC. According to IOC/WADA requirements, procedures to confirm a positive case should be based on MS analysis. Therefore, after methylation of compounds in the 1990s, GC-MS was the technique of choice in most cases. In the late 1990s, changes occurred in the detection of diuretics in the field of doping, with the introduction of more robust, reliable, and affordable LC-MS equipment. LC-MS was sufficiently selective and sensitive compared to previous methods and made it possible to simplify sample preparation as the cleanliness of urine extracts is less critical compared to previous methods. Thanks to LC/MS, detection of diuretics in urine samples is no longer a problem. Among all the methods described in the literature, MS remains the gold standard for analyzing doping agents in biological samples. Future goals of diuretic analysis should be to develop more efficient and more economical methods. Reducing the analysis time and cost for laboratories, being able to detect the sensitivity of the methods and detecting more compounds at the same time will be positive developments in this direction. Because diuretics are used to mask the presence of other doping agents, the development of methods that combine the detection of diuretics with the detection of other prohibited substances will enable laboratories to work more efficiently in doping control.

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Chapter 4

CHILDHOOD OBESITY AND A NOVEL MEDIATOR HORMONE; ASPROSIN

Mehmet Akif OVALI[1]

1 Arş. Gör. Dr., Canakkale Onsekiz Mart University, Faculty of Medicine, Department of Physiology, Canakkale, maovali@comu.edu.tr, ORCID: 0000-0001-8740-6422

1. Obesity

World Health Organisation (WHO), defined the obesity as the state of excessive and abnormal fat accumulation in the body. In adults, obesity is basically measured by BMI (body mass index). Body mass index is calculated with the formula "BMI = Weight (kg) / Height squared (m^2)". According to this measure, humans with a BMI higher than 30 are defined as obese people. Adults with a BMI higher than 25 are refered as overweight adults (WHO, 2000). Obesity is a disease that has serious cultural and psychological dimensions, affects all ages and socioeconomic groups, and is frequently seen in childhood today.

Obesity is also accepted as a chronic, progressive disease with high mortality and morbidity on a global scale. Obesity causes some fatal systemic diseases by negatively affecting the quality and duration of life. It is known that the basis of obesity is an increase in body fat mass due to overeating. The most prominent health problem in the world in recent years and leading to preventable deaths together with smoking is obesity. The obesity incidence is constantly augmenting. According to the data of the WHO, the obesity prevalence has nearly tripled in the last 40 years worldwide (WHO, 2019).

Obesity triggers many diseases today. The most common and essetial ones are cardiovascular diseases, type 2 diabetes, hypertension, dyslipidemia, cerebrovascular diseases, respiratory diseases, cancers, musculoskeletal system diseases, liver and biliary tract diseases, urinary system diseases, psychological diseases and inflammations. Considering all these, obesity actually threatens our lives seriously (Turkoglu S., 2021).

Obesity criteria is defined by WHO and given in Table 1 (Khan A. et al., 2012).

Table 1. *Obesity Criteria*

WHO Classification	**BMI kg/m²**
Underweight	< 18.5
Normal range	18.5-24.9
Overweight	25-29.9
Obese	> 30
Class I	30.0- 34.9
Class II	35.0-39.9
Class III	> 40

2. Obesity in Childhood

Although obesity is generally considered to be an adult disease, the gradual increase in obesity, especially in children and adolescents, indicates that obesity in childhood is a global public health problem (WHO, 2016). Obesity, which is increasingly seen in childhood and adolescence, is becoming a threat to public health and the health system (Ebbeling C.B. et al., 2002). It has been reported that the most important negative outcomes of childhood obesity are dyslipidemia and hypertension, and it is known that this situation threatens lifespan of the children (I'Allemand D. Et al., 2008). An epidemiological study of 276.835 individuals showed that an increased BMI in children aged 7-13 years was related to coronary heart disease (CHD) in later life (Baker J.L. et al., 2007).

Obesity in childhood can lead to many metabolic and chronic diseases at early ages. In addition, the lack of self-confidence and adaptation problems that may occur in obese children which negatively affect the lives of children. A child with a healthy weight will not only feel good spiritually, but will also make positive progress in terms of personality development. In addition, the pressures of an obese child from friends and surroundings in his own age group constitute an important obstacle to the socialization of the child (Deleş B., 2019). Orthopedic problems, respiratory abnormalities and fatty liver diseases are the most found problems in obese children. Studies report that young people who do not pay attention to their diet, stay away from physical activity and live sedentary are more prone to the development of insulin resistance (Huang J. Et al., 2011).

According to a study conducted among 2nd grade primary school students in accordance with the WHO protocol within the scope of the Turkey Childhood Obesity Survey, the rate of being overweight in 2nd grade primary school children was 14.6% and the obesity rate was 9.9% (COSI-TUR, 2018). According to the analysis method based on age and gender, a BMI of 85-94% in people aged 2-20 indicates overweight and above 95% indicates obesity. Childhood obesity basically; It occurs when the calorie intake is more than the energy expended (Kleigman R. and Gahagan, S., 2016).

The main cause of obesity is not only genetic factors, but environmental factors are also thought to be important. Modern lifestyle, increased calorie intake and decreased physical activity are the most important environmental factors (Ludwig D.S., 2007). Therefore, sedentary lifestyle and decreased physical activity, which are increasingly seen in children, are considered to be among the leading causes of childhood obesity (Ness A.R. et al., 2007).

Especially community and family-based approaches are very important in the prevention of childhood obesity. Efforts should be made to ensure that children stay away from sugary drinks, and to feed them with healthy foods. In addition, regular sleep should be encouraged by increasing the duration of physical activity. In addition, making improvements to regulate the life style within the family has a very important place in preventing childhood obesity (Yılmazbas P. and Gokcay G., 2018).

3. Obesity and Diabetes Mellitus

3.1. Obesity and Type 1 Diabetes Mellitus

The increasing incidence of type 1 diabetes (T1DM) among children and adults has been closely associated with the obesity epidemic. Type 1 diabetes, defined as an autoimmune disease, continues to be investigated, but the main reason for the prevalence of type 1 diabetes, especially in young age groups, has not yet been fully elucidated (Arora S., 2014). Baum et al., 1975 conducted the first study focusing on the relationship between type 1 diabetes and weight gain. In their study, they reported that there is a relationship between high-calorie diet and hormone secretion irregularity (Baum J.D. et al., 1975). On the other hand, Wilkin revealed in 2001 that the strong relevance between body mass gain and type 1 diabetes with a hypothesis called the "accelerator hypothesis" (Wilkin T.J., 2001). As weight gain is higher in younger children, diabetes can be diagnosed earlier. This means that rapid weight gain also accelerates the process of insulin resistance, and the development of type 1 diabetes is much faster in individuals with an increased risk of developing diabetes in terms of genetic background (Al-Goblan, A.S. et al., 2014).

Obesity incidence has increased with the incidence of T1DM (Minges K.E. et al., 2013). Moreover, most young people with T1DM are obese when compared to their peers (Liu L.L. et al., 2010, Islam S.T. et al, 2014). While the prevalence of obesity in young people with T1DM is between 25-35%, it has been reported as approximately 80% in adults. Obesity increases the risk of developing T1DM and it is starting to be seen at early ages. In recent years, it has been defined that the risks of macrovascular diseases, retinopathy and metabolic syndrome increase in obese individuals with T1DM. Combined therapies for obesity and glucose and therapeutic targets for insulin resistance in individuals with T1DM are important areas of study in the treatment of obesity (Polsky, S. and Ellis, S. L. 2015).

3.2. Obesity and Type 2 Diabetes Mellitus

Type 2 diabetes mellitus (T2DM) is defined as insulin resistance, hyperglycemia, and beta cell dysfunction. Peripheral insulin resistance is

observed, and as a result of decreased insulin secretion, hyperglycemia is observed in patients over time. T2DM is a multifactorial disease, especially including high-calorie diet and obesity. The most notable condition is obesity. On the other hand, T2DM observed in young people is closely related to many diseases, including metabolic syndrome and polycystic ovarian disease, as well as insulin resistance (Arslanian S.A., 2000, Rosenbloom A.L. et al., 2009).

Obesity is closely linked to many medical and psychological problems such as type 2 diabetes. It is estimated that 171 million people have type 2 diabetes today, and this situation is expected to increase to 360 million by 2030. Obesity is closely related to diabetes and resistance in insülin secretion. The pancreatic β-cells of the islet of Langerhans play a role in maintaining normal glucose levels by secreting sufficient insulin to overcome the declines in insulin levels in healthy people (Røder M.E. et al., 1998). But this situation is quite complicated for people with type 2 diabetes.

As a summary, diabetes and obesity are chronic diseases with an increasing incidence worldwide. It is known that an increase in body mass index above normal is closely related to diabetes and insulin resistance. As an outcome of the deterioration of β-cell function in an obese individual, insulin resistance and diabetes develop together. Being overweight at an early age is also closely related to the development of type 1 diabetes. It is very important to develop new approaches to keep diabetes under control and prevent it in obese individuals (Al-Goblan A.S. et al., 2014). The causes and the outcomes of obesity is summarised in figure 1.

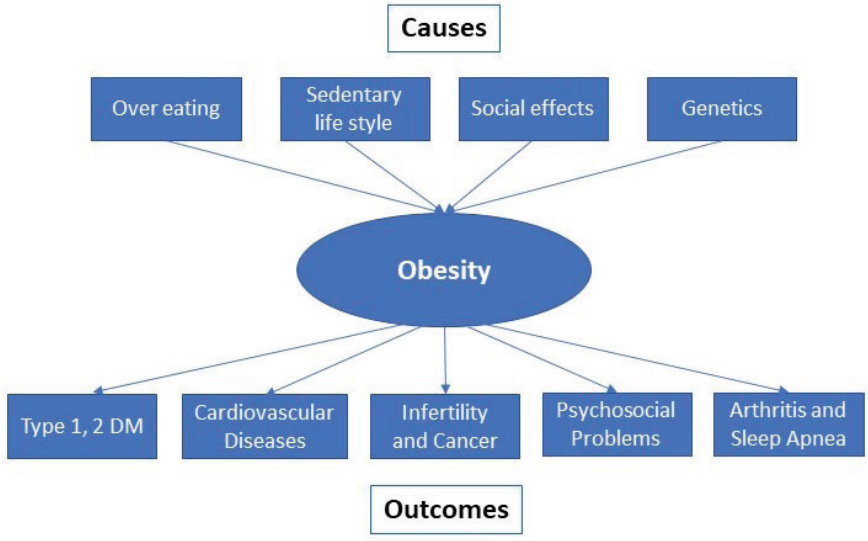

Figure 1. *Causes and outcome of obesity*

4. Asprosin; as a Novel Mediator in Obesity

Asprosin is defined as a fasting-responsive hormone which causes hepatic glucose secretion in the liver. Asprosin, is a C-terminal cleavage product of profibrilin and it's firstly identified by Romere and colleagues in neonatal progeroid syndrome patients in 2016. Patients with this syndrome are generally characterized by congenital lipodystrophy and a progeroid appearance (Romere C. et al., 2016).

Asprosin hormone is released from white adipose tissue, circulates in the blood and is taken up to the liver, and causes rapid glucose release into the circulation by activating the G protein-cAMP-PKA pathway. Humans and mice with insulin resistance show pathologically high plasma asprosin levels. Detoriated in the function of asprosin by immunological or genetic interventions has a strong glucose and insulin-lowering effect due to decreased hepatic glucose secretion. Asprosin is pathologically increased in human and mouse with insulin resistance. On the other hand, reduction of asprosin has a protective role from hyperinsulinization caused by metabolic syndrome (Duerrschmid C. et al., 2017, Romere C. et al., 2016). Mechanism of action of asprosin is summarised in Figure 2.

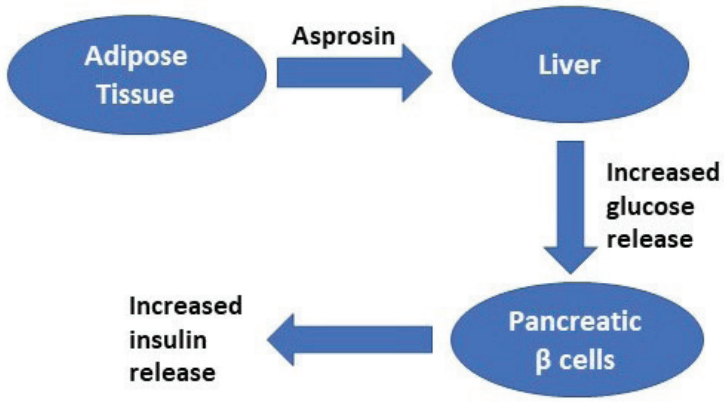

Figure 2. *Illustration of asprosin action mechanism*

It has been reported that asprosin is also an orexigenic hormone and stimulates the increase in food consumption and body weight gain (Duerrschmid C. et al., 2017). Secreted asprosin reaches to the liver and triggers glucose release in response to low dietary glucose. In response to insulin, the liver stocks excess glucose in glycogen form following by the meal. In fasting, the liver is induced to break down the stored glycogen and secrete glucose, as well as synthesizing new glucose. The secreted

glucose into the circulation so that the brain and other organs that utilize from the glucose can maintain their normal function. Glycogenolysis and gluconeogenesis are stimulated by hormones such as glucagon, which induce the cyclic AMP pathway in liver, and cAMP supports the activation of metabolic process that lead to glucose production and secretion; asprosin seems to use the same control system (Levine R., 1986, Röder P.V. et al., 2016).

One of the pathologies in which the hormone asprosin is most effective is obesity and metabolic syndrome. Metabolic syndrome affects 25% of the population in developed and underdeveloped countries and increases the risk of death due to cardiovascular diseases three times (Cardinali D.P. et al., 2011). The impaired glucose homeostasis seen in metabolic syndrome and obesity directs researchers to conduct new studies on this subject. For example, Wang et al. 2018 found in their study that there is a positive correlation between increased waist circumference, triglyceride level, HOMA-IR index and fasting plasma glucose, and increased asprosin level in those newly diagnosed with type 2 diabetes and people with impaired glucose regulation (Wang Y. Et al., 2018). Therefore, it is seen that there is a strong relationship between increased asprosin level, type 2 diabetes, and therefore metabolic syndrome and obesity.

Asprosin in the bloodstream crosses the blood-brain barrier and causes the activation of the orexigenic AgRP+ neurons using a pathway directly linked to cAMP. This situation stimulates appetite, resulting in an increase in fat and body weight (Duerrschmid C. et al., 2017).

Obesity and metabolic syndrome are closely related to the inflammation process due to inflammatory cytokines and acute phase proteins secreted from adipose tissue. Therefore, asprosin plays an important role in inflammation, cellular dysfunction and functions of pancreatic β cells. Lee et al. 2019 showed that asprosin is produced not only from adipocytes but also from pancreatic β cells, and they reported that asprosin increases β cell dysfunction (Lee T. et al., 2019).

5. Asprosin in Obese Children

Childhood obesity has become an increasingly important public health problem in worldwide. Although childhood obesity is more common in individuals with a family history of obesity, this situation continues in adulthood as well. Therefore, these children carry a significant risk in terms of diseases that may develop due to obesity in their later years (Kumar S., and Kelly, A.S., 2017, Whitaker R.C. et al., 1997, Juonala M. et al., 2011).

Obesity during childhood can be accepted as a source of life-span health problems. So that, possible factors in childhood obesity are worth investigating. Researchers found that there was no difference between boys and girls in terms of serum asprosin levels. According to the results obtained from the study, they found that serum asprosin levels increased in obese children compared to normal-weight children. So that, they declare that detecting increased levels of asprosin in obese individuals during childhood is more essetial for their health than adults. According to the research of Sunnetci Silistra, E., & Hatipoglu, H. U. (2020), the significantly higher asprosin level in obese children compared to normal-weight children indicates that asprosin may be an important biomarker for obesity. They also commented that asprosin may be a critical biomarker to avoid from the detrimental effects of obesity and short- and long-term deterioration in relation to it. In addition, it is thought that an agent that can reduce the harmful effects of asprosin may be useful in the treatment of asprosin-related disorders in obese individuals. (Sunnetci Silistre E. and Hatipoglu H.U., 2020).

Asprosin plays a pathogenetic role in the development of insulin resistance. This indicates that asprosin can be considered as an important biomarker in the onset of obesity and in the development of insulin resistance (Wang M. Et al.,2019). Romere et al. reported that in addition to pathologically high levels of asprosin in obese humans and mice, injection of asprosin stimulates hepatic gluconeogenesis and may cause diabetes development by increasing blood glucose levels abruptly (Romere C. et al., 2016). Asprosin has been shown to contribute to appetite stimulation in animal experiments, and inhibiting circulating asprosin with a monoclonal antibody reduced appetite and body weight in obese mice, as well as improving the glycemic index (Duerrschmid C. et al., 2017). However, more work is needed to clarify the underlying mechanism.

On the other hand, obesity is often related with metabolic diseases such as T2DM, cardiovascular disease, metabolic syndrome, and non-alcoholic fatty liver disease (NAFLD). Non-alcoholic fatty liver disease is a liver disease that is frequently observed in children and adolescents. The incidence of pediatric non-alcoholic fatty liver disease in obese children has been reported as 34.2% (Anderson E.L. et al., 2015). Non-alcoholic fatty liver disease is a very important disease, including hepatocellular carcinoma, which has become an increasing public health problem in children (Milić S. et al., 2014, Berardis S. and Sokal E., 2014).

There are few studies investigating serum asprosin levels in children and adolescents. It is known that level of asprosin in circulation is found higher in obese children once compared to normal weight children. In

addition, there is a positive correlation between asprosin and prominent obesity-related parameters such as BMI. Liu L et al., in their study in 2021, determined for the first time that serum asprosin levels were increased in obese children with non-alcoholic fatty liver disease. In addition, the researchers stated that serum asprosin level has a positive correlation with TNF-α and ALT. This reveals that asprosin is an important parameter in the pathological progression of NAFLD in children and adolescents. ALT levels in children with non-alcoholic fatty liver disease were statistically significant once compared to those without NAFLD. In addition, the increased serum asprosin level in adult patients with NAFLD indicates that it can be used as an important indication for the prediction of NAFLD development. Therefore, it is thought that there is a strong relationship between asprosin and NAFLD in obese children (Liu L.J. et al., 2021, Ke F. et al., 2020).

The molecular mechanism of the contribution of asprosin to NAFLD has not yet been fully elucidated. It is thought that an increase in asprosin in obese people increases hepatic glucose secretion and thus may cause insulin resistance (Romere C. et al., 2016). It is said that hyperinsulinemia and hyperglycemia induced by insulin resistance may also disrupt the lipid profile and stimulate hepatic steatosis (Asaoka Y. et al., 2013). It has also been reported that recombinant asprosin is associated with metabolic disorders (Lee T. et al., 2019).

Asprosin may has a role in the pathological process of NAFLD through TLR4. Therefore, it turns out that asprosin is highly related to NAFLD, but more molecular studies are needed to understand the mechanism of this relationship (Liu L. et al., 2021).

6. Conclusion and Future Perspective

Considering all these data, it is obviously seen that obesity is a global problem with an increasing incidence and serious health problems. In addition, sedentary lifestyle, which is increasingly common, is among the main causes of obesity, especially in children. It is stated that childhood obesity can be the source of life-threatening problems in advancing ages. Therefore, studies aimed at preventing childhood obesity and elucidating its molecular mechanisms in detail are of great importance. Research on the treatment of diabetes, which is one of the main negative outcomes of obesity, is very important in this regard. Due to its strong effect on glucose homeostasis, it is clear that the molecular mechanism of action of the hormone asprosin and its effect on childhood obesity should be investigated in detail. Recent studies also support this idea. In this sense, it is predicted that asprosin may be a potential agent for treatment, especially in childhood obesity.

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Chapter 5

THE EFFECTS OF ENDOCRINE DISORDERS ON THE MALE REPRODUCTIVE SYSTEM

Özay GÜLEŞ[1]

1 Asst. Prof. Department of Histology and Embryology, Faculty of Veterinary Medicine, Afyon Kocatepe University, Afyonkarahisar, Turkey, Orcid Id: 0000-0001-6170-1706

1. Introduction

Exposure to chemical products is increasing day by day as a result of the increase in the need for agricultural products and the increase in the consumption period of these products due to the increase in the world population. Endocrine disruptors are natural or synthetic chemicals that cause problems in animals or humans by impairing the endocrine system functions and development in the organism by affecting the production, release, binding, transport, activity, destruction, and excretion of hormones by imitating hormones (Lee, 2007; Durmaz and Giray, 2013). These substances affect the production, release, binding to receptors, transport, activity, destruction, and excretion of hormones responsible for hemostasis, reproduction, and development in the organism. (Yeşilkaya, 2008; Kabir et al., 2015; Özbek, 2019).

At the beginning of the twentieth century, most of the chemical substances which were about 1000, were of vegetable, animal, and mineral origin. Today the number of chemical substances surrounding our environment, mostly synthetic, has exceeded 100,000, and approximately 1,500 new chemical substances are added to the environment every year. Most of these compounds have been detected in the blood of adults, human breast milk, and cord blood of infants. Today, more than 3,000 chemicals are added to foods for purposes such as food preservatives and colorants. (Özbek, 2019).

When the effects of endocrine disruptors on the organism are examined, it has been determined that they affect the functions of the reproductive system and other systems through experimental studies. In this review, the effects of endocrine disruptors on the male reproductive system will be mentioned.

2. Effect Mechanisms of Endocrine Disruptive Chemicals

Endocrine-disrupting chemicals were previously known to affect nuclear hormone receptors, including estrogen receptors (ERs), androgen receptors (ARs), progesterone receptors, thyroid receptors (TRs), and retinoid receptors. However, as a result of the studies done so far, it has been revealed that it also exerts its effects through non-nuclear steroid hormone receptors (eg, membrane ERs), non-steroid receptors (eg, neurotransmitter receptors such as serotonin, dopamine, and norepinephrine receptors), orphan receptors (eg, aryl hydrocarbon receptor) and enzymatic pathways involved in steroid biosynthesis and metabolism (Diamanti-Kandarakis et al., 2009). Accordingly, endocrine disruptors exert their effects directly through the hormone-receptor complex or specific proteins that control the release of hormones.

Endocrine disruptors have a total or partial agonistic or antagonistic effect by binding to the hormone receptors. In some cases, an endocrine disruptor has an agonist in some isoforms of receptors and antagonistic in other isoforms. In addition, these substances affect their levels in the blood through acting the synthesis, transport, metabolism, and elimination of hormones (Gore et al., 2015; Kabir et al., 2015; Lauretta et al., 2019). Again, these substances adversely affect the morphologically and numerically fat cells by disrupting the endocrine regulation in the adipose tissue, thus it has been revealed that they impair energy metabolism and the feeling of satiety, and lead to obesity (Darbre, 2017; Lauretta et al., 2019). Endocrine disruptors show hereditary effects in various organ systems causing epigenetic changes and this effect is transmitted to the next generations. It is performed by methylation of cytosine residues in DNA, post-translational modification of histones, and changing microRNA expression (Gore et al., 2015).

3. Important Issues in Endocrine Disruption

3.1. Age of exposure

The effects of endocrine disruptors in the adult organism and the developing fetus or embryo may be different. A developing organism's environment is important for developing a disease or dysfunction in the same individual's adult stage by interacting with the individual's genes. Again, the development of disease or dysfunction in the individual also depends on the individual's exposure to endocrine disruptors from the fetal period to the early postnatal development period (Diamanti-Kandarakis et al., 2009). It is thought that exposure to these chemicals in the early postnatal period may be irreversible (Barker, 2004).

3.2. Latency from exposure

Diseases caused by endocrine disruptors may not occur during exposure to these chemicals. These diseases may be seen in the periods after exposure or in the old age period. The delay in the time elapsed between exposure to endocrine disruptors and onset of the disease creates a challenge in determining whether the disease is caused by endocrine disruptors (Diamanti-Kandarakis et al., 2009).

3.3. Adverse effects of endocrine disruptors may begin in the early development period and become permanent

Studies have shown that endocrine disruptors create a higher risk in the adult period than in the early development period. In addition, the studies conducted at the National Institute of Environmental Health Sciences (NIEHS) have shown that the negative consequences of endocrine disruptors may be transferred on to the next generations, even

if the organism is not directly exposed to endocrine disruptors. It has been revealed that this situation is caused by endocrine disruptors changing the gene function of the organism (National Institute of Environmental Health Sciences, 2018).

3.4. Importance of mixtures

Since environmental pollution does not contain a single endocrine-disrupting chemical, when the organism is exposed to an environmental pollutant, it is possible to be exposed to other chemicals (Diamanti-Kandarakis et al., 2009). If different endocrine disruptors are taken into the organism together, they may show a synergistic effect, or they may cause different effects. Endocrine disruptors (for example, estrogen, antiandrogenic agents, or thyroid disrupting agents) in the same group may have significant effects if taken together at low doses (Kortenkamp, 2007).

3.5. Low dose effect

In studies conducted by the Flemish Biomonitoring Campaign in 2007-2011, it was determined that exposure of the organism to cadmium and organochlorine pollutants even at very low doses causes significant changes in serum hormone level, sexual maturation, and physical development (Den et al., 2002). Apart from these studies, different studies are showing that endocrine disruptors can be effective at all doses, including low and high doses (Diamanti-Kandarakis et al., 2009).

3.6. Transgenerational, epigenetic effects

Endocrine disruptors can affect not only exposed individuals but also the next generations. This effect can occur by affecting the fetus/egg (Anway et al., 2005), DNA mutation, DNA methylation, or histone acetylation (Gluckman and Hanson, 2004; Anway et al., 2005).

4. Routes of Exposure to Endocrine Disruptors

Both humans and animals may be exposed to endocrine-disrupting chemicals from different sources. Dissemination of industrial waste materials by wastewater contaminates the environment with endocrine disruptors (Wuttke et al., 2010). Exposure to endocrine disruptors may occur from contaminated drinking water, soil, and air with substances such as pesticides, plastics, alkylphenols used in agriculture, industry, and households (Stumm-Zollinger and Fair, 1965). In addition, it can be exposed these substances by oral ingestion of contaminated food and water, skin contact, intravenously from phthalates used in intravenous materials, and biological transfer from the placenta and breast milk (Kabir et al., 2015).

5. Classification of Endocrine Disruptors and Their Effects on the Male Reproductive System

The European Union published a report in 2002 that there are 60 endocrine disruptors harmful to the environment and human health. Endocrine disruptors are divided into three groups: natural, synthetic and environmental (Durmaz and Giray, 2013).

5.1. Natural endocrine disruptors

These substances, which are in the structure of natural hormones such as estrogen and testosterone, have a very short half-life and are easily eliminated from the body without accumulating in the tissues. Foods such as soybeans, flax seeds, legumes, spinach, cabbage, apples, and strawberries are rich sources of phytoestrogens (Tabb and Blumberg, 2006; Lee, 2007). Phytoestrogens are classified as isoflavones, isoflavones, flavanones, chalcones, lignans, coumestans, macrolides, stilbenes, and sterols according to their chemical structures (Table 1) (Büyüktuncer and Başaran, 2005). Isoflavones (daidzein, genistein) obtained from soy and soy derivatives have an important place among phytoestrogens (Diamanti-Kandarakis et al., 2009). Phytoestrogens act like natural estrogens in the organism.

The consumption of these nutrients generally does not cause an adverse effect. However, intense and excessive intake of their causes harmful effects on the male reproductive system (Tabb and Blumberg, 2006; Lee, 2007).

Table 1. *Phytoestrogens (Büyüktuncer and Başaran, 2005)*

ISOFLAVONES	Daidzein, Genistein, Glysitein, Daidzin, Genistin, Glycitin
COUMESTANS	Coumestrol
ISOFLAVONES	Equol
FLAVANONES	Naringenin
LIGNANS	Enterolactone
CHALCONES	Xanthohumol
MACROLYES	Zeralenone
STYLBENES	Resveratrol
STEROLS	Cytoterole

Genistein and daidzein

Excessive consumption of soy phytoestrogens containing genistein and daidzein may cause infertility by affecting androgen hormone production, spermatogenesis, and spermatozoa capacity in the male reproductive system. In addition, it may cause abnormal changes in sex-specific physiology and behavior in the adult period by disrupting the

hormonal balance in the beginning of the prenatal and postnatal period (Sosic-Jurjevic et al., 2011).

Coumestans

Coumestans are structurally similar to isoflavones. Coumestrol and 4-methoxycoumestrol among the coumestans are the compounds with the most important estrogenic activity (Büyüktuncer and Başaran, 2005).

Equol

Equol is an estrogenic metabolite produced in vivo by the action of the intestinal microflora from soybean phytoestrogen. Exposure to Equol can cause significant biological effects in humans.

It has potential similar to genistein (Muthyala et al., 2004).

5.1.1. Experimental studies with phytoestrogens

The most studied phytoestrogens due to their effects are isoflavones. The number of studies on other phytoestrogens is few. Tanaka et al. (2009) determined that the use of soy isoflavones containing daidzein and genistein for three months in 28 volunteers decreased serum testosterone and dihydrotestosterone (DHT) levels and increased the level of sex hormone-binding globulin. As a result, they revealed that a diet of soy isoflavone would be beneficial in preventing the development of prostate cancer.

In another study, endocrine-disrupting mono-(2-ethylhexyl) phthalate (MEHP) administration to Sertoli cells obtained from 22-d-old male rats inhibited proliferation of Sertoli cells, and reduced superoxide dismutase (SOD), glutathione (GSH), and total antioxidant enzyme activity in Sertoli cells. But it increased the production of reactive oxygen radicals, the apoptotic index and the necrosis rate. It was determined that genistein (10 μmol/L) supplementation against MEHP-induced oxidative damage in Sertoli cells increased proliferation, SOD, GSH, and total antioxidant enzyme activity in Sertoli cells, and decreased apoptotic index, and necrosis rate. It has been concluded that isoflavone supplementation has protective effect against damage caused by phthalates in the prepubertal testes (Zhang et al., 2017).

Luo et al. (2019) showed that the oral administration of 150 and 450 mg/kg/day soy isoflavones for four weeks in obese rats increased testicular weight, seminiferous tubulus diameter, mean germ cell number in tubules, sperm density, sperm motility, plasma follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone levels, testicular SOD, catalase (CAT), and GSH activity, as well as antiapoptotic Bcl-2 expression level, and decreased testicular malondialdehyde (MDA) and

8-hydroxydeoxyguanosine (8-OHdG) levels. In the study, it was concluded that soy isoflavone administration has a healing effect against testicular damage caused by obesity in male rats.

Al-Maghrebi and Renno (2016) reported that genistein administration in rats with testicular ischemia-reperfusion model improved histologically impaired testicular structure, decreased caspase 3 and caspase 8 activity, terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin nick end labeling (TUNEL) positive apoptotic cell count, and increased total antioxidant capacity, SOD activity, and testicular Notch 2, Jagged 1 and hes-1 protein expression levels. In the presented study, it has been concluded that genistein administration has a protective effect against testicular ischemia-reperfusion through suppression of oxidative stress and inhibition of caspase 8 apoptosis pathway and can be applied clinically in testicular torsion.

Awoniyi et al. (1997) suggested that coumestrol administration, a phytoestrogen, at a dose of 100 μg/day (subcutaneous) to newborn male rats (postnatal 1-5. days) did not affect testes and prostate weights, testicular and epididymal sperm counts, histological structure of the seminiferous tubules, Leydig cell morphology and their locations, as well as serum FSH, LH, and testosterone levels. As a result of the findings, it was revealed that exposure to coumestrol in newborn male rats did not have adverse effects on the structure of the reproductive organs and sperm production.

Tarrago et al. (2006) showed that coumestrol administration subcutaneously at a total dose of 12.5, 25, 50, 100, 200, and 400 μg for three days in adult rats did not cause a significant change in plasma FSH and LH levels. However, it decreased plasma testosterone levels in low-dose groups (12.5 and 25 μg). In addition, they determined that it decreased the diameter and volume of the seminiferous tubule and expanded Leydig cells in all dose groups in the right testes, and there was no change in all dose groups in the left testes. Hence, the researchers deduced that coumestrol has an estrogenic effect without affecting the release of gonadotropins.

Administration of S-(-) equol and R-(+) equol at a dose of 250 mg/kg/day until 21-d-old from 10 days before coitus did not affect the number of pups born and birth weights. Also, the histological structure of the testis, prostate, and seminal vesicle was found to be normal (Brown et al., 2011).

Zhong et al. (2014) showed that the application of equol administration (0.01, 0.10, 1.00, 10.00 μmol/L) to the cell culture line prepared from mouse testicles did not cause any morphological changes in testicular cells. Also, it decreased 3β-hydroxysteroid dehydrogenase (3β-HSD), vimentin, and side-chain cleavage enzyme (P450scc) activity, which are associated with testosterone synthesis in 10.00 μmol/L dose application. Accordingly, they

concluded that exposure to equol may have potential adverse effects on testosterone production and spermatogenesis.

5.2. Synthetic endocrine disruptors

There were birth control drugs, drugs used in hormone replacement therapies, and some animal food additives in this group. These substances are produced to regulate the endocrine system (Çetinkaya, 2009).

Diethylstilbestrol

Diethylstilbestrol (DES), is the most known synthetic endocrine disruptor chemical. It has a strong estrogenic effect and was first synthesized in 1938 by Dodds. At the beginning of the 1950s, it was allowed to be used as a growth accelerator and feed additive in farm animals by the FDA. In addition, it has been used in America and Europe for many years in toxemia, the threat of preterm birth, and the prevention of fetal deaths. In 1979, the World Health Organization (WHO) banned the use as a productivity enhancer in farm animals due to its harmful effects and potential cancer risk of DES. (Lee, 2007).

5.2.1. Experimental studies with synthetic endocrine disruptors

Nair and Shaha (2003) suggested that DES injection at doses of 0.1 and 1 mg/kg to adult rats decreased testicular weight, seminiferous tubulus diameter, serum testosterone level and sperm viability, and increased TUNEL positive apoptotic cell count and density of Fas-FasL immunohistochemical staining in spermatogenic cells undergoing apoptosis. As a result, it has been revealed that DES effects negatively spermatogenesis by inducing apoptosis.

In a different study (Rahman et al., 2019), it was determined that subcutaneous low dose (3 μg/day) and high dose (30 μg/day) DES injection in adult rats decreased the number of spermatogonium, Sertoli, and Leydig cells, and seminiferous tubulus volume. On the contrary, it increased the number of TUNEL positive apoptotic cells. Thus, it has been determined that DES may cause infertility in the male by disrupting steroidogenesis and spermatogenesis.

N'Tumba-Byn et al. (2012) reported that DES administration (10^{-5} and 10^{-6} M) to fetal rat and mouse testis cell culture line for 3 days decreased testosterone syntheses and had a toxic effect on the male reproductive system.

Filipiak et al. (2009) revealed the adverse effects of DES administration at a dose of 1.25 and 12.5 mg on testicular development by significantly reducing testicular weight, seminiferous tubulus diameter and length, numbers of spermatogonium and Sertoli cells in their study on pubertal rat testes.

5.3. Environmental endocrine disruptors

These chemicals are found in industrial, agricultural, and household products such as pesticides, cleaning agents (detergents), paints, plastics, and solvents (Kelce and Wilson, 2001). In this group, there are industrial chemicals (Bisphenol -A, -B and -F, polychlorobiphenyls, alkylphenols, phthalates, dioxin), pesticides, heavy metals (lead, mercury, cadmium, arsenic, uranium), and other environmental pollutants (Keith, 1998). Most of the environmental endocrine disruptors dissolve in fat and cause harmful effects in the organism by accumulating in the adipose tissue (Solomon and Schettler, 2000; Lee, 2007). These substances can create endocrine-disrupting effects in humans and animals.

Bisphenol A (BPA)

BPA is an endocrine-disrupting chemical such as dioxins and polychlorinated biphenyls (Fenichel et al., 2013). It is lipophilic and has environmental pseudo-estrogenic activity (Vandenberg et al., 2012; Hormann et al., 2014). BPA is used in epoxy resins and polycarbonate plastics such as baby feeding and water bottles (D'Cruz et al., 2012). Epoxy resins are used for coating the inner surfaces of metal boxes used in the packaging of food and beverages such as seafood, vegetables, beer, soft drinks, and milk powder. They are also used to make storage containers containing liquids such as wine and water (Matsumoto et al., 2003).

It has a high risk of contamination in humans due to the wide range of uses. Humans are exposed to BPA through food consumption, inhalation, and dermal absorption (Vandenberg et al., 2012). BPA has significant toxic effects on the organism, especially on the male and female reproductive systems. BPA inhibits androgen production and Sertoli cell activity by causing oxidative stress and suppressing spermatogenesis (Rahman et al., 2015).

Polychlorobiphenyls (PCB)

PCB compounds, organic chlorine pollutants, were chemically detected for the first time in coal tar in 1865 and in the feathers of some birds in nature in 1914. PCBs have been widely used in the various industrial areas since the 1930s (Güvenç and Aksoy, 2007). After it was understood that they caused environmental pollution by entering the food chain and started to threaten human health, their production was banned in many countries of the world (USA, Sweden, Denmark, Norway, Finland, Iceland, Turkey) and their use was limited (Lindell, 2012). However, they are still used in industrial equipment and industrial products in many countries (Telli-Karakoç et al., 2002). PCB compounds are frequently used as additives in pesticides, printing ink, paints, plastics, adhesives,

transformers and capacitors, vacuum pumps, high voltage cable and wire, ballast capacitors of fluorescent lamps, hydraulic and machine oils, and copy paper. Since these substances are easily spread to the environment through waste materials, they also affect the ecosystem in the waters by polluting the rivers and seas. The main source of contamination is foods of animal origin. These substances were detected on sea products (Lindell, 2012). PCBs are lipophilic and resistant to degradation in the natural environment (Carpenter 2005) and are an androgen receptor antagonist (Ulbrich and Stahlmann, 2004). Many researchers have been conducted on the carcinogenic, immunosuppressive, disruptive, neurotoxic, hepatotoxic, and teratogenic effects of PCBs (Hsu et al., 2007; Murugesan et al., 2007). Exposure to PCBs during developmental stages in males causes pathological status such as cryptorchism, testicular cancer, prostate inflammation, and infertility (Schrader and Cooke, 2003).

Alkylphenols

Alkylphenols contain two groups of compounds: nonylphenol ethoxylates (nonylphenol ethoxylate, NPE) and oxyphenol ethoxylates (OPE) (Sole et al., 2000). In particular, NPE is used in the production of detergent, latex paint, plastic, and paper, as well as in skin and textile processing, and in pesticide formulation (Chokwe et al., 2017). Nonylphenol ethoxylates are spread to the environment by industrial and domestic wastewater. After being released into nature, nonylphenol ethoxylates transform to metabolic intermediates such as nonylphenol (nonylphenol, NP), nonylphenol monoethoxylate, and nonylphenol diethoxylate (Ahel et al., 1994). 4-Nonylphenol is a resistant compound to biodegradation due to the benzene ring in its structure (Brunner et al., 1988). They cannot easily remove after entering the body, and accumulate in the adipose tissue (Birnbaum and Fenton, 2003). 17β-Estradiol and 4-NP are very similar in terms of their chemical structures. Therefore, 4-NP can imitate all the functions of the estrogen hormone. 4-NP has a stronger estrogenic activity than other estrogenic chemicals such as Bisphenol A (BPA) (Lyons et al., 2014). They behave as a hormone by binding to a hormone receptor, or inhibiting the metabolic activities of estrogen, progesterone, androgen, and other hormones that play a role in reproduction and development by preventing binding to the specific receptors of hormones. Therefore they may affect sexual behaviors and the reproductive system (Christiansen et al., 1998; Zemheri and Uğuz, 2018).

Phthalates

Phthalates are found in the structure of adhesives, cosmetics, perfumes, paints, printer ink, plastic demijohns, pet bottles, and many industrial products and disappear very slowly in nature (Montouri et

al., 2008). Their presence has been detected in the biological fluids of humans and animals (Bosnir et al., 2007). Di-ethylhexyl phthalate, Butyl benzyl phthalate, Di-n-butyl phthalate, Di-n-phenyl phthalate, Di-hexyl phthalate, Di-propyl phthalate, Dichlorohexyl phthalate, Diethyl phthalate are compounds in the phthalates group (Yeşilkaya, 2008). Phthalates are ubiquitous environmental contaminants. Perfumes and cosmetics are often responsible for the multiple phthalate metabolites in women's urine, and phthalates can also be found in some foods, drugs, and dietary supplements. They pass to water from plastic carboys and pet bottles can also be contaminated with the aforementioned chemical. The phthalate levels in the water increase at high temperatures (Zaater et al., 2014). It is known that many of the phthalates are endocrine disruptors, carcinogenic, and prevent growth and adverse effect on the reproductive system (Yıldırım et al., 2020). It is stated that it may cause adverse effects on sperm viability and number in males. It is emphasized that exposure to MEHP (mono-(2-ethylhexyl phthalate) in the prenatal period causes miscarriage in the first months (Pant et al., 2011).

Pesticides

Pesticides have an important place in the endocrine system disrupting chemicals. Insecticides such as dichlorodiphenyltrichloroethane (DDT), endosulfan, dieldrin, methoxychlor, dicofol, toxaphene; herbicides such as alachlor, atrazine, nitrofen; fungicides such as benomyl, mancozeb, and tributyltin; parasitic drugs such as aldicarb, and dibromochloropropane are included in the pesticide group (Çetinkaya, 2009). A large number of pesticides are used in the production of food products. The use of some pesticides has been prohibited. Although the use of organic chlorinated pesticides is prohibited, many pesticides continue to be used in agricultural production. For example, it has been reported in various studies that atrazine, one of the triazine group herbicides still used in products such as citrus and corn, has an estrogen hormone-like activity as well as negatively affects the production of androgen hormone and the adrenal glands (Belloni et al., 2011; Mou et al., 2011). Exposure to methoxychlor, which is in the insecticide group, causes infertility and functional impairment in the ovary in females (Özden Akkaya et al., 2017).

Heavy metals

Heavy metals such as cadmium, arsenic, mercury, nickel, lead, and zinc can be released into the environment from many sources such as erosion, mining, industrial and urban wastewater, sewage discharge, pesticides, or insecticides (Jaishankar et al., 2014). Since heavy metals accumulate in the soil and plants, their permanence in nature is high. It is one of the important environmental pollutants. Heavy metals can be

taken into the organism by mouth, respiration, and skin. Even if they enter the organism in very few amounts, they are excreted very slowly from the metabolism. Therefore, they accumulate in the organism and reach dangerous doses for the organism (Farooq et al., 2008). In experimental studies, it has been reported that heavy metals have endocrine-disrupting effects and mimic the activities of androgen and estrogen hormones (Dyer, 2007; Georgescu et al., 2011). Exposure to heavy metal exposure causes adverse effects on the male reproductive system (damage to the sperm parameters and histological structure of testes, changes in serum/plasma reproductive hormone levels), female reproductive system (damage on the histological structure of ovaries, changes in serum/plasma reproductive hormone levels, increase in abort and stillbirth rates, congenital anomaly), neuroendocrine system and thyroid hormone levels (Köse et al., 2019).

5.3.1. Experimental studies with environmental endocrine disruptors

Gules et al. (2019) determined that BPA administration at a dose of 50 mg/kg/day by oral gavage for 14 days in adult rats increased testicular and serum MDA and serum BPA levels, and decreased testicular and plasma GSH activity and plasma SOD activity. In the examination of histological sections, BPA treatment decreased the stage VII-VIII seminiferous tubules diameters and epithelial heights, as well as the number of undifferentiated embryonic cell transcription factor-1 (UTF-1) positive cells, and increased the number of TUNEL positive apoptotic cells. In addition, in the examination of semen smears, it was determined that mid-piece abnormal spermatozoa rate increased, and sperm viability decreased. In the study, researchers concluded that BPA causes toxic effects on the male reproductive system by inducing oxidative stress.

Güleş et al. (2019) in a different study reported that BPA treatment (100 mg/kg/day) by oral gavage for 14 days decreased serum testosterone level and sperm viability, and increased the head and midpiece abnormal spermatozoa rate. In addition, in the histological examination, they found that the stage VII-VIII seminiferous epithelial height decreased and the number of TUNEL positive apoptotic cells increased. Thus, it has been determined that BPA causes dysfunction in testicular morphology.

On the other hand, Ozden Akkaya et al. (2021) determined that the intraperitoneal BPA injection (50 mg/kg) on days in vivo embryonal (E) 18-21, postnatal (P) 0-3, and P4-7 in rats did not affect Notch 1 protein expressions.

It was reported that the 200 μM BPA treatment to primary Sertoli cell cultures culture mediums decreased the protein levels (Connexin 43, occluding, and N-cadherin) in the blood-testicular barrier by deterioration

of the blood-testicular barrier (Li et al., 2010).

Nakamura et al. (2010) showed that 100 and 200 mg/kg BPA administered subcutaneously for six weeks in four-week-old male rats significantly reduced body weight, testis, and epididymis weights, as well as plasma testosterone, FSH and LH levels. In addition, they also observed that the P450scc enzyme and steroidogenic acute regulator (STAR) protein levels, as well as numbers of Leydig cell decreased in BPA groups.

BPA administration at doses of 480 and 960 mg/kg between the 31st and 44th days after birth in mice decreased body weight and testicular weight, and seminiferous tubules diameters, as well as increased percentage of apoptotic index in Leydig cells and germ cells (Li et al., 2009). In a different study, it was determined that BPA treat to Sertoli cell culture line increased the number of apoptotic cells (Iida et al., 2003).

Aly et al. (2009) determined that PCB administration (1.5 and 3 mg/kg/day i.p.) in male rats reduced body weight, testicular weight, epididymis weight, sperm count, sperm motility, daily sperm production, mitochondrial CAT, glutathione peroxidase (GPx), glutathione reductase (GR), and SOD activities. In addition, it has been suggested that it increases hydrogen peroxide (H_2O_2) levels and lipid peroxidation by inducing oxidative stress in mitochondria.

In a study by Murugesan et al. (2007), PCB administration to Leydig cell culture line obtained from adult rat testis decreased 17beta-HSD, SOD, CAT, GPx, and GR activities, the levels of steroidogenic acute regulatory protein (StAR), and steroidogenic enzyme activities such as cytochrome p450scc, 3beta-hydroxysteroid dehydrogenase (HSD), and increased lipid peroxidation, hydrogen peroxide, and hydroxyl radical levels. Accordingly, it has been revealed that PCB exposure suppresses testosterone production.

Hsu et al. (2007) reported that exposure to PCB in male rats for the prenatal period decreased cauda epididymis weight, epididymal sperm count and motile sperm count, and increased reactive oxygen compounds (ROS) in the spermatozoon in adulthood. In addition, it was observed that the caspase 3 and 9 activities increased, and the pro-apoptotic Fas, Bax, and p53 gene expressions decreased.

Earlier studies indicate that PCB increased apoptosis in Leydig cells (Oskam et al., 2004), testicular and prostate weight and abnormal sperm count (Faqi et al., 1998), caused degenerative changes in seminiferous tubules (Alston et al., 2003), decreased sperm motility (Hsu et al., 2003, Krishnamoorthy et al., 2007) and sperm production (Faqi et al., 1998).

Lu et al. (2014) suggested that nonylphenol administration at doses of 100 and 250 mg/kg by oral gavage for 90 days in male rats caused tubular

atrophy in the testes, and decreased epididymal SOD, and CAT activities, serum testosterone level, sperm count and motility in cauda epididymis. In addition, exposure to BPS reduced seminiferous epithelial height due to the numerical decreasing of germ cells and Sertoli cells, and increased percentage of abnormal spermatozoa, and reactive oxygen radicals and number of apoptotic cell in epididymal sperms. Consequently, it has been shown that oral NP administration damages the structure and functions of the testis, induces apoptosis and oxidative stress in the epididymis, and causes cytotoxic effects on epididymal sperm.

Ijaz et al. (2021) reported that nonylphenol administration (50 mg/kg/day) by oral gavage for eight weeks in rats decreased testicular antioxidant activities (SOD, CAT), plasma FSH, LH and testosterone levels, epididymal sperm count, and motility, increased dead sperm count, abnormal sperm count and apoptotic Bax and caspase 3 expressions. In addition, in the histological examination, they found that the seminiferous tubule diameter and the seminiferous epithelial height, and the thickness of the tunica albuginea in the testes decreased and the histopathological damage increased. As a result of the findings, it has been shown that NP induces apoptosis by inducing oxidative stress in the testis and has negative effects on the histological structure and functions of the testis.

Exposure to nonylphenol (50, 200, and 375 mg/kg) by oral gavage for six weeks increased Fas and FasL messenger RNA (mRNA) expression in the testis and the number of TUNEL positive apoptotic cells in the seminiferous tubules. As a result of this study, it was revealed that germ cell apoptosis occurs via Fas/FasL pathway (Han et al., 2004).

Balci et al. (2020) were observed histopathological changes in the fetal testes of male rats born from female rats exposed to a dose of 50 mg/kg/day di (2-ethylhexyl) phthalate (DEHP) by oral gavage during pregnancy and lactation. In addition, it was determined that proapoptotic caspase 3 and caspase 8 levels, TUNEL positive apoptotic cell numbers, and testicular autophagic protein levels increased.

Boekelheide et al. (2009) observed the inhibition of testicular somatic cell proliferation, and decreasing testis volume in the fetal testes of male rats born from female rats exposed to doses of 50, 100, and 500 mg/kg/day di(n-butyl)phthalate (DBP) by oral gavage during pregnancy

Vaithinathan et al. (2010) determined that methoxychlor administration at a single dose of 50 mg/kg by oral gavage in rats increased the number of TUNEL positive apoptotic cells in histological sections as well as activations of procaspase 3 and procaspase 9, and expressions of Fas and FasL. These findings showed that methoxychlor induced apoptosis in adult rat testis.

Intraperitoneal DDT injection at doses of 50 and 100 mg/kg/day for 10 days in adult rats decreased testicular SOD, CAT, Gpx, GR, GST, and GSH activities, and increased testicular MDA and H_2O_2 levels. In addition, it also increased the number of TUNEL positive apoptotic cells, apoptotic index, and DNA fragmentation detected by agarose gel electrophoresis. These findings showed that DDT caused apoptosis in rat testis through oxidative stress (Marouani et al., 2017).

Abarikwu et al. (2010) determined that atrazine treatment at doses of 120 and 200 mg/kg/day by oral gavage for 16 days increased MDA level in the testis and epididymis, and abnormal spermatozoa, and reduced the SOD activity, sperm viability, and sperm count in testis and epididymis. They did not observe any morphological changes in the histological examination of the testis and epididymis.

Amanpour et al. (2020) reported that intraperitoneal cadmium injection at a dose of 2 mg/kg/day for 28 days decreased mitofusin 1 and mitofusin 2 gene expression levels in testis, induced apoptosis by increasing caspase 9 and Bax expression levels in the testis. Consequently, it was determined that cadmium induced apoptosis in rat testis by mitochondrial dynamic genes and pro-apoptotic genes.

Baltaci et al. (2016) determined that sodium arsenite administration at a dose of 10 mg/kg/day by oral gavage for 15 days in adult rats increased testicular MDA level, and decreased testicular SOD, CAT, and GSH-Px enzyme activities, and serum testosterone level. In addition, it was determined that it decreased seminiferous tubulus diameter, testicular biopsy score, and immunohistochemically PCNA positivity, and increased the number of TUNEL positive apoptotic cells, and histopathological damage. Consequently, it was concluded that cadmium causes toxication in rat testis.

Almeer et al. (2020) determined that mercury chloride exposure at a dose of 0.4 mg/kg/day by oral gavage for 28 days in adult rats increased testicular lipid peroxidation (LPO), nitric oxide (NO), tumor necrosis factor-alpha (TNF-α), and interleukin-1beta (IL-1β) levels, and decreased testicular SOD, CAT, GPx, and GR activities. In addition, it was determined to cause histopathological damage in the testis.

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Chapter 6

CORIONIC VILLUS BIOPSY IN PRENATAL DIAGNOSIS

Fatih AKKUŞ[1]

1 Op. Dr. Necmettin Erbakan University Faculty of Medicine, Department of Obstetrics and Gynecology / Perinatology Clinic ORCID: 0000-0001-7037-9165

İntroduction

Chorion villus sampling (CVS) is a procedure in which a sample of small tissue is taken from the placenta, usually between 11-14 weeks, for prenatal genetic diagnosis. CVS results are available a week earlier than amniocentesis results, so It saves the family from waiting anxiously. If the results lead to a decision to terminate the pregnancy, the procedure can be done earlier in the weeks. First trimester termination of pregnancy is safer than the second trimester. However, CVS results have higher diagnostic uncertainty than amniocentesis. CVS may be a less safe procedure than second trimester amniocentesis.

CVS provides prenatal diagnosis of cytogenetic, molecular, biochemical and whole DNA analysis. The American College of Obstetricians and Gynecologists (ACOG) recommends informing all patients about prenatal invasive diagnostic testing("ACOG Practice Bulletin No. 88, December 2007. Invasive prenatal testing for aneuploidy," 2007).

İndications

The most common causes of prenatal genetic diagnosis are(Wapner, 1997):

- Maternal age at birth 35 or older
- Previous child with a chromosomal abnormality or genetic disorder
- The parent is a carrier of a balanced translocation, monogenic (single gene or Mendelian) disorder or structural chromosomal defect.
- Parents are carriers of an autosomal recessive disorder
- Mother is a carrier of a sex-linked disease
- Suspicion of congenital anomaly in first trimester ultrasound examination
- Abnormal results in aneuploidy screening

Amniocentesis is an alternative to CVS, both procedures provide basically the same genetic information. CVS results can be obtained four to six weeks earlier than amniocentesis results. The patient decides which procedure to choose. İt is necessary to inform about the risks and benefits and limitations of each technique.

Early amniocentesis (before 15 weeks) is generally not recommended as it is associated with higher pregnancy loss. CVS may be associated with greater fetal loss in early amniocentesis. While not a diagnostic test, non-invasive prenatal tests such as cell-free DNA have high sensitivity and specificity for trisomy 21. For this reason, many patients undergo cell-

free DNA screening for Down syndrome and avoid an invasive diagnostic procedure.

Contraindications

Maternal alloimmunization is a relative contraindication for CVS because fetomaternal hemorrhage from the procedure may result in more severe fetal erythroblastosis(Moise & Carpenter, 1990). There is a risk of vertical transmission of maternal infections such as HIV, hepatitis B and C. Caution should be exercised in patients with bleeding diathesis.

Procedure

CVS is an outpatient procedure usually performed in tertiary care centers under real-time ultrasound guidance. The number of procedures required annually to maintain competence is uncertain. The Royal College of Obstetricians and Gynecologists (RCOG) recommends at least 30 procedures per year. It is stated that expertise is obtained after 250 procedures(Kuliev et al., 1996). In recent years, the number of prenatal invasive diagnostic procedures has been decreasing. The use of simulators is beneficial in improving clinician's ability to perform CVS(Cordier et al., 2016).

CVS is especially done between 11-14 weeks of pregnancy. CVS is not performed before the 10th week of pregnancy because administration very early in pregnancy is associated with an increased risk of limb reduction defects. CVS can also be done after the 14. week of pregnancy. Amniocentesis is preferred at ≥15 weeks of gestation as it is technically easier and more comfortable for the patient.

Patient Preparation

Embryo number and chorionicity are determined. Ultrasound examination should be performed prior to the procedure to document fetal viability and to screen for fetal structural abnormalities. Maternal bladder must not be empty to provide an acoustic window

Technique

CVS can be done transabdominal route or transcervical. Usually placental location is effective in deciding the route of administration(Jackson et al., 1992). Transabdominal route seems to be safer than transcervical route with less fetal loss, lower vaginal bleeding and infection rates. The transabdominal route is superior, with a lower need for multiple insertions, a higher first-time sampling success rate, and less maternal cell contamination(Jackson et al., 1992; Silver, MacGregor, Muhlbach, Kambich, & Ragin, 1994). It is easier to take samples from the fundal placenta with a transabdominal CVS, and from a posterior and inferior

placenta with a transcervical CVS. According to a survey conducted in 32 centers in the United Kingdom, 96 percent of CVS is performed transabdominally(Sileo, Curado, & Bhide, 2019).

In retroflex uterus and posterior placenta, transcervical CVS is technically easier than transabdominal CVS(Smidt-Jensen, Philip, Zachary, Fowler, & Nørgaard-Pedersen, 1994). Transservical CVS is probably safer than transabdominal CVS if there are intestinal loops between the abdominal wall and the uterus.

Transabdominal chorionic villus sampling

The patient is informed before the procedure and the steps to be performed are explained, and then is placed in the supine position. Placental location is detected by transabdominal ultrasonography. The lower abdomen of the patient is prepared with plenty of antiseptic solution. Local anesthetic can be used in the transabdominal CVS procedure, however it is not very effective in reducing pain(Mujezinovic & Alfirevic, 2011).

A 19 to 20 gauge needle is inserted under the guidance of the ultrasound probe. The needle is advanced at an angle to allow advancement along the long axis of the placenta. The stylet is removed and mounted in the syringe holder. The needle tip is moved back and forth through the placenta until a sufficient sample is aspirated by the vacuum created in the syringe. At the end of the sampling process, the needle is withdrawn under negative pressure. The sample taken is sprayed into a plastic cup and evaluated under a nearby microscope.

Clinical outcomes were similar for continuous and discontinuous negative pressure needle aspiration systems(Young, von Dadelszen, & Alfirevic, 2013). Some clinicians use a double-needle technique. An 18 gauge needle is inserted first, then the stylet is removed and replaced with a 20 gauge needle to aspirate the sample(Sileo et al., 2019).

Transcervical chorionic villus sampling

The patient is placed in the lithotomy position, The external and internal genitalia are prepared with an antiseptic solution and a speculum is inserted into the vagina. A single-toothed teneculum or ring forceps is used to bring the uterus into straight axis.

If the uterus is sharply anteverted, filling the bladder can help correct the uterocervical angle. The cannula is bent to form a curve and then placed into the placenta under ultrasound guidance. The inside of the cannula is removed and a syringe containing 20 mL of medium is attached to the catheter. The chorionic villi are aspirated while the catheter is moved through the placenta. At the end of the procedure, the syringe is held under

negative pressure and the catheter is withdrawn. Alternatively, use a biopsy forceps to obtain the placental sample(von Dadelszen et al., 2005; Young et al., 2013). Placental pieces are placed in a plastic tissue culture dish and the contents are evaluated under a microscope. A light source is also used here. With aspiration, there is a higher risk of undersampling than with forceps biopsy (relative risk [RR] 3.81, 95% CI 1.52–9.56), no difference in low rates between the two techniques (RR 1.00, 95% CI 0.14-6.96)(Young et al., 2013). Clinicians should continue to use whatever technique they are accustomed to.

Evaluation of tissue sample

Usually 5 mg of villus tissue is required. The assistant can provide a quick assessment of sample adequacy, evaluate the quality of the obtained sample. If there is a large amount of blood in the sample, the blood should be removed immediately to avoid blood and villi adhesion(Brown, Abigania, Warburton, & Brown, 2006).

Genetic evaluation

chorionic villi; It consists of syncytiotrophoblast, cytotrophoblast and inner mesenchymal cell nucleus. These cells have a high mitotic index. Since it can be examined at metaphase, rapid karyotyping can be performed within 2 to 48 hours after sampling by direct examination of the cytotrophoblast. Long-term (one-week) cultures of mesenchymal cells should be done concurrently because of the risk of false positive results. Conventional karyotype or microarray is performed on cultured cells. When necessary, biochemical and molecular genetic analyzes can also be performed. The time required for analyzes depends on the specific test.

Multiple pregnancies

It is important to determine the chorionicity of multiple pregnancies. Knowing the chorionicity in multiple pregnancies is very important for correct selective termination in the future. In twins, CVS can be done with a transabdominal, transcervical or combined approach. In monochorionic twins, it is sufficient to collect fluid from only one amniotic space, but different karyotypes have been reported in monozygotic twins, so some clinicians sample both fetuses when there is an anomaly. If there are separate placentas, the procedure is the same as for a singleton pregnancy. If the placentas are fused and the chorionicity cannot be determined definitively, the tip of the needle should be placed either close to the entrance of the umbilical cord or at the border of the placenta. If results are the same and chorionicity remains uncertain, amniocentesis may be required to confirm that each fetus has been sampled. Amniocentesis is required in 6 percent of cases(Pergament et al., 1992; Wapner et al., 1993).

Patients can continue their daily work after the procedure. It is generally recommended that they do not do heavy work for a few days and avoid sexual intercourse. They are told that spotting is normal, but in case of continuous bleeding, pain and fever, they should apply to the hospital.

COMPLICATIONS

The most undesirable complication of CVS is fetal loss. Later, bleeding, infection, and failure to conclude are the conditions.

Total fetal loss rate

To determine the comparative rates of fetal loss in patients undergoing CVS and amniocentesis, patients should be allocated to the CVS or amniocentesis group early in pregnancy. This approach explains the spontaneous losses occurring at the end of the first trimester and in the early second trimester in both groups.

CVS is performed at earlier weeks than amniocentesis, comparing the spontaneous loss rate after CVS with the procedure independent of post-amniocentesis is not an appropriate method to indicate that CVS results in a higher loss rate than amniocentesis**.** In the review by Mujezinovic et al., they reported the total fetal loss rate as 0.7% within 14 days after transabdominal CVS(Mujezinovic & Alfirevic, 2007).

In a large national cohort study of 147,987 women, patients who underwent first trimester combined screening plus TA-CVS or amniocentesis had similar rates of miscarriage or stillbirth compared with patients who underwent first trimester combined screening alone(Wulff et al., 2016).

If the risk of aneuploidy was low, the risk of miscarriage increased after CVS, in contrast, when the risk of aneuploidy was high, the risk of miscarriage after CVS was paradoxically reduced. This inverse relationship is thought to be due to termination of pregnancies with major aneuploidy, which would likely result in spontaneous abortion.

Unfortunately, since the cell-free DNA screening program was implemented, the CVS procedure is not used very often. Because of this, it is becoming increasingly difficult for operators to learn and maintain technical skills related to CVS.(Caughey, Hopkins, & Norton, 2006; Silver, MacGregor, Sholl, Hobart, & Waldee, 1990).

Perinatal loss

The perinatal mortality rate after CVS is not significantly higher than after amniocentesis (7 vs 6 per 1000 live births)(Alfirevic, Navaratnam, & Mujezinovic, 2017). In only one study, they found a 4.6% lower live birth rate in patients who underwent CVS compared with amniocentesis. (86%

vs. 91%, p<0.01)("Medical Research Council European trial of chorion villus sampling. MRC working party on the evaluation pf chorion villus sampling," 1991).

Multiple pregnancy loss

The safety of CVS in multiple pregnancy is uncertain. The small number of studies, the heterogeneity of the data, and the general lack of matching between CVS and controls prevent any meaningful conclusions from being drawn about the risk of CVS in twins. In the meta-analysis limited to studies published after 2000, the overall fetal loss rate was 2 percent (95% CI 0.0-6.5%)(Di Mascio et al., 2020). From the two studies directly comparing patients with and without CVS, the overall fetal loss rate was similar between the groups (3/201 vs. 5/218).

Diagnostic uncertainty and misdiagnosis

The rate of false negative results with CVS is very, very low (0.03% in a series of over 62,000 procedures)(Hahnemann & Vejerslev, 1997).

There may be mosaicism limited to the placenta. When we encounter a mosaic karyotype, amniocentesis should be performed in case of a false positive test. The chorionic villus specimen can be evaluated by both direct genetic analysis and long-term cultures. However, long-term culture appears to be more reliable than direct examination.(van den Berg, Van Opstal, Polak-Knook, & Galjaard, 2006).

Maternal cell contamination

Maternal cell contamination is more common in CVS than in amniocentesis (3.8% vs. 0.3%, RR 12.3, 95% CI 3.8-39.7)(Alfirevic et al., 2017). Guidelines have been developed for the assessment and reporting of maternal cell contamination in long-term cultures. (Nagan, Faulkner, Curtis, & Schrijver, 2011). The direct preparation method has a lower risk of misdiagnosis than long-term culture due to maternal cell contamination because maternal decidua has a low mitotic index.

Mosaicism limited to the placenta

Restricted placental mosaicism, by definition, refers to the difference between the genotype of the placenta and the genotype of the fetus. CVS has a higher risk of limited placental mosaicism than amniocentesis (2.3 vs 0.4 percent, RR 5.7, 95% CI 1.9-16.2)(Alfirevic et al., 2017). Mosaicity in CVS has importance in terms of both diagnosis and prognosis. Because placental function will be impaired, it may lead to miscarriage, stillbirth and fetal growth restriction (Taylor et al., 2014). In type 1 placental mosaicism, mosaic cells are limited to the cytotrophoblast; In type 2 placental mosaicism, mosaic cells are limited to the mesodermal villus stroma; and

in type 3 placental mosaicism, mosaic cells contain both cytotrophoblast and mesodermal villus stroma(Kalousek & Vekemans, 1996).

Limb reduction defects and oromandibular hypogenesis

The generally accepted lower limit for CVS procedures is ten weeks of gestation. because it has been reported that the rate of limb abnormalities is increased when CVS is performed before 9 weeks. This risk is independent of the expertise of the operator, the route of the procedure, or the size of the needle or cannula used(Dolk, Bertrand, & Lechat, 1992; Mastroiacovo et al., 1992; Olney et al., 1995).

Vaginal Bleeding

Small vaginal bleeding after CVS is reported in 30% of patients(Rhoads et al., 1989).

Infection

Rarely, clinically significant infectious complications have been reported. Clinical or subclinical intrauterine infection may cause fetal loss(Brambati, Lanzani, & Oldrini, 1988; Hogge, Schonberg, & Golbus, 1986; Rhoads et al., 1989).

Mother-to-child transmission of infections such as hepatitis virus, cytomegalovirus, toxoplasmosis, and HIV can also occur during CVS.

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Chapter 7

DECODING HUMAN IMMUNODEFICIENCY VIRUS

Simay TUTAR[1]
Yasemin YÜCEL [2]

1 Simay TUTAR, Altınbaş University, School of Pharmacy Student, OrcID: 0000-0003-1518-5258, This chapter is produced from the graduation thesis "The Power of Knowledge: Decoding Human Immunodeficiency Virus", 2021.
2 Dr. Öğr. Üyesi Yasemin Yücel YÜCEL, OrcID: 0000-0002-6595-6850, Altınbaş University, School of Pharmacy, Department of Biochemistry

1. ORIGIN AND HISTORY OF HUMAN IMMUNODEFICIENCY VIRUS

The very first time Acquired Immune Deficiency Syndrome (AIDS) accepted as a new illness was in 1981, after expanding quantities of youthful gay men passed away due to unexplained opportunistic infections and rarely seen malignancies. The retrovirus, now termed human immunodeficiency virus type 1 (HIV-1), was afterwards recognized as the main initiating agent responsible for one of the deadliest and wrecking infectious illnesses that has came up in recent human history (Paul M. Sharp & Hahn, 2011). The epidemic of HIV was emerged after zoonotic diseases with simian immunodeficiency viruses from African primates. First group of people who were infected with HIV was presumably bushmeat huntsmen. The transmission of HIV-1 was from apes, sooty mangabey monkeys were the main cause of transmission of HIV-2. The subgroups M, N, O are from chimpanzees; O, P are from gorillas. N, O, P can only be seen in West Africa. The origin of world wide spreaded HIV pandemic is group M (Maartens et al., 2014). Following genomic continuances, when there were many inter-species transmitting episodes of an akin lentivirus from apes to humans, it was shown that the HIV outbreak most likely began in central Africa in the beginning of twentieth century, most possibly from hunting techniques (Melhuish & Lewthwaite, 2018). Chimpanzees (along with sooty mangabeys and a variety of other ape species) are frequently hunted and slaughtered for their meat and offered as 'bushmeat' in big metropolitan market places. Direct human contact to mucousal discharges or animal blood as an outcome of hunting or additional behaviors like eating raw infected meat appears to be the quickest and most reasonable cause of the interspecies transfer of SIVs to HIVs (P. M. Sharp et al., 2001).

Transmission must have happened via cutaneous or mucous membrane exposure to infected ape blood and/or bodily fluids, based on the biology of these viruses. Wildlife hunting is the most typical source of these kinds of exposures (Paul M. Sharp & Hahn, 2011).

2. MECHANISM OF HIV

HIV, like any other lentiviruses and retroviruses, need to merge into the DNA of the target cell. As a result, the function of the mingled virus genome, also known as provirus, is highly impacted by the target cell's biochemical and functional status, and the durability of the provirus is determined by the lifetime of the carrier cell. Extreme actions in proviral function and lifespan are more noticeable in the CD4 lymphocyte repository than anywhere else in the system. Virus multiplication is quick and effective in a stimulated T cell. The binding sites located in the viral long terminal repeat monitors the biological factors necessary for HIV

replication and they are plentiful in macrophages and stimulated T cells. The findings from HIV research established that CD4 is a receptor for viral use. CXCR4 and CCR5 chemokine (cytokine) receptors have been described as HIV-1 cell surface coreceptors (Stevenson, 2003).

Just like in different retroviruses, the core (p6, p7, p24) and matrix (p17) fundamental proteins are encrypted by the gag gene, while the viral envelope glycoproteins gp41 and gp120 are encrypted by the env gene, which identifies the exterior cell receptors. The pol gene encrypts for enzymes essential for viral duplication. The enzymes are reverse transcriptase, which transforms virus RNA into DNA, integrase, which integrates virus DNA into target chromosomal DNA (the provirus), protease, which divides big pol and gag protein precursors into their constituents.

HIV multiplication phase can be broken down into six stages; 1) fusion and entry; 2) uncoating; 3) uncoating transcription; 4) integration of viral DNA; 5) viral protein synthesis and assembly and 6) budding (Fanales-Belasio et al., 2011). The initial stage of the virus replication cycle starts with virus attachment to the target host cell and concludes with the melding of the cell and viral membranes, followed by viral nucleus transport into the cytoplasm. To begin, virions must attach to the host cell, which is accomplished by the virus envelope (Env) protein or target cell membrane proteins integrated into the virus via either of a variety of cell binding determinants. Attachment of Env to its major receptor, the target protein CD4, is the second stage of viral entry and the first vitally essential factor for infection. CD4 is an immunoglobulin superfamily constituent that generally serves to promote T-cell receptor (TCR)-interfered signaling. Env's most important duty is to merge with the CD4 attachment site (Wilen et al., 2012). To receive access, HIV binds to the CD4+ protein on the layer of these and additional cells.

There are many sorts of coreceptors for various types of cells that HIV mutations can exploit to infect host cells. CXCR4 and CCR5 are two primary chemokine receptors that have been discovered as being important in HIV entrance (Naif, 2013). The migration of the viral molecule to the location of the active membrane fusion is the fourth phase in viral penetration. According to a demonstration of several new research, the viruses employ cellular transfer channels to arrive at particular locations that are required for the virus and to make the invasion more powerful. Env-mediated membrane fusion is the fifth and last stage of the HIV entrance process. The hydrophobic gp41 fusion peptide is exposed as a result of coreceptor interaction, and it integrates into the target cell membrane. Coreceptor engagement releases the gp41 fusing compound's potential power, leading in the development of a 6HB (six helix bundle),

the entrance and stability of the membrane fusion gap, and the following transportation of virus components into the target cell cytoplasm (Wilen et al., 2012).

The binding of HIV particle to a CD4 cell takes 30 minutes to 2 hours, the conversion of the virus RNA genome into provirus DNA takes around 6 hours, and the merging into the target cell's genome takes another 6 hours. The first viral components are evident roughly 12 hours after penetration; roughly 24 hours after transmission, the first offspring virion are discharged from the infected host cell (Seitz, 2016).

3. TRANSMISSION OF HIV

The condition was called 'gay related immune deficiency' (GRID) at first but near the end of 1982, incidents of acquired immunodeficiency was seen and announced in women sexual acquintences, offsprings of affected females and in heterosexual people living in US, originally from Haiti. Later, the term acquired immune deficiency syndrome (AIDS) was embraced, due to the disease obviously not being linked only to homosexuals (Weber, 1998).

The routes for HIV to reach the body are through unharmed mucous membranes, wounded skin or eczematous or mucosa, and via parenteral injection (Seitz, 2016). The most frequently seen way of HIV transmission worldwide is exposure to cell-free or cell-associated infectious virus in body fluids or mucosal places, by virtue of sexual intercourse. Contamination through transfusion of blood and blood products, drug injections, exposure of fetus or babies from the mother are less seen ways. (Moir et al., 2011).

Getting infected with HIV through blood or organ transplant, including bone, can happen within five to six days after infection of the donor. In pregnancy, mother to child contamination has been established in the 12th week of germination. But the transmission mainly happens (> 90%) in the final trimester and especially a little while before or during birth. HIV also can be transmitted via breast milk (Seitz, 2016). Relevant sexual behaviors (e.g., anal-receptive intercourse), habits of sexual pairing, contraception preferences, and the usage of drugs that lower sexual inhibitions are examples of social standards that influence infectivity. Circumstantial aspects also influence the ratio of sexpartner transition, which can have a significant impact on the epidemic's development. The prevalence of unregulated commercial sex services, bathhouses and "crack" cocaine homes are examples of such factors, in addition to social 'standards' that influence the approximate number and frequency of sexual alliances (Royce et al., 1997).

4. DIAGNOSIS OF HIV

Following an infection causing exposure, there is a fluctuating time frame also known as the eclipse period, where no available diagnostic assay is able to identify HIV. The primary valid indicator of the infection is HIV RNA; among people who are HIV positive, 50% of them have identifiable plasma RNA occuring in around 12 days, with levels reaching maximum value in 20 and 30 days. Until day 15, HIV capsid protein called p24 can be detected in the blood. P24 containing antigens in the blood rises during days 25 to 30, and at that time initial anti-HIV antibodies might combine with floating p24; until day 50, antigen is usually completely removed from the circulation. This narrow spanned detection range of p24 is thus useful in detecting the recentness of the infection, but it also restricts its value in diagnostics (Hurt et al., 2017). To make the initial diagnosis, HIV antibody monitoring assays are used, subsequently a control test is made if the screening test gives a positive response. Granular agglutination methods are done along with ELISA (enzyme linked immunosorbent assay) or versions of this assay technique.

A virus infection can be diagnosed serologically following three weeks of exposure, but frequently after four to five weeks, based on antibody titers and the immunological response. In exceptional situations,the assays of HIV positive people with severe immunosuppression may result with HIV antibody negative, yet nevertheless they have HIV like clinical symptoms and detectable viral titers in their blood (Seitz, 2016). The findings of HIV antibodies is frequently used method to make an HIV diagnosis. Monitoring and confirmation assays are the two types of serological assays. For detecting antibodies for HIV antigens, ELISAs and quick tests are two of the most frequently used monitoring assays,since they provide rapid and accurate techniques. Western blot is typically used as an additional test to confirm the precision of antibodies found in monitoring assays (Rikhtegaran Tehrani et al., 2015).

HIV diagnostic assays come in four generations. Since they are solely sensitive enough to identify IgG, the first and second generations are titled to be ‘’IgG sensitive’’. On the solid segment of the experiment, anti-HIV antibodies collected from patient’s sample attach to fixed manufactured or recombinant HIV antigens, followed by the application of a detection indicator. The detection indicator for point of care testing is mostly a combination of colloid selenium or gold with a protein A. IgM/IgG-sensitive (previously named third generation) tests reduce the time it takes to reach the first IgM detection value, which is approximately 23 days following exposure. Similar to IgG susceptible examinations, antigens located on the solid phase attach to the antibody obtained from patient’s sample. Only difference is, except for marked protein A, additional

enzyme linked or labeled artificial HIV antigen is added for detection, in a procedure known as an 'antigen sandwich'. Antigen/antibody (Ag/Ab) combined detection tests (previously named fourth generation) combine an IgM/IgG-susceptible antibody test with concurrent, additional p24 antigen diagnosis. On the dry phase of the examination, antigen from the individual's sample is caught by the fixed anti p24 antibody, subsequently, an additional marked anti p24 antibody is added, generating an 'antibody sandwich' (Hurt et al., 2017).

The Western blot/Immunoblot test was first developed in Germany as a serological validation assay; however the test has lesser susceptibility for the beginning stages of HIV infection than p24 antigen discovery techniques or antibody assays. If the requirements for a positive Immunoblot/Western blot are met, then HIV infection is recognized as diagnosed (Seitz, 2016).

POC tests, as opposed to complicated computerized facility dependent systems, focus on one of two techniques: flow through, where the individual's sample and reagents are successively placed to a layer coated with HIV antigens, or lateral flow method, where the sample is pulled inside from an antigen embedded band by capillary action. The majority of the time, detecting method is done by using protein A colloidal gold substituents, which are either applied individually to flow through tests or remoistened from inside the lateral flow band (Hurt et al., 2017).

Quick HIV tests, which functions by making use of blood samples obtained from the finger or gathering of oral fluids, are able to give HIV infection tests result in 30 minutes and it is beneficial in some environments where patient revaluation care is difficult, like public tests at pubs or community events. Despite the benefits, most of the present authorized quick tests have restricted ability to discover acute HIV infection; if signs and symptoms indicate acute infection, better responsive fourth generation nucleic acid or antigen-antibody tests should be incorporated. It is crucial to discover acute HIV infection in order to get ahead of the disease before further transmission (Deeks et al., 2015).

The design and production of great-capacity HIV diagnostic tests is a critical strategy for fighting viral transmission and lowering the percentage of AIDS deaths. Enzyme Immunoassays have been the most frequently used monitoring techniques presently. The third and fourth generations of EIAs have great specificity, but the simultaneous discovery of HIV antibodies and antigens given by the fourth generation EIAs reduces the time frame for analysis and diagnosis (Rikhtegaran Tehrani et al., 2015).

5. SYMPTOMS AND STAGES OF HIV

HIV infection is a viral pathogenic condition that gradually destroys the affected indiviaul's immune system and capacity to combat infections or cancerous neoplastic illnesses, leading to final phase called in AIDS. Two to six weeks after the infection, around 50-70% of individuals develop an acute illness similar to mononucleosis. Following acute infection, there is a protracted duration of asymptomatic intermission phase also called clinical latency. Clinical latency does not imply viral latency because substantial amounts of HIVs are existing in the blood and even much more in lymphoid cells. At the conclusion of the pathogenic process, the immune system is destroyed, resulting in the emergence of opportunistic diseases and malignancies (Yılmaz, 2001). Most of the people who are HIV positive suffer from an acute HIV condition two to four weeks after exposure to the virus, which is described as clinical symptoms similar to common cold, coupled with high plasma viral load and, in some cases, fever and lymphadenopathy. In the lack of a immune reaction, HIV generally multiplies exceedingly quickly at this initial stage, hitting plasma viral load levels as extreme as 10 million copies each milliliter.

In cases where antiretroviral therapy is not present, climax of plasma viremia levels take three to four weeks after the infection, and then dropping gradually for few months, eventually attaining a viral baseline or steady state. The viral set point value is an essential predictor of the pace of infection development in HIV positive people who are not receiving antiretroviral therapy (Moir et al., 2011). People with HIV infection might be totally asymptomatic or have chronic widespread lymphadenopathy (identified as swollen glands at at least two or more extra inguinal spots). The glands are generally well proportioned and located in axillary,cervical and inguinal nodules. They may cooccur with manifestations like fatigue, muscle weakness, sweating and discomfort. Lymphadenopathy might be moderate in some individuals and be unrecognized (Melhuish & Lewthwaite, 2018).

After initial infection, which is accompanied with a clinical manifestation of different intensity in the most of individuals, typical progressors have a prolonged interval of clinical latency (extending to six to eight years). Regardless of the absence of symptoms, HIV illness is ongoing, as evidenced by constant viral duplication and the gradual depletion of CD4 cells. In terms of the connection between clinical manifestations and CD4 cell levels, persons with CD4 cell levels more than 500 per microliter often show no clinical symptoms, however persons with CD4 cell levels less than 500 per microliter are more prone to have

systemic abnormalities. Individuals with CD4 cell counts more than 500 per microliter who acquire Kaposi's sarcoma, persistent widespread lymphoadenopathy, or neurocognitive disorders are aberrations to this pattern. In normal progressors, the transition to clinically evident illness or AIDS-defining sickness takes eight to 10 years. As CD4 cell levels fall below 200 cells per microliter, the clinical presentation might be defined by serious and continual systemic indications and manifestations; at this number of CD4 cells, patients are more vulnerable to tumors and opportunistic diseases (Pantaleo & Fauci, 1996).

Toxoplasma gondii, Pneumocystis jirovecii, pneumococci, Mycobacterium TB and atypical mycobacteria, cytomegalovirus (CMV), *Cryptosporidium parvum, Salmonella species*, human polyomavirus JC,and herpes simplex virus (HSV) are most commonly seen opportunistic infections. Kaposi's sarcoma related with the human herpes virus type 8 (HHV-8), nonHodgkin's lymphomas, such as Epstein-Barr virus (EBV) associated B-cell lymphoma, and melanomas of the penile, scrotum, and cervix generated by human papillomaviruses (HPV) are common neoplasms seen with HIV infections (Seitz, 2016).

5.1 The Difference Between HIV and AIDS

During the period of chronic infection, the virus progresses and maintains circulating, causing immune cells to be demolished, leading to immunosuppression. Approximately in 10 years, HIV evolves to AIDS if it is left untreated. In the stage of AIDS, the immune system is critically damaged. In infected individuals, cancers, neurodegenerative illnesses, opportunistic infections ocur (Illanes-álvarez et al., 2021). When HIV positive individuals' plasma load is high and their CD4 levels are less than 200 cell per microliter, infected people begin to develop an AIDS status (Naif, 2013).

6. POSSIBLE TREATMENTS AND PREVENTIONS

The progression of the HIV disease is continual in any case, and the lack of antiretroviral treatment always leads to lethal results. With the help of antiretroviral therapy, clinical manifestations and CD4 cell destruction can be slowed down and get under control for many years. It is possible to prolong the process of the infection with or without only minor effects for several years with antiretroviral treatment (Seitz, 2016). The invention of ART combination is frequently cited as one of modern science's biggest accomplishments. Modern hybrid treatments decrease viremia levels by a great amount in a short period of time, when administered to committed patients. Because of the high level of viral repression, viral replication and the development of drug resistant variants were avoided. In theory, these medications can be effective endlessly. Without viral duplication,

the immune system regains many of the destroyed function, thus, AIDS is avoided (Deeks et al., 2015). From the time of its creation until now, the reduction in fatalities in developed countries is by 80% (Melhuish & Lewthwaite, 2018).

The drugs are distributed into six distinct classes based on their molecular mechanism and resistance profiles: (1) Nucleoside-analog reverse transcriptase inhibitors (NRTIs), (2) Non– Nucleoside reverse transcriptase inhibitors (NNRTIs), (3) Integrase strand transfer inhibitors (INSTIs), (4) Protease inhibitors (PIs), (5) Fusion (entry) inhibitors (FI), and (6) Coreceptor (chemokine) antagonists (CRA) (Table 2.6) (Arts & Hazuda, 2012).

As per the S2 recommendations, the first line of treatment for HIV should consist a mixture of two NRTI plus one NNRTI, while being safe and well tolerated. Therapy must begin when the CD4+ cell number is at or near 500 cells/microlitre, according to recommendations (Seitz, 2016).

The beginning treatment chosen is determined by the profile of the particular patient. Until considering choices for initial ART, practitioners must evaluate certain considerations for every patient to find the right match. These considerations may include: CD4 cell count, Pretreatment plasma HIV RNA (viral load), baseline drug resistance assessed by HIV genotypic drugresistance analysis, ideally at the point when HIV was diagnosed, present medical problems like renal disease,cardiovascular diseases, liver disease, coinfections like hepatitis C, tuberculosis, concominant drugs that may interact with some of the agents, like warfarin, proton pump inhibitors, methadone, inhaled corticosteroids (Johnson & Sax, 2014).

The main focuses of antiretroviral therapy are as follows: Reduce and suppress plasma viremia below existing assays' detection stage and to keep it there, increase CD4+ T cell counts, hence enhance general immune function, extend longevity, decrease the potential of transmission of infection to other people (Pau & George, 2014).

6.1. Nucleoside Reverse Transcriptase Inhibitors (NRTIs)

One of the three main enzymes produced for the human immunodeficiency virus is reverse transcriptase. Drugs that block the RNA and DNA relying DNA polymerase function of reverse transcriptase, along with prorease inhibitors, are the main elements of antiretroviral therapy, which has significantly decreased death rates in HIV positive individuals in developing nations (El Safadi et al., 2007). The earliest category of antiretrovirals, nucleoside reverse transcriptase inhibitors are also widely used as the 'core' of the treatment. They are the first classs of

drugs approved for use (Dionne, 2019). NRTIs are distributed as prodrugs, which necessitate phosphorylation and host cell entrance (Arts & Hazuda, 2012). Intracellular phosphorylation converts NRTIs to active diphosphate or triphosphate metabolites, which blocks the enzymatic activity of the HIV reverse transcriptase by inserting into the nucleotide equivalent, inducing DNA chain discontinuation, or by interacting with the virus' own substrate. This action in particular, prevents viral RNA from being converted into double-stranded DNA. First authorized drug for the individuals with severe HIV was zidovudine (AZT), in 1987, subsequently lamivudine, didanosine, stavudine, zalticabine (Pau & George, 2014). Since NRTIs are not metabolized by CYP450 enzymes, they have less compound reactions than alternative antiretrovirals. Hepatic steatosis and lactic acidosis are possible class side complications, but these were relatively frequent with older NRTIs (didanosine and stavudine) and are now uncommon with widely used inhibitors (Dionne, 2019. Abacavir and tenofovir are two of the most widely used NRTIs in present day, both administered with a mixture with lamivudine and emtricitabine (Pau & George, 2014).

6.2. Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)

The way non-nucleoside reverse transcriptase inhibitors (NNRTIs) block reverse transcriptase is greatly dissimilar to nucleoside reverse transcriptase inhibitors' (NRTIs). The agents attach to area close to active site, causing the enzyme to change adaption and block the reverse transcription (Deeks et al., 2015). The NNRTI attaching area is only available when NNRTIs are present (Arts & Hazuda, 2012). Because NNRTIs are not competitive inhibitors, this causes a conformational transition and thereby reduces the enzyme's activity (Pau & George, 2014). NNRTIs are strong, effective and simple to manufacture class of drugs, these factors make them accessible (Deeks et al., 2015). NNRTIs approved by the FDA include delavirdine, nevirapine, efavirenz, rilpivirine and etravirine (Farooq et al., 2016). Previously, efevirenz has been the most frequently used medication in rich and developed regions. While it is effective and relatively better absorbed, it may raise the potential of suicidal ideas and depression and it has a little central nervous system toxicity. A reduced dosage can be just as potent and safer (Deeks et al., 2015).

6.3. Protease Inhibitors (PIs)

In virion growth phase, the HIV protease enzyme is in charge of the gap needed for gag-pol polyprotein and viral gag precursors (Arts & Hazuda, 2012). Protease inhibitors perform their pharmacologic activities later in the duplication phase, by attaching to HIV proteases, resulting in an obstruction of the enzyme's proteolytic functions and the failure to develop

mature, contagious virions (Pau & George, 2014). Ten authorized protease inhibitors are as follows: atazanavir (ATZ), lopinavir (LPV), amprenavir (APV), darunavir (TMC114), indinavir (IDV), fosamprenavir, ritonavir (RTV), nelfinavir (NFV, saquinavir (SQV), tipranavir (TPV) (Arts & Hazuda, 2012). The medications are quickly metabolized in the liver and they are usually given together with an agent that blocks certain metabolic processes (cobicistat or ritonavir, also known as 'boosters') (Deeks et al., 2015). In treatment naive individuals, ritonavir boosted protease inhibitor treatments combined with two NRTIs are favored regimens. Mixed medications of PI and NRTI have been found to be successful not only in treatment naive individuals, as well as in people who have failed treatment with other antiretroviral drug types (Pau & George, 2014).

6.4. Integrase Strand Transfer Inhibitors (INSTIs)

Raltegravir, with a unique mechanism of action, was brought into the market as the earliest INSTI, approximately ten years after the debut of protease inhibitors and non-nucleoside reverse transcriptase inhibitors (Pau & George, 2014). Integrase strand transfer inhibitors work by blocking the HIV genome from integrating with the host genome (Deeks et al., 2015). Their mechanism of action works by attaching to a magnesium moiety located on the integrase enzyme, thus blocking the viral DNA (provirus) from entering into cellular DNA. Polyvalent cations such as calcium, zinc, aluminum can chelate the magnesium binding site, resulting with decreased absorption. To prevent this, the administration of integrase inhibitors should be two hours before or six hours later from polyvalent cation-containing medications (antacids, supplements etc.) (Dionne, 2019). Approved drugs are raltegravir, dolutegravir, elvitegravir (Deeks et al., 2015). In treatment naive individuals, the recommended regimen includes 1 INSTI + 2 NRTI drugs. (Pau & George, 2014).

6.5. Fusion Inhibitors (FIs)

Enfuvirtide is the only present fusion inhibitor authorized by the FDA for the treatment of HIV (Dionne, 2019). The fusion inhibitor enfuvirtide meddles with this fusion mechanism by sticking to the first heptad-repeat (HR1) in the viral envelope glycoprotein gp41, hence, hampering conformational changes needed for the blending of the viral and cellular membrane (Pau & George, 2014). Subcutaneous injection is the preferred way to apply enfuvirtide. In 90 mg dosage forms, twice a day. This can usually lead to injection area reactions, which can cause pain and sometimes causes nodules or induration (Dionne, 2019). Due to injection requirements, enfuvirtide is, in most cases, saved for patients who has multiple drug resistance and if at least two or three oral agents can not be used (Pau & George, 2014).

6.6. Chemokine (CCR5) Receptor Antagonist (CRA)

In the case of CCR5-using virus, the only CCR5 antagonist authorized for use in the therapy of treatment-naive or treatment experienced patients is Maraviroc. By selectively attaching to human CCR5 receptor on the cell layer, it obstructs the interaction of the CCR5 receptor and HIV gp120 for CCR5-tropic HIV. On the other hand, if the virus is CXCR4-tropic or it uses both CCR5 and CXCR4 for entry, it does not show any effect on blocking the viral access. Prior to prescription of maraviroc, the test for viral tropism must be done to affirm that the patient's virus uses just the CCR5 coreceptor (Pau & George, 2014).

6.7. Pre-Exposure Prophylaxis (PrEP)

When a person has significant risk of getting infected with HIV, starting a therapy with antiretroviral drugs prior to an exposure ('pre-exposure prophylaxis') can provide considerable 37 protection. The most intently examined regimen is daily oral tenofovir disoproxil fumarate coplanned with emtricitabine (TDF/ FTC). They are both nucleoside analogues which restrains viral replication (Deeks et al., 2015). TDF/ emtricitabine is accepted for use in PrEP as one tablet taken on a daily basis, with or without meals, for as long as the patient remains at risk of developing HIV. Excluding acute and ongoing HIV infection is crucial before prescribing PrEP, since taking PrEP during infection is in relation with quick advancement of resistance to emtricitabine. The effectiveness of TDF/emtricitabine is positively correlated with patient obedience. For individuals who were taking PrEP daily and were strictly following the regimen, in a cohort of men who have sex with men (MSM), TDF/ emtricitabine lowered transmission of HIV by 86%, and by 75% in heterosexual men and women, in comparison with randomized control groups taking placebos.

Similar to hormonal contraception, improvement of extra PrEP prescription alternatives will be valuable, considering some patients may favor the accessibility of less frequent dosing regimes, although some may consider the safety profiles of every alternative. That being said, in the foreseeable future, oral TDF/emtricitabine is the only accessible PrEP regimen (Riddell et al., 2018).

6.8. Prevention

When it comes to HIV prevention, along with several other sexually transmitted infections, condom use in men has been a vital element, as foolproof use should completely diminish the risk of transmission. Nonetheless, the effectiveness of condoms has been predicted to be more or less 80% against heterosexual contamination and 70% against male

to male sexual contamination. Over-reporting of condom use is likely a factor in the lower-than-expected efficacy rates, albeit inaccurate use and condom failure also takes a part. Furthermore, arranging 38 sterile injection supplies can also reduce HIV transmission in injection drug users to a large extent (Deeks et al., 2015).

6.9. Undetectable = Untransmittable (U=U)

In 1996, along with the development of protease inhibitors, the arrival of three-drug combinations of antiretrovirals made a huge progress in HIV/AIDS treatment. In a large proportion of patients, these treatment regimens resulted in significant reductions in viral load. Typically, results were below the detection threshold of plasma and can be tolerated for long periods of time. The achievement of a suppressed, undetectable viral load was most certainly the tipping point, which the U=U hypothesis evolved into reality.

According to an examination of scientific findings, a study from Switzerland in 2008 pointed out that patients with HIV who did not have additional sexually transmitted disease, and obtained and preserved an undetectable viral load for at least 6 months, did not transmit HIV sexually. This study was the first time the U=U equation was mentioned. With the pledge of U=U, reaching and sustaining an undetectable viral load play a huge role in giving hope for HIV positive people. Using prescribed medications as told is crucial for antiretroviral therapy (ART) to show highest effect. 6 months of antiretroviral therapy is needed to reach an undetectable viral load. The patient must continue the treatment, once viral suppression is achieved. As reported by the Department of Health and Human Services, when the plasma HIV-1 RNA level approaches undetectable (<200 copies/mL), viral load monitoring should be done every three-four months. If viral suppression and healthy immunologic status are sustained for more than two years, viral load monitoring can be reduced to every six months (Eisinger et al., 2018).

Aside from urging people living with HIV to begin and stick with antiretroviral therapy (ART), U=U can also play a part in rebalancing social expectations within non-HIV communities, since one of the biggest reasons for stigma towards HIV positive people is the risk of transmission (Okoli et al., 2021).

7. HIV STIGMA

Stigma is developed and reinforced by social and behavioral dynamics, and it is basically a social phenomenon that implies ‘us’ as opposed to ‘them.’ (Turan et al., 2017). Stigma coupled by society’s focus on proper wellbeing and sovereignty, in which the reliance on health care, medicine,

and the daily warning that you are not in good health will help to belittle an individual. The stigma can also arise from analogies often used to discuss virus illness, which inadvertently incorporate derogatory moral assessments (Hutchinson & Dhairyawan, 2017).

HIV-related discrimination and stigma are two major factors that have a detrimental effect on treatment. Discrimination and stigma remain in health-care distribution processes around the world, hence infringing on human rights and impeding access to and participation in the HIV-care spectrum. (Stockton et al., 2018). In a world of society's preexisting biases, HIV acts as a catalyst. This is what individuals refer to as HIV stigma. HIV stigma is the basis of the guilt felt by certain persons who are HIV positive. HIV stigma can manifest itself through a number of channels, each of them represent broader social perceptions. Because HIV is commonly regarded as a sexually transmitted virus, the stigma may rely on bigoted and other derogatory views about sexuality and sex; when behaviors exist that describe some sexual orientations as a standard and others as immoral and unusual, several sexual activities as natural and others as odd, the other one becomes victimized.

The beliefs about the number of sexual companions or the acceptable amount of sex for a person can associate with HIV stigma. This can provoke excessive gender specific discrimination. Particular groups in culture may have been more easily identified with HIV than others, and latent biases towards these groups may consequently be affiliated with an HIV diagnosis (Hutchinson & Dhairyawan, 2017).

An individual's feeling of poor self-esteem is the major source of depression, according to psychological concept of depression As a result, HIV associated stigma, mainly embodied stigma, is predicted to be directly linked with depressive expressions and self-condemnation in HIV-positive individuals, because ingrained HIV stigma illustrates how HIV positive individuals absorb gloomy HIV perceptions and emotions and bring those feelings like self-condemnation, embarrassment, regret, not seeing themselves as 'clean' and self-belittlement into intrapersonal contemplation and interpersonal experiences on an everyday basis (Turan et al., 2017). Regardless of the absence of national territory statistics, people who have HIV have often witnessed humiliation and unfair treatment, such as pressured housing relocation, only due to their health conditions, in more latest surveys. HIV stigma may have a bad impact on the contentment of those infected with HIV and others who are at risk of acquiring the virus. Stigma will socially separate people living with HIV and make it difficult for them to obtain social assistance. By thinking they are at fault for getting infected with HIV, people who are HIV positive may embrace the HIV stigma (Jeffries et al., 2015).

8. HIV IN TURKEY

In Turkey, the incidence of HIV infection is mediocre, but it is slowly growing. In 1985, the first ever HIV/AIDS occurrence in Turkey was recorded. Before any surgical procedure and blood donation, HIV monitoring is done, it is also in premarital screening for everybody, including licenced sex workers. Men who have sex with men, intravenous drug users and sex workers are primary risk groups. In Istanbul, HIV predominance within men who have sex with men is significant. The ratio is 12.7 percent, according to a recent survey. Between October 1985 and December 2018, there were 1736 cases of AIDS and 18,557 cases of HIV disease in Turkey, according to the Turkish Ministry of Health, from a population of 80.81 million individuals. Age segments of 30-34 and 25-29 years have the topmost pervasiveness.

As the primary factor of transmission, men population outnumbers women (78.2%), along with heterosexual sex. According to a multicenter cohort survey, late presenters with 350 cells/mm3 CD4 cell count or the ones with an AIDS-defining clinical conditions cover 52.4% of the patient population. In Turkey, HIV treatment is free of charge for all patients. The medicines are refunded by the government. In the region, CD4 and viral load (VL) tests are easily obtainable (Mete et al., 2019). Social convictions about infected people's actions, which conflicts with the social morals and ideologies of the individual making the disapproval, are the core of prejudiced views against HIV and AIDS. Actions are usually associated with socially unacceptable activities such as sexual intercourse or substance use. HIV-positive men may be thought to be bisexual, gay or to have had sex with a sex worker. Women with HIV, however, can be believed to be someone working in the sex industry, despite the fact that heterosexual interaction is responsible for nearly 80% of the HIV incidences in Turkey (Özakgül et al., 2014).

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Chapter 8

ENZYME BASED BIOSENSORS

Ümit YAŞAR[1]
Umut KÖKBAŞ[2]
Zehra Gül YAŞAR[3]

1 Ardahan Üniversitesi Nihat Delibalta Göle MYO, Laborant ve Veteriner Sağlık Bölümü
2 Nevşehir Hacı Bektaş Veli Üniversitesi, Diş Hekimliği Fakültesi, Tıbbi Biyokimya AD
3 Ardahan Üniversitesi Nihat Delibalta Göle MYO, Eczane Hizmetleri Bölümü

1. Discovery of enzymes and early studies

Organic analyzes of Neolithic remains show that humans have used enzymes to their advantage for thousands of years, for example to ferment sugars into alcohol (as early as 7000 BC). So how did we get to modern applications of enzymes that are at the forefront of chemical synthesis without knowing the basic principles of biocatalysis? (Table 1). In 1833, the first enzyme discovered was the enzyme diastase (a mixture of amylase). Subsequently, other hydrolytic enzymes such as pepsin and invertase were quickly discovered. The term "enzyme" was first used when it described the production of alcohol from sugar in yeast [1-3].

In fact, when the use of catalysis reactions and catalysis processes is considered in the history of society, there are many applications that have been used by constructions without realizing it for a long time. Examples of these applications are fermentation, cheese and soap.

In the following periods, scientists revealed catalysis and catalysis processes. The Swedish chemist Berzelius (1836) observed the existence of a "force", which he thought was responsible for the acceleration of the reactions, leaving the reactions unchanged and made the first early definition of catalysis. In time, catalysts have emerged in order to both reduce the cost of materials and products produced by the society and to produce them in sufficient quantity and quality [2-4].

Enzymes, also called biocatalysts, are catalysts that accelerate cellular reactions in living things. These biological molecules can be purified from living cells and these purified catalysts can then be used in catalysis processes under appropriate conditions. Looking at their basic structure, they consist of amino acid components. Each of these components has the same groups except for side groups (-R) and a hydrogen atom (-H). Groups that are the same are amino (-NH2) and carboxyl (-COOH) groups.The basic structure of all amino acids is the same, but their side groups differ in size, shape, electrical charge, and hydrophilic or hydrophobic nature. Amino acids attract and repel each other, forming twisted structures, which causes the formation of 3-dimensional (3D) structures of the enzyme. Some side groups of these 3D amino acids accumulate within a certain domain, forming an active site. The sites where biochemical reactions occur after the substrates and coenzymes bind together are called active centers [3-5].

Table 1: *Timeline of major developments in enzymology, molecular biology, and biocatalysis (Partially retrieved) [2].*

Year	Development
1833	Discovery of the first enzyme
1894	key lock model
1897	Discovery of cell-free fermentation
1905	Discovery of the first cofactor
1926	Discovery of enzymes and proteins
1934	Asymmetric microbial synthesis of L-PAC
1951	Identification of insulin sequences
1950s	First immobilization of proteins
1952	Discovery of plasmids
1953	Structural elucidation of DNA
1958	Identification of the first protein structure is reported
1966	Genetic code decoding
1968	Discovery of restriction enzymes
1972	Immobilized penicillin acylase used at Bayer for the manufacture of semi-synthetic antibiotics
1972	First Recombinant DNA
1977	DNA Sequencing
1970s	Glucose isomerase immobilization
1978	Regional mutagenesis
1978	Insulin: the first recombinant protein
1979	Recombinant expression of penicillin acylase
1980s	Polymerase chain reaction
1991	Directed evolution
1997	BASF lipase process to produce chiral amines
2010	Engineering a transaminase for the synthesis of sitagliptin
2016	C-Si bond forming enzyme engineering
2020	Nine-enzyme cascade to produce islatravir

1.1. The power of enzymes

In order to understand the enormous power of enzymes, it is important to first learn about their catalytic activity. The primary factor in enzyme kinetics, which is similar to a chemical reaction, is the reduction of the activation energy required for the reaction to occur. In addition, the "Turnover" defined in enzyme catalysis is the number of substrate molecules consumed by an enzyme per unit time, depending on the product. The preferred unit time for detecting this frequency is usually the number of substrates converted to seconds or minutes. For better understanding, turnover numbers of different enzymes per unit time are given in Table 2. Considering these results, the conversion success of enzyme molecules into products per second is remarkable.

Table 2. *Examples of enzyme and turnover frequency [5].*

Enzyme Name	**Turnover frequency (Mole Product S^{-1} mole $enzyme^{-1}$)**
Carbonic anhydrase enzyme molecule	6.10^5
Catalase enzyme molecule	93.10^3
β-Galactosidase enzyme molecule	2.10^2
Chymotrypsin enzyme molecule	1.10^2
Tyrosinase enzyme moleculeenzyme molecule	1

1.2. Catalysis specificity of enzymes

Although they have similar and group specificity (for example, alkaline phosphase enzyme catalysis using different substrates), it has been determined that enzymes with high catalysis power are actually enormous specific molecules. Therefore, they often catalyze a single biological reaction. The substrate molecule converted to the product in the catalyzed reaction is carried out only by the enzyme specific to it. One of the best examples of this specificity is glucose oxidase. This highly specific enzyme shows almost no activity with other monosaccharides. These properties of enzymes are used in many diagnostic and analysis studies in medicine. It is especially preferred in the development of biosensor devices. Thus, they can be used to measure the target analyte in samples containing many different analytes such as blood and urine (eg glucose measurement) [5].

1.3. Enzyme classification

When naming enzymes, reference can be made to the reaction they catalyze. However, the suffix –az is generally preferred in naming. According to the reaction of chymotrypsin and trypsin enzymes; Enzymes such as dehydrogenase and oxidase can be given as examples of general nomenclature. While naming, reference is made to the substrate molecule used by the enzyme molecule in the reaction. For example, pyruvate decarboxylase, glucose oxidase. In addition, some enzyme nomenclatures provide little information about reactions. For example, diastase, catalase.

Today, many enzymes and isoenzymes have been identified. The known types of enzymes are increasing day by day and their three-dimensional structures are being defined. This situation revealed the necessity of a more rational naming as it does not cause any confusion in the naming of enzymes. In order to eliminate this naming inconsistency, the International Biochemistry Association established the "Enzyme Commission". The Commission presented the first systematic approach to enzyme nomenclature in its report published in 1961. Enzymes, the number of which is increasing day by day, while there were 3200 different

enzymes in the 1992 report, today this number has increased to over 5000. The enzyme commission defines enzymes in four parts in this systematic nomenclature (Tables 3, 4, 5).

In this definition, the first part indicates the reaction catalyzed, the next part is the hydrogen donor, the third part is the hydrogen acceptor, and the last part is which enzyme in the current category. For example, in the lactate dehydrogenase enzyme, the enzyme commission number is 1.1.1.27. This example tells us that the Lactate dehydrogenase enzyme is the 27th enzyme in the NAD+ oxidoreductase enzyme category [5,6].

Table 3. *Enzyme classes by enzyme commission [5].*

First Enzyme Commission Part	Enzyme Class	Reaction
1.	Oxidoredutase	Oxidation/Reduction
2.	Transferase	Atom transfer / Group transfer
3.	Hydrolase	Hydrolysis
4.	Lyase	Group sticker
5.	Isomerase	Isomerization
6.	Ligase	Pyrophosphate bond breaking and incorporation into the molecule

Table 4. *Enzyme Classification: Secondary classes of oxidoreductase enzymes in the EC system [5].*

Oxidoreductase: Second Enzyme commission part	Electron/Hydrogen Donor
1.	Alcohol
2.	Aldehyde/Ketone
3.	-CH-CH
4.	Primary Amine
5.	Secondary Amine
6.	NADPH/NADH

Table 5. *Tertiary classes in enzyme Commission system oxidoreductases [5].*

Oxidoreductases: Third Enzyme commission part	Electron/Hydrogen Acceptor
1.	$Nadp^{+}$/ Nad^{+}
2.	Fe^{+3}
3.	O_2
4.	Other

1.4. Enzyme catalysis

Examines the changes in reaction rates of changes made on experimental parameters in enzyme kinetic studies. Measurement of enzyme activities in body fluids and tissues in medicine is an indispensable application of

biochemistry and analysis. The rate of a biochemical enzymatic reaction at a given temperature and pH depends on the enzyme concentration and the substrate concentration. It is expressed according to the amount of the product formed or the substrate converted to the product per unit of time (1 minute or 1 second) by the action of the enzyme. It is the rate of reaction catalyzed by the enzyme in micromoles. In other words, the rate of the reaction is directly proportional to the amount of active enzyme present in the medium.

The specific activity unit is used instead of the amounts of enzymes; the different rates of various enzymatic reactions are explained by the different activities or activities of the enzymes involved. Activity is the expression of the speed of the enzyme during catalysis to transform into a product in a certain time and optimum conditions with the effect of the enzyme. It is the number of units per milligram of protein. The most commonly used unit of enzyme activity is IU. 1 IU of enzyme activity refers to enzyme activity that, under optimal conditions, replaces 1μmol of substrate in 1 minute, which corresponds to the conversion of 16.67 nmol of substrate to product in 1 second. The number of substrate molecules converted to the product per unit time by a single enzyme molecule at optimal pH, 25 oC temperature and saturating substrate concentration is called the conversion number of the enzyme and is briefly represented by the symbol -kcat-. The specific activity for an enzyme is the number of enzyme units (IU or catal) per 1 mg of enzyme protein. The higher the enzyme purity in the biological sample for which the activity is determined, the higher the specific activity. [7].

In biochemical reactions, the reaction rate increases proportionally with the substrate concentration, then a limiting Vmax rate value is followed. Although residual substrate is added, the velocity increases less and less and remains constant at a given Vmax. The reaction rate at the moment when the enzyme molecules are saturated by the substrate molecules is called Vmax. Vmax is difficult to measure. On the other hand, in an enzymatic reaction, the substrate concentration can be measured at the point where half of the enzyme molecules are saturated with the substrate and thus the half-maximal rate (1/2 Vmax) is observed. The substrate concentration at the point where the half-maximal velocity is observed is called the “Michaelis-Menten constant” and is denoted by Km [5,7].

1.5. Enzyme inhibition

All drugs designed for inhibition of specific reactions can be detected using strategies based on enzyme inhibition. The rate of enzyme-catalyzed reactions can be reduced by some substances called inhibitors. Inhibitors

reduce the reaction rate by preventing the normal formation of the enzyme-substrate complex. Inhibition studies using inhibitors can provide information about the active site, substrate specificity and functional groups of the enzyme studied.

There are two types of inhibition, irreversible and reversible. In irreversible inhibition, the Inhibitor inactivates the enzyme by binding to the active center with covalent bonds; as an inhibitory substance substrate analog in reversible inhibition; They can cause inhibition by a) binding to the enzyme active center (competitive), b) binding of an enzyme inhibitor only to the complex formed between the enzyme and the substrate, (uncompetitive), or c) It can cause inhibition by binding to the inhibitory enzyme or ES complex from a point outside the active site (noncompetitive) [3, 8].

1.6. Enzyme immobilization

Enzyme immobilization has emerged to prevent the loss of expensive enzymes, to provide enzyme stabilization, to use stabilized enzyme molecules in biosensors, and to reduce both the shelf life and enzymatic response times of the developed biosensors. The use of stabilized enzymes can be in fluid or stationary states. Mixing the enzyme and support material under optimized conditions and after an incubation period are the main procedures for immobilization.

While designing the biosensor, the appropriate immobilization method should be selected depending on the desired biosensor usage. The classification of immobilization methods is summarized in Figure 1. Among the immobilization methods, cross-linking method, covalent bonding, trapping and encapsulation are the most studied methods. These methods have their own advantages and disadvantages [3,8].

Chemical modification of the enzyme is not required in the adsorption method; hence it is easy, usually cheap and recyclable. As disadvantages, it can be said that there may be poor binding and leakage of enzymes, poor and absent stability, and non-specific binding. The main advantage of the covalent bonding method is that there is a strong bond between the enzyme to be immobilized and the binder. It can be rational control of enzyme amount and microenvironment. As disadvantages, this method is often expensive and chemical modification of enzymes can result in reduced activity by the carrier. No chemical modification of the enzyme is required in the trapping and encapsulation method; so it can be an easy method. This method is more suitable for cells. On the other hand, trapping and encapsulation methods are mainly dependent on environmental conditions and may cause undesirable results in stability. There is no need for a carrier in the cross-linking method, which is another immobilization method;

There is a tight bond between the enzyme and the crosslinking agent. The main disadvantages of this method are the necessity to crystallize the enzymes and it is difficult to control the particle properties [3].

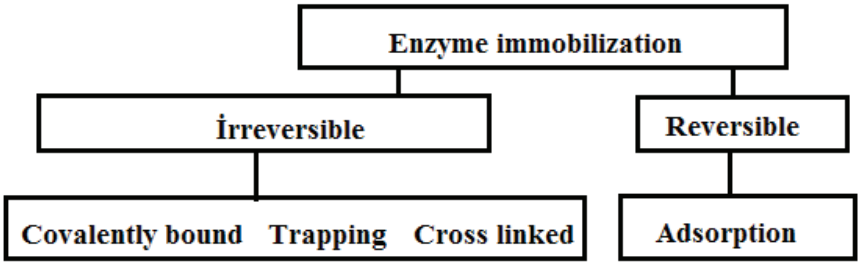

Figure 1. *Enzyme Immobilization [3].*

2. Understanding biosensors

Our nose, ears, eyes, tongue and fingers are prime examples of a sensor. The litmus paper used in laboratories is the most well-known type of sensor; It gives information about the acidity and alkalinity of a medium. Chemical or electrical responses are often converted into a signal that our eyes can observe. On the other hand, the electrical response that occurs as a result of potential changes in pH meters turns it into a response in digital indicators. The component of a sensor that processes such a conversion is called a "transducer". Biosensor classification according to transducer elements is shown in Table 6.

Table 6. *Biosensor classification according to transducer elements [3].*

Transducer system	Measurement mode	Typical application
Conductometric	Conductivity	Enzyme catalysis reactions
Enzyme electrode	Amperometric	Enzyme, substrate and immunological systems
Field effect transistors	Potentiometric (volts)	In ion, gas, enzyme, substrate and immunological systems
Ion selective electrodes	Potentiometric (volts)	Enzyme electrode, immunological electrode, ion and biological media
Gas-sensitive electrodes	Potentiometric (volts)	Gas, enzyme, organelle, cell and tissue electrodes, enzyme immunoelectrode
Impedimetric	Impedance	Enzyme imminosensor
Piezoelectric crystals, surface acoustic devices	Mass change	Volatile gas, vapors, immunological analytes
Optoelectronics, fiber optic devices	Optical	pH, enzyme substrate, immunological analytes

Thermistors, diodes	Thermometry/ colorometric	Enzyme, organelle, cell tissue substrate, analytes (vit, anibiotic.. etc).

3. Enzyme-based biosensors

Biosensors are analytical devices. These devices enable detection of target analyte with physical transducer and biorecognition elements. The most widely used biosensing materials in biosensors are enzymes. Enzymes have popular uses as bioreceptors (such as protons, electrons, light, and heat) as they can produce many measurable reaction products when they participate in chemical reactions. In enzyme sensors, enzymes are commonly used with amperometric, potentiometric, chemiluminescent and thermal transducers. In enzyme-based biosensors, it reacts selectively with the immobilized enzyme substrate. Redox enzymes that facilitate electron transfer in respiration and synthesis reactions are preferably preferred in enzyme-based sensors.

Redox enzymes are generally used in electrochemical biosensors. This is because they can convert biological stimuli into electronic signals in biochemical reactions. One of the best examples of this type of sensors is blood glucose detection sensors that improve the lives of billions of people. For example, the enzyme used in the biosensor for glucose measurement is the most studied and the most commercially developed. The system in the device is the catalysis of glucose by glucose oxidase (the formation of hydrogen peroxide and gluconic acid formed as a product) and the electron transfer signals that occur. The search for problems and solutions continues for the production of such a sensitive and stable bioelectrochemistry device.

The electronic connection between the redox enzymes and the electrodes can be realized according to different mechanisms in different generation biosensors. Particularly preferred linkages are: biosensors, biosensors based on the electroactivity of a substrate or product of the enzymatic reaction; Biosensors based on mediated electron transfer use redox mediators (relays), which are small electroactive molecules that move electrons between enzyme active sites and an electrode. These may be freely available mediators or may be attached to the side chains of their polymers. Biosensors that require an immobilized catalyst to re-oxidize (recycle) the freely diffusing electron acceptor; Biosensors that provide electrical communication via direct electron transfer (DET) from an enzyme active center to an electrode can be mentioned [9].

Biosensors have various applications in different fields. Health care, environmental monitoring, food analysis, defense and military applications are some of them. Enzyme-based biosensors can be used in the detection

of industrial toxins and food contamination, in the agricultural and food industry, in the detection of pesticides, glucose, ethanol in food beverages, in the detection of viral, fungal and bacterial diseases. Table 7; As can be seen from Figure 4, research on electrochemical enzyme-based biosensors has increased significantly in recent years for food and drug analysis [3,8,9].

Table 7. *Some recent applications of enzyme-based biosensors in the food and pharmaceutical industries (partially) [3].*

Destek/transducer	**Metot**	**Analit**	**Tespit sınırı**	**Uygulama**
AChE/Fe3O4eCH/GCE	CV	Carbofuran	3.6 nM	Cabbage
Lac/AuNPs/AuE	Square wave voltammetry	Formetanate	0.095 µM	Mango and grape
AChE/AuNPs-CSs/BDD	DPV	Methyl Parathion	$4.9x10^{-13}$ M	Cucumber juice
XOx/CHT/Pt/PANI/Fe3O4NPs/CPE	Chronoamperometric	Xanthine	0.1 µM	Fish and chicken meat
AChE/Pt@UiO66-NH2/GCE	DPV	Organophosphorus pesticides	4.9 _ 10_15 M	Human urine
GRO/AuNPs/PVA/HFB1/LDH/GCE	DPV, Chronoamperometric	Pyruvate	8.69 nM	Serum
MNPs/IrOxNPs/Tyr/SPE	Chronoamperometric	Methimazole	0.006 µM and 0.003 µM	Human serum
AChE/Chitosan/ZnO/Pt E	Cyclic Voltammetry	Melamine Urea	3 pM for Melamine, 1 pM for urea	Adulterated milk samples
Sol-gel/DAAO/AueNF/MWCNT/ GCE	Linear sweep voltammetry, Cyclic Voltammetry	D-alanine	20 nM	Human serum
PPO/RGO-AgNPs/Gr	Chronoamperometric	L-dopa	1.85 µM	Urine

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Chapter 9

BASIC LIFE SUPPORT (BLS) TRAINING AND SKILL ACQUISITION METHODS

Dilek YILDIRIM TANK[1]
Banu GÜLSOY[2]
Nurten TAŞDEMİR[3]

1 Araş. Gör. Dr. Dilek YILDIRIM TANK, Zonguldak Bülent Ecevit Üniversitesi Sağlık Bilimleri Fakültesi, Hemşirelik Bölümü
2 Banu GÜLSOY, Zonguldak Bülent Ecevit Üniversitesi Sağlık Bilimleri Enstitüsü Hemşirelik Anabilim Dalı
3 Doç. Dr. Nurten TAŞDEMİR, Zonguldak Bülent Ecevit Üniversitesi Sağlık Bilimleri Fakültesi, Hemşirelik Bölümü

INTRODUCTION

According to the World Health Organization, sudden cardiac death (SCD) is defined as natural death due to cardiac causes manifesting with the sudden loss of consciousness within one hour of the onset of acute symptoms (Koponen and Lantz, 2015). Sudden cardiac death occurs in more than 19 million people worldwide every year, and cardiovascular system diseases are the most common cause of death (Grandmaison and Geoffroy, 2006). In Europe, 350 to 700 thousand people die every year due to sudden cardiac arrest. Sudden, unexpected deaths, which we also call natural death, occur frequently outside the hospital, and 80% of them occur at home (Topuzoğlu, 2018). Sudden cardiac deaths are responsible for 15-20% of natural deaths and approximately 50% of cardiovascular deaths in the United States and Western Europe (Myerburg and Robert, 2001). According to the 2018 data of the Turkish Statistical Institute, it is reported that 38.4% percent of the deaths in Turkey are caused by circulatory system diseases (TÜİK, 2018).

After out-of-hospital cardiac arrest, the overall survival rates are 2-10% (Nolan and Jerry, 2015). Survival rates increase if trained individuals who witness cardiac arrest can initiate cardiopulmonary resuscitation immediately. Unfortunately, a tiny proportion of patients with sudden cardiac death find a chance for cardiopulmonary resuscitation, and only 1-3% of patients can be brought back to life (Myerburg and Robert, 2001). According to the International Committee for Resuscitation Liaison, Basic Life Support (BLS) is the provision of adequate circulation and respiration until advanced life support conditions are met to save lives and prevent worsening of the patient's disease in the event of cardiopulmonary arrest. BLS, which is carried out outside the hospital, includes interventions that are simple but require knowledge and experience, meet the emergency oxygen needs of the tissues by replacing the heart and lungs of the person, and prevent the irreversible damage that may occur in vital organs due to lack of oxygen, enabling the person to return to life (Uyanık, 2013). While the probability of survival of the individual is around 29% with the BLS procedure, which is initiated within the first four minutes of the patient, this rate drops to 7% in the interventions made after the fourth minute, and neurological damage begins in the brain in the anoxic period (Çetin and Nurdan, 2021). Therefore, early recognition and intervention of cardiac arrest increase the survival rate. While discharge is 10.81% in out-of-hospital cardiac arrests, it varies between 22.3-25.52 in in-hospital cardiac arrests. For this reason, international associations such as the European Resuscitation Council (ERC) and American Heart Association (AHA) carry out the necessary studies to increase the quality of basic life support and publish updated guidelines every five years.

Basic Life Support for the Adult Patient

Basic Life Support (BLS) is the effort to minimise the damage that may occur in the patient by noticing the person whose breathing or circulation is thought to have stopped and by providing the patient with sufficient air and heart pressure until the professional rescuers arrive.

Ensuring Environment Security

When a person lying motionless on the ground is seen, the first thing to do is to check whether the environment is safe and if there is an element that may endanger safety, remove it from the environment.

Questioning the Consciousness of the Patient

After the environment's safety is ensured, the rescuer should give a stimulus to the patient by shaking the patient's shoulders and asking loudly and clearly, "Are you okay?" If the patient responds, it means that there is breathing and circulation. If the patient does not respond to stimuli, it is assumed that he is unconscious and that the emergency response system is activated.

Evaluation of Respiration

The look, listen and feel method used in respiratory control was seen as a waste of time with the American Heart Association (AHA) update made in 2010, and it was stated that respiratory control should be done simultaneously while the patient's patient's consciousness status was questioned. Breathing is being evaluated, the patient is placed in the supine position and after the airway is opened, the patient is approached to his/her face and breathing is felt, presence of respiratory sound has listened, whether thoracic cage rises or not is visually monitored. If there is a gasping type of breathing, the patient is considered to have no breathing.

Providing Airway

Evaluation is made in terms of the presence of an object obstructing the patient's airway. If an object obstructing the patient's airway is noticed, the rescuer removes the object from the mouth by using his index finger like a hook.

If there is no obvious trauma, the patient is placed on a flat surface, and the head and chin position is given. If the patient has trauma, the patient is not moved as much as possible. In the position it is in, the head-chin position is given.Two different head and chin manoeuvres are used to ensure the patient's airway patency.

Checking the Heart Rate

In a patient who is thought to have Cardiopulmonary Arrest, the most immediate and practical pulse evaluation area is the carotid artery in the

lateral region of the neck. If the rescuer is trained in pulse assessment or has previous experience, he/she uses the index and middle fingers of the hand to evaluate the pulse for a maximum of 10 seconds from the carotid artery in the lateral region of the neck and considers whether the patient has a pulse. If the patient's pulse cannot be taken, the CPR procedure is started immediately with chest compression.

According to the latest regulation published in 2020, trained or inexperienced rescuers should practice CPR to the patient even if they cannot feel the patient's pulse fully and accurately. This update was carried out because the harm caused by using CPR on the patient in the studies is more harmless than CPR that is not applied to the CPA patient.

Activating the Emergency Response System

If the patient is unconscious and not breathing normally, the emergency response system is activated, and the scene and the patient's condition are reported to the experts with explicit expressions. The latest guidelines recommend remote use of the mobile phone in this case. Thanks to the mobile phone, the experts' instructions have been listened to, and the necessary interventions are implemented for the patient's survival until the team arrives.

Chest Compression

When it is understood that the patient does not have a pulse and the application of chest compression is certain, the patient is placed on a flat and hard surface in the supine position, if possible, and quickly kneels next to the rescuer. The heel of the dominant hand is placed in the middle of the rib cage (lower half of the patient's sternum). The freehand is placed on the hand placed on the chest of the patient and the fingers of both hands are interlocked. Care is taken that the fingers do not come into contact with the patient. By adjusting the distance between the patient and the patient so that the elbows and shoulders are straight and tense, the rescuer begins to apply chest compressions rhythmically at a depth of 5-6 centimeters with the help of his body weight. After each compression on the patient's chest, 100-120 compressions per minute are continued to allow the chest to relax. 5 cycles are continued with a heart pressure of 30 and 2 breaths, and at the end of the 5th cycle, pulse and respiration are checked for no more than 10 seconds. If there is no pulse or respiration, if there is a second rescuer, he continues to press the heart. In chest compression cycles, care is taken to change the rescuer to minimise time loss. If there is only one rescuer, he will perform the chest compression cycles. Chest compression cycles are continued until the automatic external defibrillator (AED) arrives, the rescuer is depleted and feels inadequate, the healthcare professional asks you to stop, or until the patient wakes up and begins to breathe normally.

AED Use

The rescuer immediately turns on the device and places the electrodes on the patient's chest as soon as the AED arrives. The AED is a device that guides the rescuer with both visual and verbal stimuli. After the electrodes are connected correctly, the device performs rhythm analysis. If the device gives a warning that shock is required, it is ensured that no one touches the patient and the shock button is pressed. After the shock delivery process is finished, the CPR process is resumed quickly as possible. If the device gives the warning that shock is not recommended, the CPR steps are renewed at 30:2, and the device's warnings are followed.

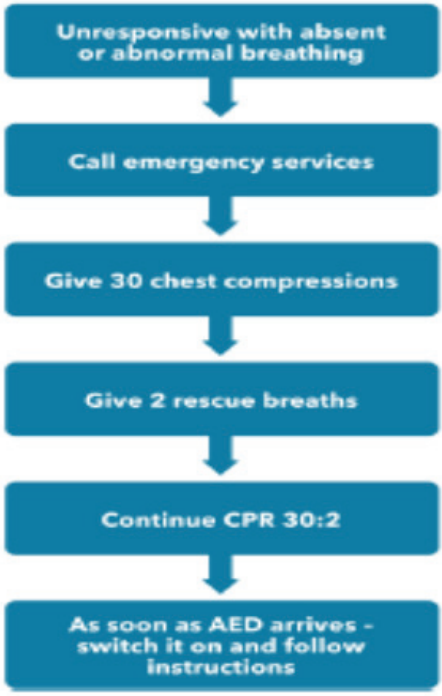

Fig. 1 BLS algoritm (ERC,2021)

Within the hospital, nurses are often the first professional group to define CPR, achieve successful cardiopulmonary resuscitation, and reduce death rates from sudden cardiac arrest (García-Suárez, 2019). Therefore, competence in basic life support is critical in recognising cardiac arrest, activating emergency systems, initiating effective CPR, and using the defibrillator safely (Perkins and Gavin, 2015).

The American Heart Association emphasises that healthcare team members should be competent in cardiopulmonary resuscitation practices

to reduce the rate of sudden cardiac arrest (Neumar and Robert, 2015). Providing basic life support education and practice skills training significantly increases nursing students' development, resuscitation success, and self-efficacy (Kose and Selmin,2019). Anxiety and lack of self-confidence are observed in nursing students who do not have sufficient skills in basic life support practices (García-Suárez,2019). Instructional techniques such as interactive videos and high-quality 3D simulation scenarios in CPR training are more effective in developing knowledge and motor skills than the traditional method of instructor-led instruction. (Boada and Imma,2015).

SKILL ACQUISITION METHODS

The traditional acquisition and retention of skills in BLS are based on theoretical and practical training on a mannequin or task-trainer, given by one instructor according to the following sequence: theoretical background in BLS, the chain of survival, correct CPR performance with emphasis on chest compressions, automated external defibrillator use, and accurate positioning of the victim after recovery. Training includes standardised assessment of performance (with feedback given by the instructor), either during the training with a form assessment, or at the end of the training, during which the key learning outcomes have to be successfully practised.

Fig. 2 BLS training with manikins and insructor

In education, inanimate mannequins and models used the application of practical knowledge provides. However, the applied model and post-application feedback from manikins only apply the CPR attempt (Türker and Tanrıkulu, 2020). In traditional CPR training, the decision of a student's proficiency typically depends entirely on judgments made by an instructor. Although there is a remarkable similarity when the trainers' data are compared with the data of the dummies recorded during CPR, the trainers' judgments alone are not reliable and sufficient to determine the compression proficiency of the students (Lynch, 2008).

Innovative technological environments such as e-learning, virtual reality, and simulation provide users with roleplaying opportunities, improving learning outcomes and helping to achieve permanent learning (Bogossianae et al., 2018; Durmaz Edeer and Sarıkaya., 2015; Smith and Hamilton, 2015; Tobase et al., 2017; Park and Yu, 2018). Simulator devices classified according to their technological level, virtual reality practices or haptic systems can be counted among these methods. The use of dummies with voice commands leads to a significant increase in the quality of CPR performed by nursing and medical students because it allows them to correct their mistakes or refresh their knowledge independently and is reported to improve their knowledge and skills (Nielsen, 2010).

It has been reported that intermittent video-assisted training after CPR training given previously to occupational groups other than the nursing profession does not adversely affect CPR quality (Hamilton, 2005). CPR training, which is carried out through different simulation programs with high accuracy, basically provides better results in gaining theoretical knowledge (Aqel, 2014).

Learning with high-quality simulation increases the effectiveness of education as it provides realistic environments and is student-oriented (Weidman, 2010).

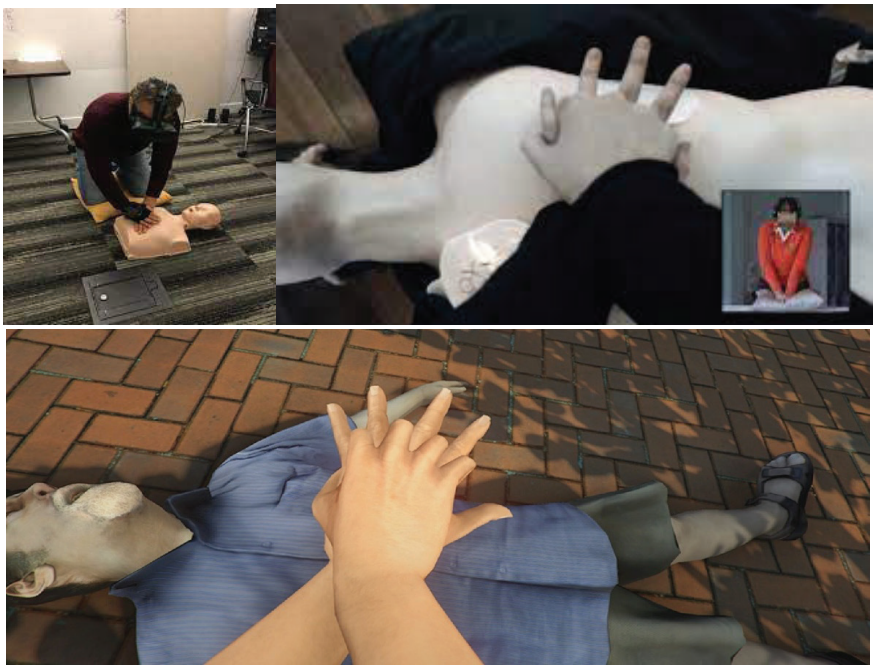

Fig.3 Examples of Virtual Reality Simulation in BLS

Tecnology Based Systems

For BLS, the main areas of interest are applications to locate AEDs, smartphones and smartwatches as an aid for first responder and providers to reach the patients, and CPR feedback in real-time and video communication for video dispatch. The new 'sci-fi' technology describes the potential impact of drones and artificial intelligence on the chain of survival. Smartphone and video communication play an important role in modern society. Traditionally, dispatchers give audio-only instructions; newly developed technology enables dispatchers to provide video CPR instructions through the caller's mobile phone.

Social media and smartphones apps for engaging the community Mobile phone technology is being increasingly used to engage bystanders in out-of-hospital cardiac arrest (OHCA) events (Semeraro et al, 2021) . There is growing interest among researchers in integrating smartphones and smartwatches in education and training in cardiopulmonary resuscitation and defibrillation, and for improving the response to OHCA with dedicated apps. Initially, apps were developed to provide educational content on resuscitation. Following the technological evolution of the last years, smartphone apps have been used to provide feedback on CPR quality by exploiting the built-in accelerometer. Such systems can provide real-time audio-visual feedback to the rescuer through the speakers and the screen.

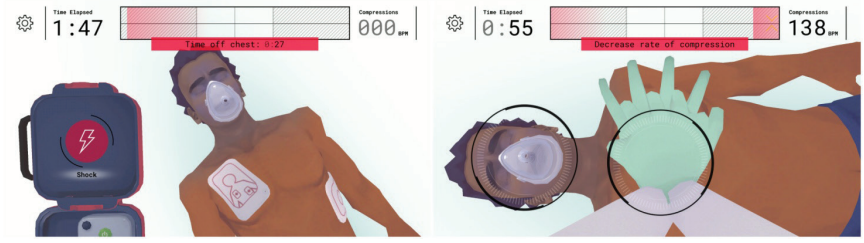

Fig. 4 Example of smartphone app for BLS

Low Resources (Low-Cost Materials/ Homemade Manikins)

The difficulty of accessing mannequins requiring high resources in educational environments where the transition to e-learning is attempted causes an increase in the existing knowledge and skills gap. In addition, studies have reported that the effectiveness of BLS training decreases over time. It is expected that the education method supported by e-learning and accessible materials will contribute to the permanence of education by allowing the application to be repeated independently of time, place, and expensive materials. Handmade models developed with low-cost, limited

materials for CPR training began to be discovered many years ago but were not widely used.

These models, obtained from recyclable materials, will also help raise awareness by drawing attention to recycling. Gozuen et al. (2020) also drew attention to this issue in their study and reported that CPR training with plastic bottle models was sufficient.

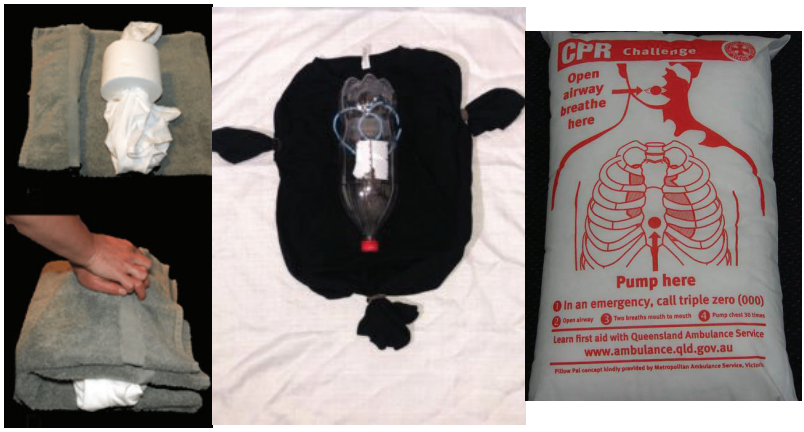

Fig. 5 Examples of BLS training with low resource materials (*pillow, plastic bottle, toilet paper)*

It is insufficient to evaluate the effectiveness of the training provided by the trainer using traditional methods with the help of dummies giving voice commands in the development of training skills, with the help of classical dummies that do not report data on CPR. Simulation training has a significant role in developing skills and increasing the quality of CPR. The knowledge and skills gained through simulation training should then be supported by video training. In addition, the quality and effectiveness of CPR should be increased by repeating CPR training, which nurses encounter more often in clinical settings and requires early intervention, in parallel with the updated information.

The rescuers need to grasp the information published by the European Resuscitation Council (ECR) and the American Heart Association (AHA) every five years regarding the patient's connection to life. Raising more trained rescuers in the community will increase the rate of correct and timely intervention.

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Chapter 10

DNA METHYLATION IN HEALTH AND DISEASE

Meliha Merve ÇİÇEKLİYURT[1]

1 Meliha Merve ÇİÇEKLİYURT, ORCID ID:0000-0003-4303-9717, Department of Medical Biology, Faculty of Medicine, CanakkaleOnsekiz Mart University, Turkey

Epigenetic programming is a heritable phenotypic variation resulting from DNA methylation, histone modifications, and RNA-mediated processes, structure without changing the DNA sequence (Dupont, Armant, and Brenner 2009; Feinberg 2018; Jackson et al. 2016). These epigenetic mechanisms are involved in many cellular process such as development (cell differentiation, parental imprinting, X chromosome inactivation), gene activity regulation (cell-specific gene expression, genomic stability and structure).That process starts from the embryonic period and continues until death. The epigenetic process is crucial for mammalian development and maintaining the specific functions according to the cell type.

Global methylation, especially on C (cytosine) base, which occupies specific positions in the DNA molecule, is the best-understood mechanism among epigenetic regulations (Bestor 2000). Methylcytosine is also called the fifth base, depending on the fact that most common DNA methylation in genomes occurs on the cytosine base. The current literature knowledge indicates cytosine methylation is related to many genetic functions, including timing of the cell differentiation, regulation of gene expression by gene silencing, imprinting, transgene expression as well as gene transposition, transcription and foreign gene expression in the cell (Hsu et al. 2021; Smith and Meissner 2013; Suzuki et al. 2006). Epigenetic mechanisms contain essential information about the environment and exposure of the living thing, and it provides the adaptation of the cell to the environmental environment(Ficz 2015; Jackson et al. 2016).

Generally, differentiated somatic cells have a stable and heritable methylation profile, but epigenetic reprogramming occurs in germ cells and preimplantation embryos (Reik, Dean, and Walter 2001; Xu et al. 2021). In early embryonic development, the massive demethylation process (hypomethylation) occurs immediately after fertilization; then, the epigenetic profile changes again after fertilization through implantation (Osman, Franasiak, and Scott 2018; Xu et al. 2021). After implantation, DNA begins to methylate again, and this process is logically compatible with embryonic pluripotency. That mechanism organizes the transcriptional order that regulates developmental gene activation by providing molecular memory to control the first cell fate until lineage-specific gene expression (Weinreb et al. 2020).

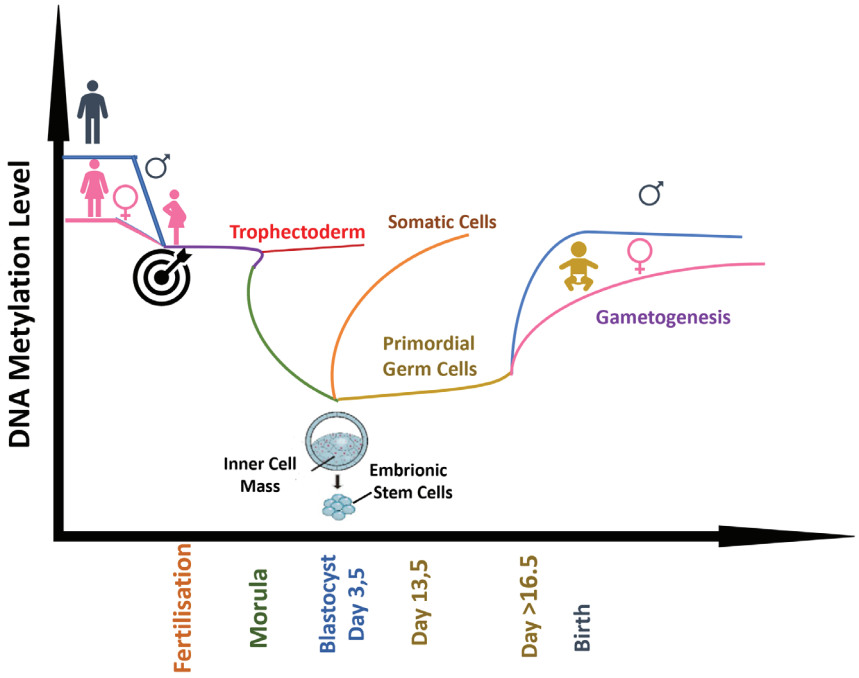

Figure 1 Remodeling of 5'methyl-cytosine during embryogenesis (Adapted from (O'Neill 2015))

The methylation profile of the genome at birth is not the same as in the 20s. Genomic and environmental factors change the methylation profile. These environmental factors include many factors such as stress, drug use, exercise, smoking and alcohol use, diet, toxins, pathogens. Several lines of evidence suggest that epigenetic alterations have a strong association not only with disease development also disease response. For that reason, DNA methylation can be a potent biomarker for disease status together with future therapeutic strategies(Cavalli and Heard 2019; Ling and Rönn 2019).

DNA methylation profile is formed as a result of the balance between methylation and demethylation. DNA methylation period includes the active operation of DNA methyltransferases and DNA demethyltransferases. DNA methylation is determined by many factors. The interaction of trans-acting receptors (TR) activates histone modification enzymes such as histone deacetylase (HDAC) and histone methyltransferase (HMTASE). When HDAC and HMTASE enzymes are activated, DNMTs and DNA-binding proteins are also activated (Szyf 1994). As a result of these activations and interactions, the methylation process begins. On the other hand, Trans-activating factors (TAF) activate histone acetyltransferases (HAT) of specific genes. Activation of HATs causes acetylation of histone

proteins. The tight chromatin structure begins to loosen, then due to the loosened chromatin structure, demethylase enzymes enter the chromatin structure and turn the balance in the direction of methylation(Razin 1998; Szyf 1994)(Figure 1).

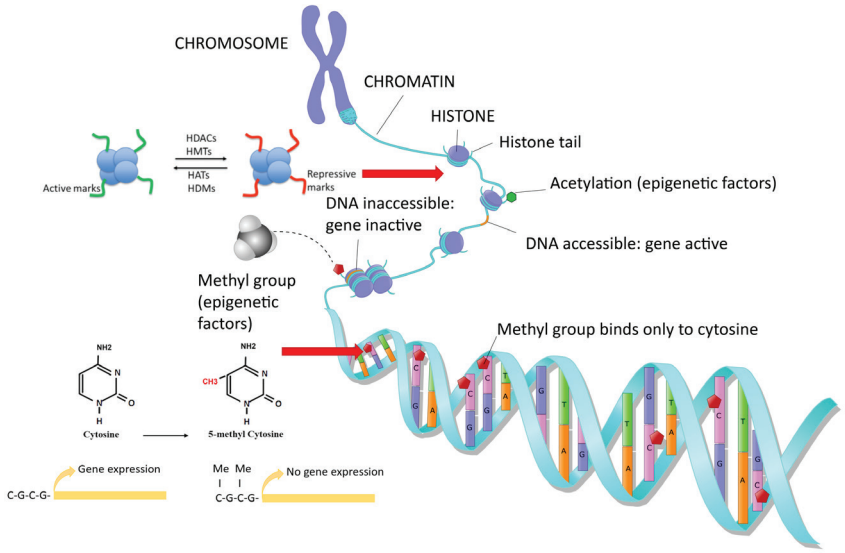

Figure 2 Model for epigenetic programming by histone modifications, global DNA methylation, and promotor-specific modifications. Epigenetic marking alters gene expression during the life cycle and physiological stress conditions.

DNA methylation is a covalent modification catalyzed by a family of DNA methyltransferases (Dnmts). These group of enzymes transfers a methyl group to the fifth carbon of a cytosine residue via from S-adenyl methionine (SAM) to 5mC(Cao, Zhang, And Du 2013). The attached methyl group does not affect base pair formation, however, it affects DNA-protein interaction within the major groove of DNA(Momparler and Bovenzi 2000). Nowadays, five identified DNA methyltransferases are Dnmt1, Dnmt2, Dnmt-3a, Dnmt-3b, and Dnmt-3L.

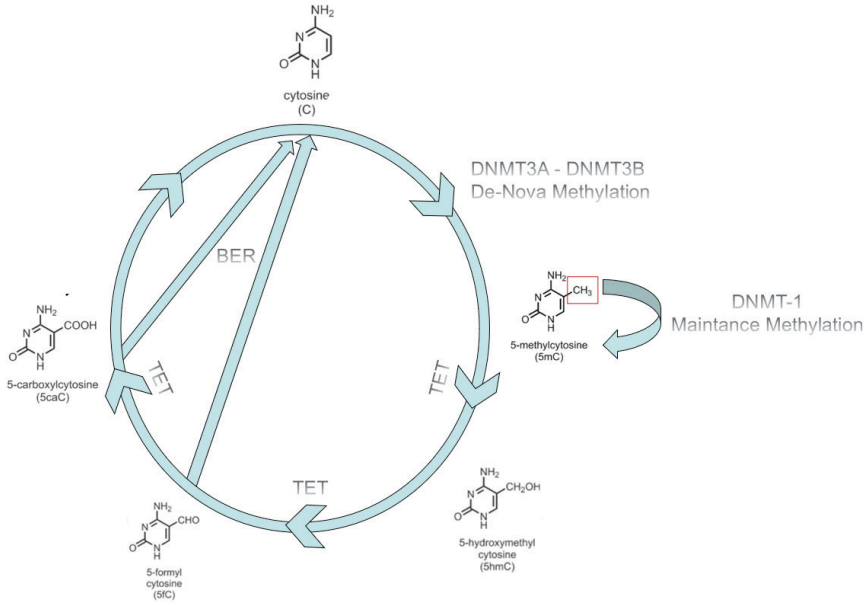

Figure 3. DNA methylation pathways. A family of DNA methyltransferases (Dnmts) (a) Dnmt3a and Dnmt3b are de novo Dnmts and (b) Dnmt1 " maintaining the DNA methylation"enzyme.

The mammalian DNA methyltransferases, maintenance methyltransferase "Dnmt-*1"* and de-nova Dnmt-*3a*/Dnmt-*3b* are essential for mammalian development and active during embryonic development (Okano et al. 1999). During replication, Dnmt1 preserves the methylation profile by transferring the methylation profiles of the synthesized to the newly synthesized one in the same way. In general, the Dnmt1 enzyme consists of two parts; the first is a relatively conserved carboxy-terminal region and an amino-terminal region(Bestor 2000). The carboxy-terminal area plays an essential role in catalytic activity, nuclear localization, and protein-protein interactions(Bestor 2000; Robertson 2001). The feature of the carboxy-terminal region is that it contains catalytic activity for all DNA methyltransferases. The region at the C terminal binds to unmethylated DNA, the region at the N terminal binds to methylated DNA(Bestor 2000; Robertson 2001).

Although Dnmt-2 is structurally similar to DNA methyltransferases, it uses RNA, not DNA, as a substrate. The Dnmt-2 gene generally encodes a protein responsible for methylation of the aspartic acid tRNA at the cytosine-38 residue in the anticodon cycle(Goll et al. 2006).

Dnmt-3 has different isoforms; Dnmt-3a, Dnmt-3b, and Dnmt-3l. The Dnmt-3a is a classical DNA methyltransferase enzyme that adds a methyl group to the cytosine base found at CpG sites in DNA

and is responsible for de-nova methylation(Jurkowska, Jurkowski, and Jeltsch 2011). Its particularly involved in cell differentiation, embryonic development, transcriptional regulation, heterochromatin formation, and X-inactivation(Chédin 2011). Dnmt-3l is a cofactor for Dnmt-1, Dnmt-3a, and Dnmt-3b and is active in embryonic and germ cells, responsible for the immobilization of male germ cells (Barau et al. 2016).

Two different DNA methylation patterns can be observed in disease pathogenesis. The first one, global DNA methylation status changes (global hypomethylation), and another one promotor specific rmethylation. The global DNA methylation machinery is strongly linked with cell cycle control by transcriptional regulation and chromatin structure, so alterations in the process cause neoplasm (Madakashira and Sadler 2017). Normally, tissue-specific active genes are hypomethylated in functional tissues and hypermethylated in non-functional tissues. Tissue-specific house-keeping genes are hypomethylated, thus expression is continuously throughout life. Previous studies have been shown that aberrant or accidental methylation of CpG islands in promotor region of tumor supressor genes or hypomethylation of oncogenes trigger tumoregenessis (Jiang et al. 2013; L. Lin, Cheng, and Yin 2020; Van Tongelen, Loriot, and De Smet 2017).

The hypomethylation status causes genomic instability, whereas hypermethylation of the promotor region causes the inactivation of the gene(L. Lin et al. 2020). DNA methylation usually occurs in G+C isochores and the CpG hot region, which is in the linear 5′ to 3′ sequence of DNA(Bird 2002; Jones 2012). The CpG islands are generally located in the first exon in the promoter regions of genes, and approximately 3-4% of the cytosine bases in the genome are methylated. CpG islands are not highly methylated because they are in the promoter region. If those CpG islands are methylated, its means the methylated region represses the transcription of the gene expression. However, in cases such as aging, metabolic disease, and cancer, the methylation status of these regions are changed to hypermethylation or hypomethylation(Beggs et al. 2013; Jones, Goodman, and Kobor 2015; L. Lin et al. 2020; Ling and Rönn 2019; Wei et al. 2016).

Thus mutations in DNA methyltransferases results in diseases such as ICF (immunodeficiency, centromere instability, facial anomalies) Rett syndrome, ATRX (X-linked, α-thalassemia mental retardation), and fragile X syndromes (Robertson 2002). At the end of 1969, it was observed that there was a decrease in 5-mC numbers in tumor cells. This condition, called undermethylation of DNA (hypomethylation), has been demonstrated in the genomes of benign and malignant tumors. If hypermethylation in CpG islands occurs in the promoter region of the tumor suppressor gene region, the promoter activation is blocked and this triggers the blocks tumor suppressor gene expression. Especially, hypomethylation in repeat

sequences of the genome cause activation of the gene and has been implicated in tumorigenesis (Jiang et al. 2013).

Cancer Development and DNA Methylation

Tissue-specific active genes are hypomethylated in functional tissues and hypermethylated in non-functional tissues. Tissue-specific house-keeping genes are hypomethylated throughout life.Currently, we known that neoplastic cells contain 20-60% less methylated DNA when compared to normal cells. This means hypomethion trigger to re-programming of the genome to break the regulatory tools. DNMT1 is linked with maintance and de-novo methylation of tumor supressor genes. Also, its reported that DNMT1 overexpression cause de novo methylation in cancer cell lines.

Nowadays, its important to profile the global methylation and expression analysis of the specific-type cancer genes. Both genome-wide and gene specific metylation status highlighted to cancer state. For example, genome-wide de novo metylation as well as gene specific hypomethylation status have been demostrated in many experiments for ovarian cancers (Michaelson-Cohen et al. 2011) Matsumura and collegues have been shown that 68 genes metylation status changes due to tumor histology(Matsumura et al. 2011). Also, both ATG4A and HIST1H2BN gene hypomethylation initate ovarian cancer (Liao et al. 2014).

Epidemiological evidence suggests that the human diet directly associated with the colorectal cancer development. For taht reason, the epigenetic hallmark of colon cancer is widely studied both in changes due to the stage of colon cancer and nutrition-specific methylation. S-adenosyl methionine reactions is dirven by folat, thus folic acid supplementation is possitively correlate the enzyme activity and increases DNA hypomethylation indirectly. In that frame, folic acid supplementation and colon cancer initiation was investigated 3 different human colorectal cancer (CRC) cell lines (HCT116, LS174T, and SW480) and shown the association between folic acid supplemantion and colon cancer (Farias et al. 2015). Similar to previous study, Boughanem and collegues ahce been shown that low serum vitamin B12 status cause global high DNA methylation through long interspersed nuclear element-1 (LINE1) in colocteral cancer patients(Boughanem et al. 2020).

The current knowledge on aberrant methylation profile both in global and gene-specific methylation of DNA illustrated that global and gene-specific methylation status able to use as biomarker for cancer stage (Micevic, Theodosakis, and Bosenberg 2017).

Neurological disease-linked alterations in DNA methylation

Baets and collegues has been reported DNMT1 mutations cause aggresome-induced autophagy related protein degregation and trigger central and peripheral neurodegeneration which results neurological disorders(Baets et al. 2015). Hutnick and collegues have been shown that DNMT1 specific conditional knowck-out mice has 20-30% lifespan hypomethylated DNA and cause hypomethylation in cortical and hippocampal neurons which meaans neuronal cell death induced neurobehavioral defects in learning and memory behaviours in adult mice(Hutnick et al. 2009). Extensive studies examining aging and age related neurodejenerations was highlighted the link between global metyhlation and neurologic disorders. Nowadays, its speculated that age related DNA methylation changes can be use as a potential chronological age marker to evaluate health status of a people(Xiao, Wang, and Kong 2019).

The lower B12 and folate and high homocyctein level in blood of patients with Alzheimer disease associated with the S-adenosylmethionine cycle and age-related increase in neurological disease results(Coppieters and Dragunow 2011) thus extensive studies has been performed metylation status of patients of Alzheimer disease(Coppieters and Dragunow 2011; Scarpa et al. 2006). As a results of animal and clinical studies modifications in DNA methylome especially mC profile of genomic DNA is linked with Alzheimer disease. Francesco and collegues shown that increase global DNA metylation profile due to higher DNA methyltransferase 1 (DNMT1) gene expression and protein levels in human peripheral lymphocytes(Di Francesco et al. 2015).

Chouliaras and collegues have been shown that both DNA methylation and hydroxymethylation markers, 5-methylcytidine (5-mC) and 5-hydroxymethylcytidine (5-hmC), is decreased in the hippocampus of AD patients when compared to healthy controls(Chouliaras et al. 2013). Bollati and collegues has been observed the similiar results. bisulfite-PCR and pyrosequencing of of Alu, LINE-1 and SAT-α sequences is compared between Ad patients and healty controls. As a results, LINE-1 hypermethylation is obeserved in AD patiensts blood sample(Bollati et al. 2011). The similiar results has been shown in LINE-1 repetative sequences in late-onset Alzheimer disease (LOAD) in Colombian patients(Hernández et al. 2014). Depend on the results of the repetitive elements methylation it could be speculated global metylation status may be use as non-invesive marker for AD disease. Grossi and collegues have been reported promoter methylation levels of PSEN1, BACE1, DNMT1, DNMT3A, DNMT3B, and MTHFR are linked to circulating levels of folates, hcy, and vitamin B12(Grossi et al. 2016, Stoccoro and Coppedè, 2018). In contrast, gene

specific methylation of myloid-beta peptide production (PSEN1 and BACE1), DNA methyltransferase (DNMT1, DNMT3A and DNMT3B), and Methylenetetrahydrofolate reductase (MTHFR) have not shown difference between AD patients and healthy controls(Tannorella et al. 2015).

Depression is the most common psychiatric disorder in the world. It is thought to be the disease that will contribute the most to the disease burden in the world by 2030. Despite the use of antidepressant drugs from many different groups, 30-40% of patients still do not respond to treatment. Despite extensive research, the exact cause of depression at the neurobiological level and the mechanism by which it can be treated are not fully understood. None of the gene is directly associated with depression in GWAS studies(de Moor et al. 2015; Otowa et al. 2016). The enviromental stress able to cause epigenetic alterations thus early or adult stress factors may be trigger depressive disorders (Chmielewska et al. 2019). In the literature, DNA metyhlation mechanism is linked with depression(Bakusic et al. 2017; Januar, Saffery, and Ryan 2015; Story Jovanova et al. 2018). Meta analyses of the genes related to depression illustrated CDC42BPB, ARHGEF3 genes and intergenic CpG site associated with major depression in eleven population who European and African originated (Story Jovanova et al. 2018).

Massive epigenetic changes occours during embriyonic development and intrauterin life. Maternal life style and stress are major player that effects the embriyogenesis thus maternal depression has influence of prenatal environment. In a study, maternal depression is found increased methylation level of the promotor region of glucocorticoid receptor (NR3C1) gene in cord bood samples(Oberlander et al. 2008). Lester and collegues have been shown that NR3C1 methylation status in placenta is associated with poor neurobehavioral integrity and stress response(Sheinkopf et al. 2016). Nemoda and Szyf indicated changes in DNA methylation in the promoter of the glucocorticoid receptor and the serotonin transporter positively correlated with maternal depression in different tissue samples of newborns(Nemoda and Szyf 2017). Maternal depression is another issue that has big impact on infacts' behavioral, emotional, and cognitive development, thus post-partum depression (PPD) is important both for mother and her newborn(Payne and Maguire 2019). Oxytocin is a neuropeptide known to function in uterine contraction at birth and milk ejection during breastfeeding. Exogenous oxytocin has been found to affect social relations and bonding behavior in primates through the central nervous system. Kimmel and collegues have been shown that DNA methylation variation in the Oxytocin-receptor (OXTR) is directly linked with postpartum depression(Kimmel et al. 2016). Bell

and collegues' pyrosequencing experiment shown the OXTR metylation status is associated with both genotype of the mother and PPD(Bell et al. 2015). In addition, OXTR exon 1 DNA methylation status decreassed in female patients with depression(Reiner et al. 2015).

Metabolic disease-linked alterations in DNA methylation

Overweight and obesity, the prevalence of which is increasing in the world, can be defined as "increase in the amount of fat that poses a risk to health". Musculoskeletal diseases, hypertension, coronary heart disease, diabetes mellitus (type 2) development and an increase in the incidence of some malignancies are observed in obese individuals. In addition, psychological disorders arising from obesity occur in obese individuals, and their self-esteem weakens. Knowledge of obesity-related risk factors is essential for prevention and treatment. Alterations in the specific genes directly related to obesity, changes occur in the carbohydrate and fat metabolisms of individuals. Nutration also influence the epigenetic regulation of the genes. In an example, folate, vitamin B12, choline, betaine and methionine can be donors to provide methyl groups, thus maternal diet linked with methylation and methyl donors in utero modifies gene expression and is passed on to new generations(Johnson and Belshaw 2008). Interactions between nutrition, gene and environment constitute one of the most important environmental parameters. Fernandez-Twinn speculated that obesity is linked with intrauterine environments which influence epigenetic modifications and results obesity and diabets(Fernandez-Twinn et al. 2019).

Today, cardiovascular diseases are the most common cause of death in developed countries. It has been shown that oxidative stress and inflammatory processes are closely related to cardiovascular diseases such as the development of atherosclerosis, ischemic injury and IR injury. Several cancer related studies has beeen illustrated DNA methylome of cells is directly linked with oxydative stress(O'Hagan et al. 2011; Weitzman et al. 1994). In this perspective, changes in DNA methylation profile trigger atherosclerosis and ischemic injury throught inflammatory processes(Tabaei and Tabaee 2019; Tao et al. 2021). Zaina and collegues have been shown that HOXA6, HOXA9, MIR23b, PDGFA, PLAT, PRRX1, and PXDN genes promotor CpG metylation differs between normal and atherosclerotic aortic human tissue(Zaina et al. 2014). Another study reported that atherosclerosis-linked DNA methylation has been observed in ACTA2 (aorta α2 smooth muscle actin), ELN (elastin), MYOCD (myocardin), C9orf3 (miR-23b and miR-27b host gene), and MYH11 (smooth muscle myosin) genes (Lacey et al. 2019). The enviromomental exposure of l ead (Pb) or cadmium (Cd) linked with atherosclerosis previously. Lin and collegues has been demostrated that Pb exposure cause changes in global

DNA metyhlation, and that mechanism trigger Pb-associated subclinical atherosclerosis (C.-Y. Lin et al. 2020).

Discussion

Epigenetic alterations in disease is currently popular topic to both understand disease mechanism and investigate personal response to disease treatment. In addition, the epigenetic effects of various drugs on living cells are a subject of many research. In the future, epigenetic markers can be used as preventive, diagnostic, and therapeutic markers.

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Chapter 11

THE RELATIONSHIP BETWEEN BRAIN AND INSULIN[1]

Pınar BAYRAM

Selina AKSAK KARAMESE

1 Kafkas University, Faculty of Medicine, Department of Histology and Embryology, Kars, Turkey.; pinar.bayram@yandex.com ORCID: 0000-0001-9924-7051; selin.atauni@gmail.com ORCID: 0000-0002-4820-2096.

History of Insulin

The first disease that comes to mind when insulin is mentioned is indisputably diabetes. It is a fatal disease that has existed since ancient times. The Greek physician Areteus was first described it as "a melting down of the flesh and limb into the urine… life is short, disgusting and painful… thirst unquenchable … the kidneys and bladder never stop making water… it may be something pernicious, derived from other diseases, which attack the bladder and kidneys" (Ghasemi, Haeri, Dargahi, Mohamed, & Ahmadiani, 2013).

In 1869, Paul Langerhans announced that the pancreas consists of two cell systems. In 1889, two German physiologists found signs of diabetes in dogs whose pancreas had been removed, demonstrating the role of the pancreas in diabetes. Since then, many researchers have been tried to use pancreatic extract for the treatment of diabetes. First, the entire pancreas was given to the patients. Then, Rennie and Fraser isolated the Langerhans islands from the pancreas of fish; boiled the extract and applied it on patients; however, it did not give satisfactory results (de Leiva Hidalgo, Brugués, & de Leiva Pérez, 2009).

Eugène Gley performed some studies to support his hypothesis that "the Langerhans islands secrete a substance which can prevent the urinary excretion of glucose", and used diabetic and pancreatectomic dogs to prove this hypothesis. He even injected the two sources separately to find out whether the effect was due to the exocrine part of the pancreas or the Langerhans islands. He noted that when he gave aqueous pancreatic extract to dogs, glycosuria decreased and their diabetes symptoms improved significantly. He wrote down his findings and sent the "Société Francaise de Biologie" in 1905, but sealed it so that it would not be opened until he asked. After 17 years, Banting and his colleagues repeated the same experiment and they isolated insulin. Upon hearing this news, Eugène Gley realized that he had accidentally discovered insulin, then asked for the letter to be opened (Vecchio, Tornali, Bragazzi, & Martini, 2018). However, 18 months after the first patient treated, Banting and his friends were awarded the Nobel Prize in 1923 for the discovery of insulin (Ghasemi et al., 2013).

Insulin and Brain

Since it was thought that insulin could not cross the blood-brain barrier (BBB), the idea that the central nervous system (CNS) was insulin-independent was dominant. Therefore, the effects of insulin on glucose metabolism in the brain have not been adequately studied. After it was proven that insulin can cross the BBB and affect the CNS, Havrankova et al. showed insulin receptors in the CNS on rats in 1977. He was then continued to investigate whether the insulin in the CNS was produced by the

neurons themselves or not. The analyzes revealed that insulin or a peptide very similar to insulin is found in the CNS (Havrankova, Schmechel, Roth, & Brownstein, 1978).

In a review published by Plata-Salamán in 1991; Insulin in the cerebrospinal fluid (CSF) was thought to be the local synthesis of the CNS. In addition, the possibility that insulin can pass from the peripheral blood to the brain via the BBB and circumventricular organs has also been discussed. It has also been hypothesized that the choroid plexuses forming the blood-CSF interface may be a nonspecific pathway for rapid insulin transport to the CSF. Additionally, it has also been mentioned that insulin can pass from CSF to peripheral blood by absorption of arachnoid villus, so that insulin transfer may occur bidirectionally both from blood to brain-CSF and from brain-CSF to peripheral blood (Plata-Salaman, 1991).

Following studies had focused on investigating the source of insulin in the CNS. Higher levels of C peptide and immunoreactive insulin were detected in human cadavers. In addition, C peptide has been found in the brain tissues of elderly people and Alzheimer's patients, and the link between decreased insulin receptor numbers and C peptide has been demonstrated for the first time. Insulin II mRNA (secreted during the pre-pancreatic period of embryonic development) has been demonstrated in fetal, neonatal and adult rat brains by RT-PCR (Real-time Polymerase Chain Reaction) technique. It has been demonstrated that insulin-like mRNA and insulin-like substance, which are no different from real insulin, are produced and secreted in fetal neuron cultures by immunohistochemical techniques and in situ hybridization (Blazquez, Velazquez, Hurtado-Carneiro, & Ruiz-Albusac, 2014). Insulin mRNA was determined in neuroglia cells in the rat cortex by single-cell quantitative RT-PCR studies (Gray, Meijer, & Barrett, 2014).

Brain Glucose Utilization

The brain is the body's main consumer of glucose. Although the brain is only 2% of body weight, it uses 20% of the energy originated from glucose (Mergenthaler, Lindauer, Dienel, & Meisel, 2013). The uptake of glucose in peripheral tissues is insulin-dependent. When blood glucose level increases, insulin is secreted from pancreatic beta cells and peripheral tissues take up glucose via insulin-sensitive glucose transporters (GLUTs) and blood glucose level decreases. The glucose uptake of brain is insulin independent in contrast to peripheral tissues and takes the glucose it needs from the blood. After glucose crosses the BBB, it enters into the cell via GLUTs (Banks, Owen, & Erickson, 2012).

Brain prefers glucose instead of using other energy sources such as fatty acids, amino acids. Brain competes with muscle and adipose tissue

for using the current glucose. It is the most important for brain to win this competition considering the only energy source of the brain is glucose. Astrocytes, which are glial cells of the CNS are the biggest supporter of brain to use glucose as an energy source (Fehm, Kern, & Peters, 2006). Astrocytes surround the capillaries in the CNS with their foots. GLUT1 is abundant in the foot processes of astrocytes. When energy demand occurs in the brain, glutamate is released into the synaptic cleft by excitatory cortical neurons. In astrocytes, one molecule of glucose is taken into the cell with each glutamate. Thus, astrocytes play a crucial role in the "energy on demand" process (Peters et al., 2004).

GLUT1 is expressed by SLC2A1 gene. It is found in almost every tissue with different expression levels in different cell types. GLUT1s in the brain have isoforms that may vary according to the region where they found in. 45 kDa isoform of GLUT1 is expressed in astrocytes and oligodendrocytes (De Vivo et al., 1991), while 55 kDa isoform is expressed in capillary endothelium (Vannucci, 1994). GLUT1s are not expressed in neurons.

GLUT2 is expressed by SLC2A2 gene. In brain, GLUT2 is less expressed than GLUT1. Many studies have been performed about the areas where GLUT2 is localized in the brain. It has been reported in limited studies that GLUT2 is found in astrocytes, tannicides and limbic areas (Arluison et al., 2004; Dwyer, Vannucci, & Simpson, 2002; Garcia et al., 2003; Manolescu, Witkowska, Kinnaird, Cessford, & Cheeseman, 2007; Young & McKenzie, 2004).

GLUT3 is expressed by SLC2A3 gene. Its expression level is higher in the brain compared to muscle and adipose tissue (Bell, Burant, Takeda, & Gould, 1993). GLUT3 is expressed primarily on the cell membrane and is "insulin-insensitive" (Scheepers, Joost, & Schurmann, 2004). It is expressed in neurons' soma, axons and dendrites (Simpson et al., 2008). Mentioned neurons are found in neocortex, thalamus, midbrain, cerebellum and ventricles ependymal layer (Simpson, Carruthers, & Vannucci, 2007).

GLUT4, an insulin sensitive transporter, is expressed by SLC2A4 gene. It is particularly sensitive to insulin and increases the expression of insulin in the brain. It has been stated that GLUT4 is localized in neurons of hippocampus, hypothalamus, and motor cortex regions (Piroli et al., 2007).

GLUT5 has a low glucose transport capacity; however, it has a high capacity of fructose transportation (Funari, Crandall, & Tolan, 2007). A study have shown that fructose is used as an alternative energy source when there is not enough glucose in the brain (Shu, Isenberg, Cormier, Benz, & Zorumski, 2006). GLUT5 is expressed in brain ependymal cells,

Purkinje cells of the cerebellum, microglia and a few astrocytes. It is also expressed in neurons' dendrite, presynaptic and postsynaptic membranes (Kojo, Yamada, & Yamamoto, 2016).

GLUT6 is expressed by the SLC2A6 gene. There are limited studies about this protein. Martinez et al. showed in their study that it is expressed in both hypothalamic β2 tannicides and ependymal cells. They also stated that it plays a key role in transferring glucose from the CSF to the hypothalamic ventricular spaces (Martínez, Cifuentes, Tapia, & Nualart, 2019).

GLUT8 is expressed in hippocampus, cerebellum, hypothalamus, thalamus and cerebral cortex (Schmidt 2008). GLUT8, an intracellular transporter, is also localized in the cytoplasm of choroid plexus ependymal cells, vasopressin in the secretory granules of neurons, and nerve terminal endings (Mashima et al., 2017).

GLUT10 is expressed by the SLC2A10 gene, and its amino acid sequence is nearly identical to its GLUT9 homologue; however, it is longer than other members of GLUT family. The expression of GLUT10 in the brain, were shown by northern blotting. The function and localization of this protein in brain is not known (Doege, Bocianski, Joost, & Schurmann, 2000).

Brain-Insulin Signaling Pathway

Insulin supports neuronal surviving, sustains in synaptic plasticity and regulates memory, cognition, learning and mood. Insulin resistance is defined as "the failure or decrease the response to insulin". Central or peripheral insulin signaling occurs by binding insulin to the insulin receptors (IR) and phosphorylation of IR. Insulin binds to the α-subunit; the α-subunit activates the β-subunit; at the end of this activation the insulin receptor substrates (IRS) is phosphorylated (Zhao, Chen, Quon, & Alkon, 2004). The subunits of IRs in the CNS have lower molecular weight than peripheral IRs (Akintola & van Heemst, 2015).

Neurotrophic factors and hormones that ensure long-term survival of neurons, protect their integrity and prevent their loss, protect the brain, and insulin-like growth factor 1 (IGF1) plays a key role in these processes (De Magalhaes Filho et al., 2017). IGF1 is produced locally in the brain and can cross the BBB. Insulin and IGF1 are similar in their structural and functional functions, so they can also bind and activate both IR and insulin-like growth factor receptor IGF1R (Bedse, Di Domenico, Serviddio, & Cassano, 2015).

IR signaling activates two different intracellular signaling pathways. The first one is the canonical PI3K and protein kinase B (Akt) pathway.

The second one is the mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) pathways (Petersen & Shulman, 2018). The PI3K/Akt pathway promotes neuronal growth and survival (Long et al., 2021). The MAPK/ERK signaling pathway promotes differentiation and migration of neurons (Albert-Gasco, Ros-Bernal, Castillo-Gomez, & Olucha-Bordonau, 2020).

Although there are some methods to measure the peripheral insulin sensitivity, it is impossible to use those methods in the brain. As it is known, accessibility to the brain is difficult, so there is no parameters that can define brain insulin resistance yet (Talbot, 2014). There are also differences between studies and biomarkers which are used for evaluation the brain insulin resistance. The imbalance between brain insulin receptors is used for proving the development of brain insulin resistance (Belfiore et al., 2017).

Preclinical studies with obesity have suggested that free fatty acids (FFA) which trigger obesity, impair insulin signaling and then brain insulin resistance occurs by triggering inflammation (Sripetchwandee, Chattipakorn, & Chattipakorn, 2018). Diaz and colleagues established that saturated free fatty acids (sFFAs) induced insulin resistance in the brain. They incubated the hypothalamic neurons with palmitate. Then, they found a reduction in phosphorylation of insulin signaling protein (Diaz et al., 2015). In a study performed with rats fed a high-fat diet (HFD), brain insulin signaling pathways were disrupted and brain insulin resistance developed after the 12th week (Pratchayasakul et al., 2011). Additionally, it has been shown in the transgenic mouse model of Alzheimer's disease (AD) that a high sucrose diet triggers insulin resistance, memory deficits and amyloidosis (Cao, Lu, Lewis, & Li, 2007).

Alzheimer's Diseases and Insulin

The insulin hormone plays an important role in whole body glucose homeostasis. Although insulin receptors are found in many target tissues such as liver skeletal muscle and adipose tissue, it has been reported that it is also found in the brain in the highest concentration (Blazquez et al., 2014). Studies have shown that insulin in the brain can be synthesized not only from pancreatic β-cells, but also in neurons and glial cells (Scheepers et al., 2004). In addition to insulin's effects on food intake, appetite, and brain energy homeostasis on the brain, it has been shown to modulate neuronal activity through various molecular mechanisms. For example, it regulates neuronal survival, synaptic plasticity, and brain functioning, including memory, cognition, learning, and attention (Talbot, 2014).

It is known that AD has become a type of dementia with a prevalence of 60-70% and is characterized by gradual decline of memory and cognitive

functions associated with the accumulation of neurotoxic amyloid-β (Aβ) in the brain. Although the mechanisms to associate insulin-resistant states with AD are not sufficient, changes in cerebral glucose utilization and defects in insulin transmission have been reported in the early stages of AD in recent years (Caselli, Beach, Yaari, & Reiman, 2006). İmpaired neuronal insulin signaling in Alzheimer's disease is described in detailed Figure 1. In a study performed on post-mortem brain tissues of patients with Alzheimer's, they determined that the expression of genes encoding insulin, IGF-I and IGF-II, insulin receptor and IGF-I receptors were dramatically decreased. Additionally, they suggested that Alzheimer's may represent a neuroendocrine disorder similar to but distinct from diabetes mellitus (DM) (Steen et al., 2005).

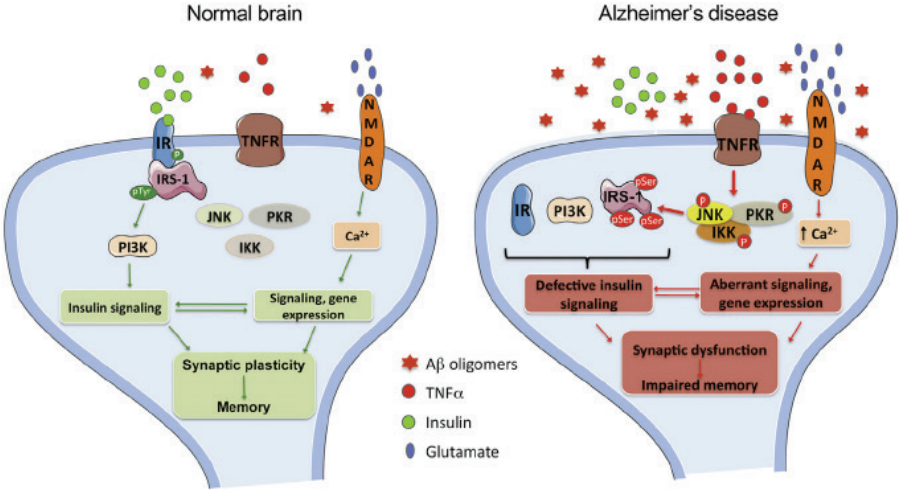

Figure 1: *Impaired neuronal insulin signaling in healthy brain and Alzheimer's disease's brain. Binding of insulin to the cell surface receptor (insulin receptor [IR]) under normal conditions triggers autophosphorylation of the IR and subsequent tyrosine phosphorylation of the insulin receptor substrate 1 (IRS-1). The activation of phosphoinositide 3-kinase (PI3K) signaling pathway and downstream of cellular responses are the results. Activity-dependent calcium (Ca^{+2}) activates the expression and signaling of some genes which are involved in synaptic plasticity and memory. This IR-dependent signaling may regulate the actions of insulin on memory. The accumulation of amyloid-β (Aβ) oligomers may cause an increase in the level of TNF-alpha and stress kinases such as (double-stranded ribonucleic acid [RNA]-dependent protein kinase [PKR], IκB-α kinase [IKK], and c-Jun N-terminal kinase [JNK]). Aβ oligomers trigger more removal of IRs from the cell surface and redistribution to the cell body. This combination can results with the blockage of neuronal insulin signaling. By the way, activation of NMDARs results in excessive Ca+2 influx, neuronal oxidative stress, and disrupted signaling. Finally, activation of protein tyrosine phosphatases may inhibit IRS-1 signaling; then, may cause synapse impairment and memory failure (De Felice, Lourenco, & Ferreira, 2014).*

Systemic administration of streptozotocin causes DM with neurodegeneration (Altunkaynak et al., 2012). In a study, it was reported that intracerebroventricular streptozotocin injection in rats caused insulin resistance, insulin deficiency and AD type neurodegeneration in the brain, but did not cause systemic diabetes mellitus (Lester-Coll et al., 2006). Finally, some studies reported that the neuroprotective effects of GLP-1 (stimulates insulin action), IGF-1 (slows down brain aging), and calorie restriction (reduces insulin resistance) were detected (Li et al., 2012; Mattson, 2010; Tarantini et al., 2021). Therefore, it was supported the idea that AD is a metabolic disease of the brain diabetes type.

Insulin and Cognition

In middle- and low-income countries, it is reported that the majority of neuropsychiatric disorders (ND) begin before the age of 25. Current literature evidence is that ND may take place with the downregulated of the insulin signaling pathway in brain (Hamer et al., 2019). In diseases such as major depressive diseases, Type 2 diabetes and Alzheimer's, brain insulin resistance occurs and the risk of cognitive impairment increases. Mansur and colleges suggested that glycemic control and cognitive dysfunction are closely related. Additionally they suggested that risk for cognitive impairment increase, with metabolic abnormalities (Mansur et al., 2018).

Hyperinsulinemia and obesity both occur as a result of insulin resistance and are among the risk factors of insulin resistance. Moreover, hyperinsulinemia and obesity induce oxidative stress, neuro-inflammation. It increases pro-inflammatory cytokines while decreasing anti-inflammatory cytokines, thereby altering hippocampal synaptic plasticity and spatial learning (Cetinkalp, Simsir, & Ertek, 2014).

Most of GLUTs are insulin-insensitive. However, GLUT4 is insulin-sensitive in some area such as cortical areas and hippocampus. In hippocampal-dependent cognitive functions, insulin stimulates the translocation of GLUT4 to the cell membrane and glucose enters the brain. As a result of chronic exposure to insulin, the membrane localization of GLUT4 in the hippocampus is downregulated and hippocampal-mediated cognition is affected (Pearson-Leary, Jahagirdar, Sage, & McNay, 2018). In the light of this information, it can be interpreted that the insulin signaling with the use of glucose may play role in learning and memory together.

Conclusion

Until about 40 years ago, the brain was thought to be insulin-independent, but today, the powerful neuromodulator, neuroprotective and neurotrophic effects of insulin on the brain have been revealed. And through these effects, the damage caused by T2DM, AD and cognitive

diseases in the brain is tried to be reduced by using insulin.

Although studies on the CNS insulin mechanism still cannot answer many questions, the problems that arise with insulin disorders are tried to be proven with evidence. With decreased insulin functions in the brain; the relationship between cognitive disorders and late neurodegeneration problems has been demonstrated. Although there is no consensus on the common parameters to be used for the proof of insulin resistance in the brain, researchers use different parameters for diagnosis. After the discovery of the common parameters, the symptoms of brain insulin resistance will be reduced. This intersection of insulin and neurodegeneration holds great promise in terms of preventing possible symptoms by providing early diagnosis of diseases.

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Chapter 12

CURRENT MATERIALS USED IN COMPUTER AIDED DESIGN AND MANUFACTURING (CAD/CAM) SYSTEMS IN DENTISTRY'

Şule Tuğba DENİZ

1. Introduction

Computer aided design and manufacturing (Computer Aided Design / Computer Aided Manufacturing - CAD / CAM) systems have been used in dentistry since 1985, and continue to develop until today. CAD/CAM systems used today are preferred to be used at the chairside, aiming to finish the treatments in a single session.[1]

The CAD/CAM technique is based on the conversion of data collected through optical scanners into three-dimensional designs using computer software. With the data collection unit in the system, information about the prepared teeth is scanned directly inside the mouth in some systems, and through the model in others, and the digitalized data is transferred to the computer. In this way, three-dimensional designs are created. Then, these designs are transferred to the instruments connected to the system and desired restorations are obtained by milling porcelain blocks prepared by various manufacturers from feldspathic or castable ceramics. Following the milling process, occlusal alignment, polishing and cementation are performed.[2]

The stage of collecting and saving data differs according to different CAD/CAM systems. Data collection is done using mechanical and optical digitizers. The mechanical digitizer maintains the position of the scanner relative to the tooth, creating a map of the entire prepared tooth surface. This type of digitizer is often used to obtain data from negative surfaces such as the silicon impression of the tooth. However, since deformation can be seen in the marginal areas during scanning, it is recommended to use mechanical digitizers after the model is obtained by taking measurements.[3,4] Optical digitizers are generally motion sensitive. Therefore, while collecting data with intraoral optical scanners, a slight movement of the patient may affect the fit of the prepared restoration. Optical digitizers enable fast and high resolution data to be obtained. However, shadowing is the disadvantage of extraoral optical digitizers. In newly developed scanners, the position of the model can be changed on 3-5 different axes, so that the desired area can be scanned exactly.[5] The data recorded in the computer environment is then converted into a virtual model consisting of dots by means of computer software.[6] After the design of the restoration is completed, the CAD software converts the virtual model into a set of commands that control the CAM unit.[7]

2. Advantages of CAD/CAM Systems

- Traditional measuring techniques have disappeared.
- It can be obtained in a shorter time.
- The error potential is considerably reduced.

- The opportunity to trade in a single session has arisen.[8,9]

3. Disadvantages of CAD/CAM Systems

- The most important disadvantage of CAD/CAM systems is the production cost.

- Optimal aesthetics may not be achieved due to the use of monochromatic blocks. However, this problem gradually disappeared with the development of blocks of different colors.

- Problems may occur while performing deep subgingival scanning. Therefore, a good gingival retraction is essential.[10]

4. Indications of CAD/CAM Systems

Today, CAD/CAM systems cover a wide range of indications such as inlays, onlays, laminate veneers, partial crowns, full crowns and fixed partial prosthese, skeletal structures of removable partial dentures, design and production of stents used in implant surgery.[11] These systems are also used in the preparation of maxillofacial prostheses. CAD/CAM technology is also applied in the design and production of abutment, crown-bridge and hybrid prosthetic infrastructure in implant supported prostheses.[12,13]

5. Materials used in CAD/CAM Systems

5.1. PMMA (Polymethylmethacrylate) Based Materials

5.2. Composite Resins

5.3 Resin Matrix Ceramics

5.3.1. Resin-based ceramics

5.3.2. Hybrid ceramics

5.4. Silica Ceramics

5.4.1. Feldspathic ceramics

• Traditional feldspathic ceramics

• Leucite reinforced glass ceramics

5.4.2. Lithium silicate ceramics

• Lithium disilicate ceramics

• Lithium silicate ceramics reinforced with zirconia

5.5. Oxide Ceramics

5.5.1. Aluminium oxide

5.5.2. Zirconium dioxide ceramics

• Cubic Zr

• Y-TZP

5.1. PMMA Based Materials

These materials are used as long-term temporary restorations. In some cases, restorations are produced from resin material and the margin adaptation or aesthetic appearance is ensured, and then the production of ceramic blocks with a much higher cost is started. There are monochromatic and polychromatic blocks with superior optical properties used for this purpose, as well as materials that do not contain polymethylmethacrylate in their content, have been put on the market by the manufacturers. In addition, with the use of acrylic resin-based blocks in CAD/CAM systems, infrastructure models and surgical plates that can be cast without leaving any monomers can be prepared.[14]

Telio CAD: Telio-CAD-blocks are applied for the fabrication of long-term (up to 12 months) temporary restorations using the CAD/CAM technique.

Vita CAD-Temp Monocolour Blocks: Vita CAD-Temp blocks consist of a fiber-free, homogeneous, high-molecular and cross-linked acrylate polymer. It contains 14% micro SiO2 particles as a filler in its structure. These inorganic micro-fillers polymerize into the network structure and form a completely homogeneous structure. This special structure is called MRP (Microfilled Reinforced Polyacrylate) material by Vita.

Vita CAD-Waxx: An acrylate polymer that can be baked without leaving any residue for the production of wax-assisted computer fabrication and drilling templates.

Merz Dental ArtBloc Temp: ArtBloc Temp is a monochrome, tooth-colored block made from cross-linked interwoven OMP-N (Organic Modified Polymer Network). Because of its plaque resistance, artBloc Temp is the optimal therapy for soft tissue management. It can also be used to reconstruct the occlusal support area for therapeutic restorations while correcting temporomandibular disorders.

5.2 Composite Resins

Long-term temporary restorations with superior aesthetic and biological properties can be prepared by using composite resin-based blocks, which are more successful than acrylic resin-based blocks due to the advantages provided by their structural, physical, biological and aesthetic properties. In addition, it is also possible to prepare single-tooth crown restorations and inlay onlay restorations by using such materials that both cause less wear on the opposite tooth and absorb chewing forces,

especially in patients with bruxism problems.[15]

Paradigm MZ100 3M: 3M Paradigm MZ100 blocks are CEREC blocks that allow quick and easy use, have high abrasion resistance and can be used for aesthetics.

Tetric CAD: Tetric CAD is an esthetic composite block for the efficient fabrication of single tooth restorations.

5.3 Resin Matrix Ceramics

5.3.1. Resin Based Ceramics

Caresmart Lava Ultimate 3M ESPE: Caresmart Lava Ultimate is a restoration material with high durability and fracture resistance, produced for CEREC systems that can stay in the mouth for a long time. According to a study, Caresmart Lava Ultimate has better workability than Celtra Duo.[16]

HC Block CAD Shofu: This material has realistic light diffusion properties with long-term stability and bending strength.

Brilliant Crios: Brilliant Crios have almost the highest bending strength on the market and have a tooth-like modulus of elasticity. It can be repaired later and does not require baking. The multimodal combination of dental glass and amorphous silica combined with a reinforced resin matrix makes Brilliant Crios an ideal material for permanent single tooth restorations.

5.3.2. Hybrid Ceramics

The ceramic network, which is dominant in the structure of these blocks, is reinforced with a polymer network that is fully integrated into each other. In this way, the positive properties of ceramic and composite materials are gathered together in materials of this structure. Ceramic structure constitutes 86% by weight and 75% by volume. The polymer network consists of surface modified polymethylmethacrylate (PMMA). Crack propagation problem, which is frequently encountered in ceramic materials, is reduced thanks to the polymer network structure. Thanks to their high loading capacity, they are used especially in crown restorations to be made in the posterior region.[17]

Vita Enamic: It is a hybrid ceramic with a double ceramic network structure. It is a hybrid restoration resistant to chewing forces. It is suitable for minimally non-invasive restorations as it can provide the elastic hybrid ceramic wall thickness. Restructuring is possible in a shorter time and at less cost, as it does not require polishing. In a study comparing Vita Suprinity, IPS Emax and Vita Enamic, Vita Enamic was shown to have significantly lower stiffness and rigidity than IPS Emax and Vita Suprinity. The crack pattern is more common in Vita Suprinity and IPS Emax, while

the minimal rupture and type 2 crack pattern is more common in Vita Enamic.[18]

3.4 Silica Ceramics

3.4.1 Feldspathic Ceramics

3.4.1.1 Traditional Feldspathic Ceramics

Feldspathic ceramic-containing blocks are the first blocks used in CAD/CAM systems in dentistry. In a 10-year study with these blocks, a very high success rate of 90.4% was observed.[19]

In the glass matrix, there are 30% and homogeneously dispersed feldspar particles in the size of 3-4 micrometers. Their breaking strength is 150 Mpa and their modulus of elasticity is 45-63 Gpa.[20]

There are 3 different feldspathic ceramic blocks as monochromatic, dichromatic and polychromatic. Efforts to develop monochromatic blocks led to the development of dichromatic and polychromatic blocks. Dichromatic blocks have a spherical dentin core and a translucent enamel layer around it. In these blocks, the color transition is prepared in the form of an arc in 3D in order to imitate dentin and enamel. Polychromatic blocks can imitate natural tooth tissue due to their different color saturation and light transmittance. Thus, it is ensured that the existing natural dentition and restoration form a whole by copying the optical properties of the natural tooth.[21]

These blocks are suitable for making inlays, onlays, laminate veneers, partial crowns and full crowns. Due to the high glass content, they can be roughened with hydrofluoric acid and give more successful results in adhesive cementation than oxide ceramics. Their mechanical polishability is quite good. These blocks are very suitable for chairside (single session applications per patient) applications thanks to all these features.[19,22]

Vitablocs Mark 2: They are monochromatic blocks containing tooth-colored feldspathic cavities.

Vitablocs Trilux Forte: These are blocks containing multi-colored and feldspathic spaces with shadow feature, produced to ensure the natural harmony of colors.

CEREC Blocks: CEREC blocks are feldspathic ceramics used for the production of inlays, onlay crowns and veneers.

3.4.1.2 Leucite Reinforced Glass Ceramics

Leucite crystals in ceramics are produced by multi-stage fabrication processes by creating controlled crystallization in a glass matrix. The leucite-based glass ceramic material used in the system basically consists

of silicon oxide (SiO_2), aluminum oxide (Al_2O_3) and potassium oxide (K_2O).[23] 30-40% of the volume of silicate glass matrix is composed of 1-5 micrometer leucite crystal phase.[24] While the semi-permeability and abrasion effect of the material are similar to natural teeth, its resistance to bending is 160 Mpa.[25]

The effect of leucite crystals on the resistance of the material occurs as a result of two different mechanisms. The first of these; leucite crystals change the direction of the crack and stop the crack propagation.[26] The other mechanism is the formation of residual compressive stress in the glass matrix during the cooling of the ceramic. The expansion coefficient of leucite crystals, which is 40% in the structure, is higher than the glass matrix it is in. While the ceramic is heated and cooled, the leucite crystals shrink, pulling the glass matrix towards itself, and thus the internal pressure formed in the structure stops the microcracks from progressing.[27]

The properties of these materials such as colour, translucency, fluorescence, opalescence, abrasion and abrasion resistance are similar to natural teeth. The resistance of restorations depends on successful adhesion to tooth tissue and adhesive cementation is required. Indications are limited to the anterior region crown and laminate veneer.[28]

In a study, glass ceramics and hybrid ceramics were compared, hybrid materials have been found to have lower flexural strength than glass ceramics.[29]

IPS Empress CAD: IPS Empress CAD restorations show excellent light-optical properties and exceptional flexural strength.

5.4.2 Lithium Silicate Ceramics

5.4.2.1 Lithium Disilicate Ceramics

In order to expand the indications of glass ceramic restorations, there was a need to develop materials with higher strength and fracture resistance. In this system, there is a much higher amount of crystal content compared to leucite glass ceramics in order to strengthen the infrastructure ceramics. Lithium disilicate crystals are used at the rate of 70% in the material, and the superstructure ceramic consists of fluoropatite crystals.[30,31]

Due to the very difficult milling of the lithium disilicate material and the fragility of the material, different procedures are required in the production phase of the blocks prepared for use in CAD/CAM systems. In the production process of lithium disilicate reinforced glass ceramics used with CAD/CAM systems, the ceramic is partially crystallized. The purpose of partial crystallization is to ensure that the blocks are milled easily and quickly, and to provide sufficient resistance to the ceramic during the

milling process. The basic crystalline phase in partially crystallized blocks is lithium metasilicate ($Li_2Si_2O_3$). This material has very low chemical and mechanical resistance. After the crystallization process at 850˚C, lithium metasilicate turns into resistant and tooth-colored lithium disilicate.[32]

Lithium disilicate CAD blocks with three different light transmittance are available. Blocks with high translucency can be used in the construction of inlay and onlay restorations due to their ability to absorb the color of the surrounding tissues (chameleon effect) and aesthetic properties. Blocks with low translucency can be used in the construction of full anatomical restorations with various color options. In the treatment of discolored teeth, the use of multi-blocks with the layering technique is appropriate. In more aesthetic areas, superstructure porcelain can be applied with the cut-back technique.[33]

Thanks to the infrastructure prepared in 0.8 mm thickness in line with the recommendations of the manufacturers, the fracture resistance has been increased up to 400±40 Mpa. In this way, in addition to single crown restorations, the construction of 3-member bridge prostheses is possible, and the indication area is limited to the anterior region of the second premolar teeth.[34]

IPS Emax CAD: IPS e.max CAD is an innovative lithium disilicate glass-ceramic for CAD/CAM applications. The wide range of transparency degrees, colors and block sizes provides flexibility in use. Appropriate cementation materials complete the system.

5.4.2.2 Zirconia Reinforced Lithium Disilicate Ceramics

Lithium disilicate reinforced glass ceramics were one of the first blocks used in CAD/CAM systems. Today, these blocks have been developed mechanically and zirconia infiltrated lithium disilicate ceramic blocks have been produced. There are 56-64% SiO_2 15-21% Li_2O 1-4% K_2O 3-8% P_2O5, 1-4% Al_2O_3 and 8-12% ZrO_2 in the ceramic structure. The fracture strength after milling is 210 MPa, while the fracture strength after crystallization reaches 420 MPa.[35]

Vita Suprinity Pc: Zirconia reinforced high-strength glass ceramic blocks are produced. In a study comparing the translucency and flexural strength of Lava Ultimate, Vita Enamic, Vitablocs Mark II, Vita Suprinity and IPS emax CAD materials; Vita Suprinity has been shown to have the highest translucency and flexural strength of any other material. This showed the advantage of zirconia reinforced lithium disilicate ceramics over other types.[36]

Celtra Duo: The superior properties of zirconia-reinforced lithium silicate are a function of its unique microstructure. The presence of 10%

zirconia in atomically dissolved form in the glass phase provides high strength and safe and long-lasting restorations.

5.5 Oxide Ceramics

5.5.1 Aluminum Oxide

These ceramics are in a presinterized state and are fired at 1520˚C after the restoration is produced. They are semi-sintered, highly durable oxide blocks containing 100% aluminum oxide crystals. Its fracture strength is over 500 MPa, its bending strength is 610 MPa on average, and its elastic modulus is 380 GPa. It does not require glass infiltration after milling. These blocks are monochrome; but they can be colored with coloring liquid according to the porcelain color that will be piled on it later.[37]

5.5.2 Zirconium Dioxide Ceramics

Zirconium, which stands out with its high mechanical resistance, chemical and dimensional stability, is the most used material in the construction of the infrastructure of full porcelain restorations.[38] According to their production methods, zirconium dioxide blocks are examined in three groups.

Unsintered zirconium dioxide blocks are produced by pressing the zirconium dioxide powder without pressure without any sintering process at the production stage. They are easily abraded and then used by sintering.[39]

Semi-sintered zirconium dioxide blocks are produced by pressed zirconium dioxide powder into a block by putting a binder into the structure. Zirconium dioxide powder is compressed by the manufacturer without applying heat, and pre-sintered at 1350-1550˚C.[22]

Fully sintered zirconium dioxide blocks are first sintered at 1300˚C and reach a density of 95%. Since the resulting structure is very hard, the etching process takes a long time.[39]

Katana Zircoia Blocs: This block offers better mechanical properties than lithium disilicate glass ceramics and minimizes restoration wall thickness.

IPS e.max ZirCAD: IPS e.max ZirCAD is a yttrium-stabilized zirconium oxide block. It is suitable for indications requiring high strength such as posterior bridges.

6. Conclusions

CAD/CAM systems, which find widespread use in dentistry with their wide product range, are current treatment options. Existing CAD/CAM systems have different features that provide different advantages but also have limitations. Studies investigating the mechanical tests of

the materials used in many CAD/CAM systems, the physical changes that may occur after heat treatment and their compatibility with natural teeth are still limited. In the future, in addition to studies aiming to eliminate these deficiencies, in vitro studies investigating the production techniques of different CAD/CAM systems, the sensitivity of digital readers and reproducible productions, and clinical follow-up studies of restorations prepared with these systems should be planned.

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Chapter 13

LPS-INDUCED SEPSIS AND ACUTE KIDNEY INJURY

Nihal İNANDIKLIOĞLU[1]

1 Asst.Prof., PhD., Department of Medical Biology, Faculty of Medicine, Yozgat Bozok University, Yozgat, TÜRKİYE. nihal.inandiklioglu@yobu.edu.tr, ORCID ID: 0000-0001-7137-3929

1. Sepsis

Sepsis comes from the Greek word “sepo” meaning “to decay”. Sepsis has been defined as life-threatening organ dysfunction caused by a dysregulated host response to infection. Severe sepsis is an infectious disease leading to septic shock, organ dysfunction and organ failure. (1). Severe sepsis is an infectious disease leading to septic shock, organ dysfunction and organ failure (2). Sepsis continues to be the most important cause of mortality in intensive care units today, despite increasing knowledge, use of new technological devices, and advances in diagnosis and treatment. According to the data of the “Surviving Sepsis Campaign”, the mortality rate was determined 41% in Europe and 28.3% in the USA (3). In a retrospective analysis of data from 1553 studies between 1979 and 2015, an incidence of 437 per 100,000 people per year was defined for sepsis (4). In 2011, the cost in hospitals was calculated as 20.3 billion dollars. This amount constitutes 5.2% of all hospital costs. (5).

Risk factors for sepsis include being in the intensive care unit, advanced age, bacteremia, immunosuppression, diabetes/obesity, malignancy, community-acquired pneumonia, genetic polymorphism, and previous hospitalization. Approximately 50% of the patients hospitalized in the intensive care unit have hospital-acquired infections, which poses a high risk for sepsis. The use of drugs that suppress the immune system, diabetes and obesity change the immune system and increase the risk of sepsis (6).

Sepsis can develop due to bacteria, viruses, fungi, parasites and rickettsia. It can also develop due to non-infectious events such as severe trauma and pancreatitis. An increase in the frequency of sepsis due to gram-positive microorganisms has been reported over time. In recent years, it has been emphasized that sepsis due to Gram-negative microorganisms is common. (7). Endotoxin, which is in lipopolysaccharide (LPS) structure and located in the cell wall of Gram-negative bacteria, teichoic acid/peptidoglycan complex found in the cell wall of Gram-positive bacteria, mannan, zymosan-like structures in fungi, various antigenic structures found in viruses and parasites activate the development of inflammatory response in the host. As a result of infection and traumatic damage in the tissues, the humoral system is activated in the body and various cytokines are released (8). In other words, the basis of the physio-pathological events in sepsis is the activation of the host immune system due to the antigenic structures of the microorganisms entering the body or the resulting toxins. During the inflammatory response, the deterioration in the inflammatory-anti-inflammatory, coagulant-anticoagulant, oxidant-antioxidant, apoptotic-antiapoptotic balances, which are important in maintaining the internal balance in our body, ultimately results in organ failures. (9).

2. Inflammation in Sepsis

Our immune system protects our body with two types of responses against millions of microorganisms and foreign substances. It protects with the natural (innate) immune system, which is the first protective barrier when foreign matter enters the body, and the responses created by the adaptive immune system that develops specific to the pathogen. In the innate immune response, the first response against pathogens or damage occurs when the cells of the immune system act as a barrier. In the adaptive immune response, antigens and B lymphocytes are stimulated by humoral immunity, while B lymphocytes turn into antibody producing plasma cells after stimulation. Cellular immunity is formed by T lymphocyte cells (10) (Figure-1).

In sepsis, various cells such as macrophages, neutrophils, endothelial and epithelial cells are activated. Activation of these cells can result in the release of a number of mediators, including cytokines, chemokines, platelet activating factor, complement prostanoids, and proteases. This sequence of events leads to immune cell activation with the release of reactive oxygen species (ROS) (11). Antigenic structures and toxins of microorganisms bind to CD14 receptors on the surfaces of various inflammatory cells (monocytes, macrophages). After the signal formed as a result of this binding, it is transmitted to the cell by toll like receptors (TLR) and transcription factors are activated. Sepsis is firstly caused by excessive secretion of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6) and interferon-γ (IFNγ), creating a cytokine storm. They activate IL-1 and IL-6 T cells secreted from monocytes and cause the secretion of γ-interferon, IL-2, IL-4, granulocyte monocyte colony stimulating factors (GM-CSF) from these cells. Many biological effects of TNF-α and IL-1 are common and show synergistic effects. These cytokines are the most important cytokines that play a role in the pathogenesis of fever, hypotension and shock in sepsis. Apart from IL-1 and IL-6, tumor necrosis factor-alpha (TNF-α), IL-8 and platelet activating factor (PAF) are also released from monocytes (12,13,14). The balance of pro-inflammatory (TNF-α, IL-1, IL-2, IL-6, IL-8, IFNγ, PAF) and anti-inflammatory (IL-6, IL-10) cytokines regulates the activation of nuclear factor-kappa B (NF-κB) (15). NF-κB is a redox-sensitive transcription factor required for gene expression of inflammatory mediators. By activating NF-κB, they lead to the production of various proinflammatory cytokines and nitric oxide (NO). In this context, pathogen is recognized through pattern recognition receptor (PRR) in sepsis and inflammatory processes are triggered. Apart from the production of molecules such as NF-κB, cytokines, chemokines and NO, it can inhibit apoptosis and prolong inflammatory cell survival (12). Following the release

of mediators, the coagulation system, complement system, fibrinolysis and quinine system are activated. In sepsis, the target damage occurs in the vascular endothelium. Endotoxin, TNF-α, IL-1, PAF, leukotrienes, thromboxane-A2, and NO increase endothelial permeability. Activation of the complement system also contributes to the formation of endothelial damage (11,12,15). Activation of complement impairs directly by vessel permeability or indirectly by activating neutrophils. With the activation of complement, lymphocyte proliferation, macrophage activation and cytokine release occur. Cytokines, NO, intracellular adhesion molecules, prostaglandins and leukotrienes play an important role in the pathogenesis of septic shock (16).

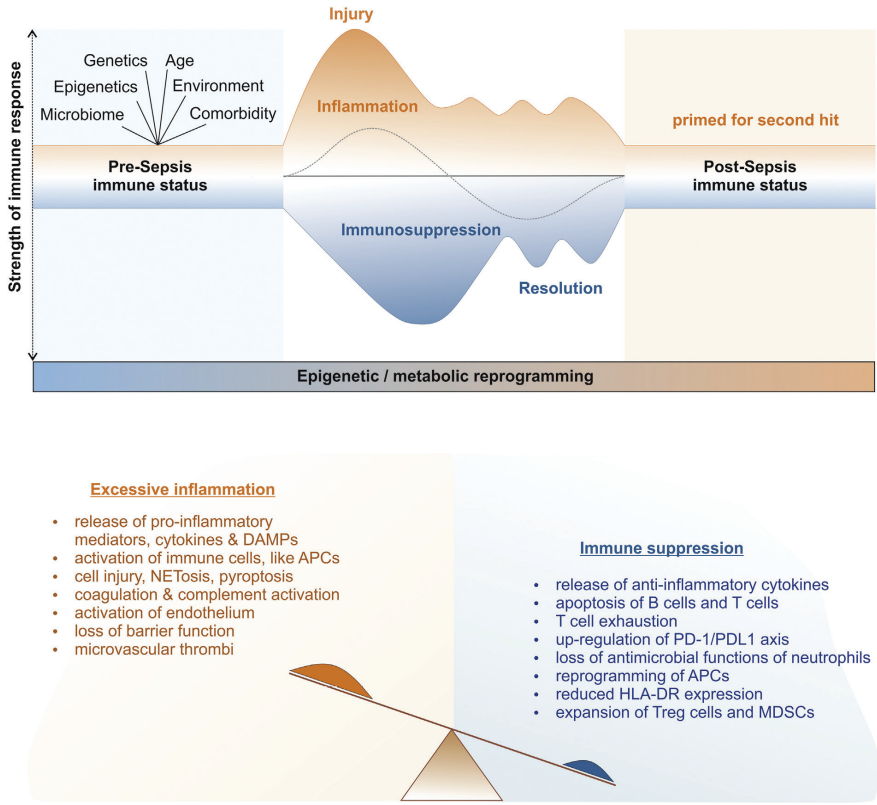

Figure-1: *Inflammatory and anti-inflammatory phases in sepsis (Steinhagen, 2020; Reference: 17).*

3. Lipopolysaccharide-induced Sepsis

Gram-negative bacteria have two membranes, an inner membrane that surrounds the cytoplasmic components and an outer membrane that separates the cell from its environment. In Gram-negative bacteria, the outer membrane serves as the first line of defense against environmental

threats. Unlike many biological membranes, the outer membrane of most Gram-negative bacteria is not in the form of a phospholipid layer, but instead has LPS molecules in the outer layer. These endotoxin glycolipid structures that form the outer membrane of gram-negative bacteria are closely related to the pathogenesis of sepsis. Therefore, the LPS infusion/injection model is widely used in sepsis research. LPS is obtained by using many gram-negative bacteria such as *Escherichia coli, Salmonella typhimurium, Klebsiella pneumoniae, Pseudomonas aeruginosa* (18).

During sepsis, pro-inflammatory cytokines and chemokines released due to pathogen-related molecular structures such as LPS and exotoxins activate the coagulation system and suppress important physiological anticoagulant mechanisms. This leads to the development of microvascular thrombosis as a result of coagulation activation. Disseminated intravascular coagulation (DIK) then occurs, which can lead to multi-organ failure and ultimately death (19).

In order for LPS to initiate the septic process, the presence of LPS-binding protein (LBP) and CD14 opsonic receptor in host cells is required. CD14 is divided into mCD14 (in the cell membrane) or sCD14 (in the circulation) according to its location. Cells such as dendritic cells, fibroblasts, and smooth muscle cells that do not have CD14 receptors on the cell surface interact with sCD14 and are stimulated with LPS. sCD14 is also present in the serum of healthy individuals. However, their levels increase significantly in sepsis. The complex formed by LPS with LBP binds to the TLR4 receptor, resulting in the inflammatory response. Again, the LPS-LBP complex responds to infection by binding to the membrane CD14 receptor, with activation of monocytes and endothelial cells. (12,20). TNF-α, IL-1, IL-6, IL-8 and PAF are released from monocytes. IL-1 and IL-6 activate T cells, causing the secretion of IFN-γ, IL-2, IL-4 and GM-CSF. After the release of mediators, the coagulation, complement, fibrinolysis and quinine systems are stimulated. In sepsis, cytokines play an active role in coagulation-related events (Figure-2). While proinflammatory cytokines such as IL-1 and IL-6 strongly stimulate coagulation, IL-10 tries to suppress tissue factor release from monocytes to regulate coagulation. While these cytokines are very useful in defeating local infection, their synthesis and release into the circulation in large quantities result in extensive endothelial cell damage. Disruption of inflammatory-anti-inflammatory, oxidant-antioxidant, coagulation-anticoagulation and apoptotic-antiapoptotic balance, which has an important place in ensuring homeostasis, causes irreversible organ failures (16,20).

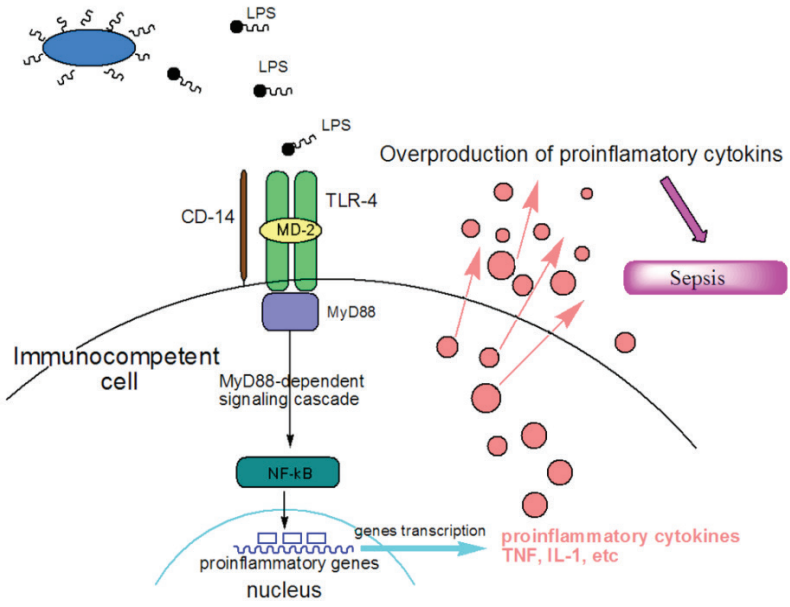

Figure-2: *Mechanism of sepsis induced by LPS effect. (Solov'eva, 2013; Reference: 21)*

4. Sepsis and Acute Kidney Injury

Acute kidney injury (AKI) is a clinical syndrome in which the glomerular filtration rate (GFR) suddenly decreases within hours to weeks, together with accumulation of waste materials such as blood urea nitrogen (BUN) and creatinine, and/or decreased urine output. AKI often requires dialysis. It often accompanies sepsis and increases the risk of mortality in sepsis. It is seen in approximately 35% of intensive care patients. Sepsis and septic shock are the most important causes in more than 50% of AKI. Mortality of sepsis-associated AKI varies between 20.9% and 56.8%, depending on the severity of the injury (22,23). Hemodynamic changes in the kidney, endothelial dysfunction, inflammatory cell infiltration into the kidney parenchyma, intraglomerular thrombosis, obstruction of tubules by necrotic cells and wastes play a role in the pathogenesis of sepsis-associated AKI (24,25).

Mechanisms that cause sepsis-associated AKI can be listed as ischemia-reperfusion injury, direct inflammatory damage, coagulation-endothelial cell dysfunction, and apoptosis (26). In LPS-associated sepsis, the blood concentration increases and there is a corresponding release of cytokines and NO. The LPS-LBP complex binds to CD-14 causing interaction with the cell surface TLR4-MD2 (myeloid differentiation protein 2) complex on monocytes, macrophages and neutrophils. This complex binds to the

renal tubule epithelial cell (27). These cells are then stimulated to produce cytokines by the myeloid differentiation primary response gene (MyD88) dependent and MyD88 independent pathway. Proinflammatory cytokines such as TNF-α, IL-1, and interferon that emerge after LPS exposure bind to TNF receptor 1 in kidney glomerular endothelial cells and TNF receptor 2 in tubular epithelial cells (28). After a series of reaction chains, the formation of inducible NO synthetase (iNOS) takes place. With the effect of increasing NO, arterial vasodilation and a decrease in systemic vascular resistance occur. Baroreceptors are stimulated due to a decrease in arterial pressure. Stimulation of baroreceptors causes sympathetic activation and release of arginine and vasopressin in the central nervous system. At the same time, the renin-angiotensin-aldosterone system is activated. As a result, circulating levels of norepinephrine, arginine, vasopressin and angiotensin increase. The increase in these hormones increases cardiac output and causes renal vasoconstriction to maintain circulatory integrity. Intrarenal vasoconstriction and a decrease in glomerular filtration rate are observed with sodium and water retention (29,30,31).

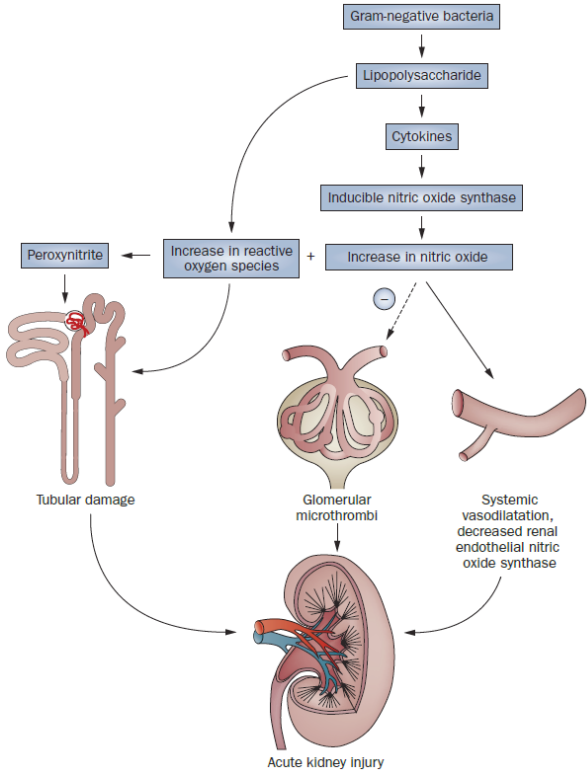

Figure-3: *Development of acute kidney injury in sepsis. (Heemskerk, 2009; Reference: 32)*

As a result of systemic vasodilation and renal vasoconstriction, renal blood flow decreases and hypoxemia occurs. Initially, prerenal acute renal failure occurs and the renal tubules are not affected. As the condition progresses, apoptosis and necrosis of tubular cells are observed due to renal ischemia. The damaged tubular epithelium is excreted in the urine and causes microobstruction in the urine stream. Increase in free radicals, changes in the coagulation system and complement system cause kidney damage and ultimately loss of kidney function (Figure-3) (31,32,33).

TLR4, which is the most researched and known TLR in humans. It plays a role in the recognition of LPS. Apart from LPS, TLR4 is known to recognize many molecules present and absent in the host. The recent identification of TLRs and related co-effector proteins such as CD14 and MD2 in kidney tissues raises the possibility of an as yet undiscovered mechanism of AKI in Gram-negative bacterial sepsis. The presence of TLR4 in the kidney was thought to result from both systemic and local inflammation (34). It has been shown that overstimulation of these receptors is associated with ischemic kidney diseases, AKI, end-stage renal failure, acute tubulointerstitial nephritis, and renal transplant rejection (35). When the kidney and endothelial structure are stimulated with LPS, TLR-4 expression, which is particularly sensitive to LPS, is increased. TLR-4 triggers signaling pathways that result in the activation of gene transcription factors in which NF-κB is central. As a result of pathways activated in this way, cytokine and chemokine levels such as IL1-β, IL-6, IL-8, IL-10, TNF-α and macrophage inflammatory protein 2 (MIP-2) increase. This increase results in cell, tissue and organ damage in the future (30,35). Although NF-κB activation contributes to inflammatory tissue damage during sepsis, it also has a critical role in host defense and other protective cellular responses. Therefore, the net effects of agents planned to inhibit NF-κB should be evaluated in models where survival is an endpoint. The degree of NF-κB activation in septic patients appears to be related to patient survival in septic shock. While excessive NF-κB activation may cause pathophysiological consequences, insufficient stimulation may lead to increased morbidity in septic shock (36).

Various protective agents have been tried against organ damage as a result of sepsis induced by LPS, and positive results have been obtained in some of them. When the effects of N-acetyl cysteine and deferoxamine administration on lung and kidney tissue in rats with sepsis were examined, it was found that antioxidant therapy was particularly effective in the lung (37). The antioxidant effect of sildenafil in kidney and lung tissue of rats with sepsis was found to have positive results for both tissues (38). In the LPS-induced sepsis study, apilarnil has been found to have a protective effect on kidney damage as a result of both histological and genetic analyzes

in kidney tissue of rats (39). It has been observed that propolis, another bee product, causes a decrease in serum MDA levels and an improvement in histological findings (mild ischemic injury, tubule epithelial vacuolization, vascular occlusion, and glomerular atrophy) (40). Although there are many biomarkers studied for early recognition of AKI today, studies on how to use these biomarkers by clinicians continue.

5. Conclusion

Since the mortality rate is very high in sepsis-associated AKI, rapid diagnosis and initiation of appropriate treatment is an important process. Discussions about the pathophysiology of septic AKI are changing from hemodynamic mechanisms to immunological/toxic/inflammatory mechanisms, from ischemic vasoconstriction to vasodilation, from acute tubular necrosis to acute tubular apoptosis. As the pathophysiology is better understood, treatment approaches will be shaped accordingly. More studies are needed to better understand the role of these multiple mechanisms in the pathogenesis of sepsis-induced AKI and to improve outcomes in the patient population.

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Chapter 14

OPTICAL COHERENCE TOMOGRAPHY IN DENTISTRY

Sedef KOTANLI[1]

Yasemin YAVUZ[2]

Mehmet Sinan DOĞAN[3]

1 Affiliation: Harran University, Faculty of Dentistry, Department of Dentomaxillofacial Radiology, Şanlıurfa /TURKEY, ORCID: 0000-0002-0827-0991, Mail: sedefakyol@harran.edu.tr

2 Affiliation: Harran University, Faculty of Dentistry, Department of Restorative Dentistry, Şanlıurfa /TURKEY, ORCID: 0000-0001-5961-4996, Mail: yyavuz@harran.edu.tr

3 Affiliation: Harran University, Faculty of Dentistry, Department of Pediatric Dentistry, Şanlıurfa /TURKEY, ORCID: 0000-0002-3089-1305, Mail: drmsdogan@harran.edu.tr

INTRODUCTION

Optical coherence tomography (OCT) is a non-invasive imaging technique that is widely used in ophthalmology and provides cross-sectional examination of biological structures. With OCT, it is possible to examine the oral mucosa, periodontal structures, and demineralisation and remineralisation of the hard tissues of the tooth (Mandurah et al. 2013; Tezuka et al. 2016). Many different methods are used to examine the mineral change of dental hard tissues; however, no method can detect the variation of mineral type and amount of mineral with very high sensitivity. Successful use of OCT in the examination of dental hard tissues in an increasing number of studies in recent years; It has made it possible for its use in dentistry to become widespread.

OCT can provide information that cannot yet be obtained by other methods, such as the diagnosis of periodontal diseases, the diagnosis of caries, and the evaluation of the integrity of restorations (Colston Jr et al. 2000). OCT can be used to detect early enamel caries, show the depth of caries, and quantitatively monitor the demineralisation and remineralisation of dental tissue (Amaechi et al. 2003).

OCT System

OCT is a partial coherence interferometer. In OCT, infrared laser light from a super luminescent diode laser (SLD) device is used, and so far this laser light has no reported harm (Aydin and Bılge 2007; Colston Jr et al. 2000). Although it is widely used in ophthalmology today, its use in dentistry is new. It was first introduced in 1991 by Huang et al.(Huang et al. 1991). Today, Different types of OCT devices such as Doppler OCT (DOCT), polarization sensitive OCT (PS-OCT), acoustic OCT, time domain OCT (TD-OCT), spectral domain OCT (SD-OCT), fourier domain OCT (FD-OCT), swept source OCT(SS-OCT) are used (Hsieh et al. 2013).

Light of different wavelength from the OCT device is sent to the tissue to be examined. The light is split in two by the splitter, as in the Michelson interferometer. The first of the light beams that split into two is directed to the fixed reference mirror and this is the reference light beam. The other beam is directed to the tissue, not to another mirror, unlike the Michelson interferometer. The light beam going to the tissue returns with different intensity and delay depending on the structure of the tissue layers it passes through. This is called scattering. The light going to the reference mirror reaches the detector at a known distance with a known delay time. By taking the reference light beam from the reference mirror, whose distance and delay time are known, as a reference, the light beam from the tissues containing reflections depending on the number of tissue layers can be defined. OCT calculates the amplitude and frequency of the light scattered

from the tissue, as well as the delay and reduction in intensity of the light reflected from its different layers. Because light scattered from the deep layers of tissue travels a longer distance than that scattered from the surface, it will have a delay time and return with less intensity (Clarkson 2014; Hsieh et al. 2013). The wavelength, amplitude, intensity, frequency of the light used in the OCT device, the distance of the reference mirror and the speed of the 33 light are known. According to this knowledge, it is possible to create structural images of the tissue according to the intensity of the light scattered from the tissue and the delay time of the light scattered from the deep layers. Different types of OCT devices used today can use different wavelengths of light and may differ in terms of scanning speed and mechanics of the device. As a result, all different types of OCT devices are Michelson interferometers that make the same measurement with reference to the variables in the basic mechanism described above (Clarkson 2014).

Use of Optical Coherence Tomography in Dentistry

The use of OCT for dental purposes, which is used for imaging transparent and translucent structures such as the retina, has been easily possible due to the structural similarity of the tissues in the oral cavity to the retina. The main advantage of OCT in terms of dental diagnosis is the visualization of both microscopic structures and surface contours of alveolar bone tissue and soft tissue (Otis et al. 2000). The first use of OCT technology in imaging dental tissues was realized by in vivo and in vitro studies by Colston et al (Colston Jr et al. 2000). After, Warren et al.(Warren et al. 1998) used OCT to determine the structural features of healthy and decayed teeth in vitro.

Nowadays, polarization sensitive OCT appliances have been developed. In this way, information about the demineralisation-remineralisation process in carious lesions could be obtained and the diagnostic potential of OCT in dentistry was increased.

Examination of the Oral Mucosa with Optical Coherence Tomography

In the study of Feldchtein et al.(Feldchtein et al. 1998) in which they examined the oral mucosa tissue with different keratin contents, such as the chewing mucosa or the covering mucosa, with OCT technology, it was observed that the images of the chewing mucosa were different from the tissues with weak or no keratinization.

Keratinization reduces contrast and makes it difficult to distinguish lamina propria (LP) and submucosal tissues from epithelial tissue. In non-keratinized mucosal epithelium, the contrast of the LP and

submucosa is more prominent, and muscle and bone tissue adjacent to the mucosa can be observed. With OCT images, it is also possible to monitor blood vessels and gland structures under the LP and mucosa (Feldchtein et al. 1998).

Examination of Dental Structures with Optical Coherence Tomography

In OCT images of hard dental tissues (enamel, dentin, cementum) in the oral cavity, it is possible to distinguish all these hard tissue layers from each other and to determine the enamel-dentin junction (Feldchtein et al. 1998; Colston Jr et al. 2000).

Examination of Caries Lesions with Optical Coherence Tomography

Dental caries formation is a multifactorial pathological process that starts with a local hard tissue demineralization and then progresses to morphological cavitation and tissue damage, causing lesion formation. There are many methods for the classification of dental caries. These classifications are categorized according to the tissue where the caries is localized (enamel, dentin, etc.) or the tooth surface that it affects (occlusal, facial, etc.) (Feldchtein et al. 1998).

Clinical examination and intraoral radiographs are among the methods used for the diagnosis of dental caries. Although the popularity of advanced imaging methods is increasing day by day, ionizing radiation is used in most of these methods and a quantitative assessment of the lesion depth cannot be made (Wenzel et al 1991; Ricketts et al. 1995) . Electronic caries imaging technique, which is an alternative to ionizing radiation and based on the electrical resistance measurement of the tooth, has been used in some studies, but has not yet become widespread (Rock and Kidd 1988; Ricketts, Kidd, and Wilson 1995). Fiber-optic transillumination (FOTI) technique, an advanced optical method, has yielded superior results than clinical examination and radiography images in detecting caries lesions (Wenzel et al. 1991; Wenzel et al. 1992). The depth of the caries lesion, which could not be determined even with all these advanced techniques, Amaechi et al.(Amaechi et al. 2001) could be evaluated quantitatively by OCT and also determined the demineralization stage during the formation of the lesion. In the OCT method, which allows quantitative evaluations, caries lesions are observed as a strong light reflection area on the tooth. The use of OCT is particularly important when carious lesions cannot be detected clinically. In the diagnosis of secondary caries developing between dental tissue and restorative material, the importance and advantage of the OCT method is revealed once again. In addition, caries lesions, which are observed as a small cavitation in clinical examination,

but whose actual depth is greater than that seen, can be easily detected by OCT technique and dimensional measurements of the actual depth can be made. Feldchtein et al obtained images of decayed teeth using both OCT and X-ray and digitally recorded these images using CCD cameras (Feldchtein et al. 1998). When the images obtained by these different methods were compared, it was determined that OCT images gave more information about the details than radiographic images.

Examination of Restored Teeth with Optical Coherence Tomography

Many different materials are used in dentistry to restore dental tissues. The physical properties of these materials (pressure strength, elasticity, thermal expansion-expansion coefficients, refractive index) are required to be similar to the properties of the tooth tissue. With the OCT method, important criteria such as the integrity, continuity and edge adaptation of the restorations can be evaluated(Otis, Colston, et al. 2000; Otis et al. 2000).Thanks to this technique, it is possible to monitor the application stages of the restorative material. With the images taken from the same area, it can be observed during the application of the restorative material the application stages of a restoration and whether the factors that will affect its success are provided. These factors are whether the caries is completely cleaned, the smear layer formed on the cut dentin, whether the smear layer is eliminated after the applied etching process (Otis, Colston, et al. 2000). In addition, the marginal harmony of the occlusal restorations with the enamel, the harmony between the dentin-composite and the occlusal wall contour of the cavity preparation can also be observed. The labial margins of class V composite restorations can also be monitored with the OCT method (Otis et al. 2000).

Examination of Periodontal Tissues with Optical Coherence Tomography

Three basic methods are used in the evaluation of periodontal tissues: clinical examination, periodontal probing and radiographic imaging. The periodontal attachment is evaluated by placing the probes between the soft tissue and the tooth. Thus, the penetration depth of the probe (pocket depth) is measured and the localization of the soft tissue attachment is determined. While periodontal probing can harm the patient, the debate about its diagnostic accuracy continues today. The force applied during probing, the level of inflammation of the tissue, and the anatomical variations in the tooth contours are counted as the problems and disadvantages of this technique(Soğur et al. 2005). It is possible to visualize the morphological features of alveolar bone,

which cannot be detected by clinical examination, with radiographs. Although demineralization areas on the teeth and alveolar bone loss are partially determined by radiographs, the inadequacies of 2D images of 3D formations are known. At the beginning of these deficiencies are the difficulties in the diagnosis of active and inactive periodontal disease. Radiographic diagnosis of periodontal diseases is possible only in cases of significant bone loss. Since only 2 dimensions can be observed with radiographs, the position and depth of the bone defect cannot be precisely localized. Currently, there is no method that can display soft tissue changes in the presence of gingival or periodontal diseases (Otis, Colston, et al. 2000).

The OCT technique stands out in terms of periodontal as a method that can provide us with high resolution (<20 μm) images on many important issues such as periodontal tissue attachment localization, morphological changes in the gingival tissue, degenerations of dental tissue, and structural integrity of dental restorations (Huang et al. 1991). The ability to visualize changes in connective tissue attachment and alveolar bone loss with the progression of periodontal disease with OCT is an important step in the development of periodontal diagnostic methods. Considering that OCT shows the micro-level structural details of periodontal soft tissues, the ability of this new imaging modality to diagnose active periodontal disease without substantial alveolar bone loss is emerging (Otis, Colston, et al. 2000). OCT images at the onset of the disease can be stored in patient records, so that the prognosis of the disease and its response to treatment can be visually documented.

CONCLUSION

The OCT technique will provide significant contributions and support to both scientific research in dentistry and many clinical applications, especially early diagnosis and treatment planning. With the widespread use of the OCT technique, the use of imaging techniques using ionizing radiation may be gradually abandoned.

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Chapter 15

SYSTEMIC FACTORS AFFECTING DENTAL IMPLANT TREATMENT

Selver Suna BAŞAK[1]
Funda BAYINDIR[2]

1 Assist. Prof. Dr. Selver Suna Başak, Kütahya Health Sciences University, Kütahya, ORCID ID: 0000-0003-1373-9579
2 Prof. Dr. Funda Bayındır, Atatürk University, Erzurum, ORCID ID: 0000-0001-5699-2879

In dentistry, implants are widely used in the treatment of single tooth deficiencies, total and partial edentulism, restoration of the entire mouth, as well as repair of congenital and acquired jaw and facial deformities (Özcan et al., 2015; Atala et al., 2019). A good osseointegration is required for the success of the implant treatment and the long-term stability of the implant. Osseointegration refers to the direct and tight relationship between implant and bone. On the other hand, some factors that affect clinical success of dental implant treatment one of which is the general health status of the patient.

Today, the role of systemic diseases in the continuity of oral health has been understood. The purpose of this review is to evaluate the success of implant treatment in patients with systemic diseases. Within the scope of this review, systemic disorders encountered in dentistry and their effects on implant treatment will be discussed.

Introduction

Today, dental implants are an alternative treatment option to traditional fixed and removable prostheses, which are frequently preferred and can be applied successfully. The success of implant treatment depends on a good osseointegration. Branemark et al. first described the concept of osseointegration in 1969 as a direct structural and functional connection between regular and living bone tissue and the surface of a load-bearing implant. (Branemark, 1985; Nandal, 2014; Uzun and Keyf, 2007). There are a number of factors that affect the success of dental implant treatment. These factors include the biocompatibility of the implant material, the macroscopic and microscopic structure of the implant surface and design, the health of the implant site and its healthy bone quality, the surgical technique applied, a healthy healing phase and loading conditions (Parith Denmarklaignan & Padmanabhancorresponding, 2013; Nandal, 2014). In addition to these, in a successful implant application, the implant should be immobile / immobile when clinically tested. There should be no peri-implant radiolucency when evaluated with a healthy radiography, the average vertical bone loss should be less than 0.2 mm after one year of use, three should be no pain, discomfort and infection and it should have a success rate of at least 85% at the end of 5 years and at least 80% at the end of ten years. (Özcan et al., 2015; Nandal, 2014) In addition, age, uncontrolled diabetes and smoking are other factors that affect the success of treatment. Patients undergoing dental implant treatment should be systematically evaluated in terms of age and genetic factors, heart diseases, anemia, diabetes mellitus, pregnancy, osteoporosis, ectodermal dysplasia and scleroderma, dry mouth and Sjögren's syndrome by undergoing chemotherapy or radiotherapy (Özcan, 2015). Dental implant treatment should be performed within safe limits in implant surgery. Therefore,

there are a number of serious systemic conditions that constitute absolute contraindications to implant surgery. These systemic conditions include myocardial infarction or cerebrovascular event within 6 months, heart valve implant or transplantation within 6-12 months, patients who have high risk of bleeding with INR value greater than 3-3.5 and platelet count greater than 50000/mm^3, patients with severe immunosuppression, with a total white blood cell count of 2,500-3,000 cells/mm^3, active cancer therapy and intravenous bisphosphonate therapy. Under the aforementioned conditions, failure in implant surgery should not be at a high level. However, the surgical procedure performed under these conditions can also endanger general health. In addition to these, psychiatric disorders should also be carefully evaluated because cooperation problems in this group of patients can prevent people from understanding the treatment to be applied and accepting the prescribed treatment, and also poor oral hygiene in these patients can affect the success of the treatment (Donos & Calciolari, 2014).

In this review, systemic diseases that may be encountered in implant patients and their effects on implant success will be evaluated.

Age

The age of the patient appears to be an important factor in dental implant success. In a retrospective study conducted in 2017, maximum implant failure was shown in 5200 patients over the age of 60. Under 40 years of age, moderate failure has been demonstrated in the lowest 41-60 age group. As the age of the patients increases, the failure rate tends to increase (Raikar, 2017). On the contrary, there is also a study reporting that marginal bone loss is the same in healthy young patients and healthy elderly patients with the same implant superstructure length, and therefore chronological age does not have a direct effect on dental implant osseointegration (Papež et al., 2018). Theoretically, as the age progresses, the recovery time of the patients increases, systemic problems occur and bone quality deteriorates. New bone formation rate and volume around the implants decrease with age (Ikebe, 2009). Age is a risk factor for elderly patients (Nandal, 2014). However, there is literature stating that advanced age can be seen as a risk factor in implant treatment, and it does not constitute a contraindication for dental implant treatment. With aging, some physical, metabolic and endocrine changes occur in the body. Mineral bone density in the human body is at its highest value at the ages of 25-30 and increases until the age of 30.

Depending on the advancing age, bone tissue weakens in elderly patients. On the other hand, the decrease in the amount of estrogen in men and women with age also causes a decrease in bone mass (İkebe, 2009). Boboeva et al. in their study conducted in 2021, reported that there was no

statistically significant difference in implant success between the young and old groups (Boboeva et al., 2021). In addition, Jemt T, in his study in 2019, stated that younger patients, rather than older patients, have a statistically higher risk of implant failure (Jemt; 2019). It also supports the observation of another study in partially edentulous patients that some middle-aged patients (45-65 years) may show a higher risk of implant failure than elderly patients. Moreover, they stated that both young and elderly patients might show a lower risk of failure than middle-aged patients (Jemt, 2019). Age-related implant failure is also a topic discussed in the literature. There are studies concluding that age alone is not a factor leading to implant failure. In contrast, Salonen et al. and Moy et al. reported a relationship between advanced age and implant success (Salonen et al., 1993; Moy et al., 2005). Studies comparing the results of dental implant treatment between young and elderly patients have demonstrated high survival rates in both groups.

Today, the tendency towards implant treatment is increasing in elderly patients and especially those with systemic disorders. In the literature, the success rate between older patients and younger patients with dental implant treatment is approximately equal in the long-term period. In patients with systemic disease, conditions such as decreased bone repair ability, immunodeficiency and xerostomia can reduce the success rate. However, dental implant treatment is not contraindicated in elderly patients with controlled systemic conditions (Kiani et al., 2015).

Osteoporosis

Osteoporosis is one of the most common bone disorders and a skeletal disease that can affect implant osseointegration and has a widespread clinical picture worldwide. Osteoporosis is defined as low bone density in human bone tissues. Many people in the worlwide are affected by osteoporosis especially elderly women. There is a defective bone formation in osteoporosis that cause deterioration in the microstructure of trabecular bone and an increase in cortical porosity, bonefragility, and the possibility of fracture. There is a positive correlation found between systemic bone mass, osteoporosis and jaw bone resorption. There are two types of osteoporosis as primary and secondary. Primary osteoporosis is the decrease in bone density due to aging, post-menopausal state and idiopathic osteoporosis. Secondary osteoporosis occurs in patients with predisposing factors such as other endocrinopathies and some drug consumption.

When osteoporotic bone is examined histopathologically, a decrease in thickness, irregularity in trabecular structure, decrease in mineral content and increase in carbonate / phosphate ratio are observed. The most important complication of dental implant treatment in osteoporotic patients is bisphosphonate-associated osteonecrosis of the jaw that

affects osseointegration. Bisphosphonates are widely used in many bone-related diseases because they reduce bone formation and destruction by osteoclastic activity (Kiani et al., 2015; Yılmaz Altıntaş et al., 2016).

On the other hand, the most important effect of osteoporosis on oral and maxillofacial structures is periodontitis, which manifests itself with a decrease in bone volume and density. People with low systemic bone density also have fewer teeth. It has been reported that as systemic bone density decreases, individuals experience more clinical attachment loss and more advanced periodontitis.

Decreased systemic bone density may be a risk factor in the progression of alveol bone loss. As a result, an environment more prone to periodontal destruction may occur. In edentulous patients, systemic bone density is lower than in patients with natural teeth (Ofluoğlu & Ergun, 2015). Osteoporosis may affect maxilla. However, there is no definite conclusion regarding the effect of osteoporosis on maxillary bone tissue.

Patients with osteoporosis are more likely to have implant failure. However, osteoporosis is not an absolute contraindication for dental implant applications. De Medeiros et. al. concluded that dental implants placed in patients with osteoporosis did not show higher failure rates compared to patients without osteoporosis. They also reported that implants placed in patients with osteoporosis showed greater marginal bone loss. However, the results are limited by clinical parameters. Therefore, the results of these studies should be carefully analyzed (De Medeiros et al., 2018). According to Tabrizi et al. reported a correlation between bone density T-score and marginal bone loss in female patients. However, there is not sufficient evidence to prove the relationship between marginal bone loss and osteoporosis. (Tabrizi et al., 2020)

A successful dental implant treatment can be achieved by giving more time to primary recovery in patients with osteoporosis and undergoing dental implant treatment, and the use of auxiliary drugs such as calcium, multi-vitamins, vitamin D, fluoride, estrogen and calcitonin (Küçükkurt & Alpaslan, 2014; Peker et al., 2012).

Bisphosphonates

Bisphosphonates are osteoclast inhibitors. It is the first treatment option for diseases that affect bone metabolism. There are two main groups of bisphosphonates. These are nitrogen-free bisphosphonates, which are rapidly metabolized, and nitrogen-containing bisphosphonates, which are more potent and not metabolized. When bisphosphonates accumulate in bone, very small amounts are released into the circulation. As a result, the half-life of bisphosphonates in bone is estimated to be approximately 2 years.

Bisphosphonates are analogues of pyrophosphates, which are abundant in bone matrix and are natural regulators of bone metabolism. Although their mechanism of action is based on inhibition of bone resorption, they are not yet fully understood. Bisphosphonates are used in the treatment of many bone diseases such as osteoporosis, metastatic bone tumor and Paget's disease in the prevention of bone mass loss in humans. Bisphosphonates act by suppressing osteoclasts or reducing bone resorption. Bisphosphonates have a very short half-life in the blood. However, these drugs are absorbed into the bone tissue and can remain in the tissue for a long time. A radiolucent lesion or bone exposure may occur after oral surgery in patients using bisphosphonates.

Currently, intra-venous bisphosphonate therapy is considered as a major risk factor for osteonecrosis of the jaw bones associated with bisphosphonate therapy. Elective oral surgery, including the application of dental implants, is generally contraindicated in patients using bisphosphonates. Essentially, the risk of osteonecrosis with oral bisphosphonate administration is much lower than with intravenous administration. However, this risk has been found to increase with the duration of bisphosphonate therapy.

There is no risk for a dental procedure to be performed in the first 3 months of the patient receiving oral bisphosphonate therapy. If the patient has been using oral bisphosphonates for less than 3 years, tooth extractions and surgical procedures involve minimal risk. A systemic disease such as steroids, anti-angiogenic agents or concomitant diabetes mellitus increases the risk of osteonecrosis even if the patient has been on oral bisphosphonate therapy for less than 3 years. Patients, treated with oral bisphosphonates for more than 3 years, have a higher risk of osteonecrosis after surgical intervention. However, in the literature, osteonecrosis associated with oral bisphosphonates has been observed in patients receiving oral bisphosphonates for more than 10 years. Many researchers recommend antibiotic prophylaxis before invasive procedures such as implant application. Stopping oral bisphosphonate therapy 2-3 months before the procedure and until osseointegration is complete should be evaluated in terms of risks and benefits (Küçükkurt et al., 2014; Mendes et al., 2019).

Cardiovascular Diseases

The relationship between cardiovascular disease and implant survival has rarely been studied to date. No significant difference was found between those with and without heart disease in the literature. Patients with cardiovascular indications have a higher risk of peri-implantitis than healthy individuals. However, in general, heart disease does not constitute a contraindication for implant treatment (Diz et al., 2013). In addition,

some cardiovascular conditions, such as recent myocardial infarction, stroke, and cardiovascular disease, may constitute a contraindication for implant surgery.

In the literature, no significant difference was found between patients with a history of cardiovascular disease and healthy controls. Therefore, hypertension and coronary artery disease are not among the local and systemic risk factors affecting implant success. Cardiac disorders do not constitute a contraindication for dental implant treatment. However, in these patients, other problems such as bleeding or cardiac ischemia should be taken into consideration during the placement of dental implants and patients should be consulted medically before surgery.

Diabetes mellitus

Today, the percentage of diabetic patients is increasing. According to the studies conducted by the World Health Organization (WHO), the number of diabetic patients is expected to reach 300 million in the first quarter of the 21st century. According to the results of the Turkey Diabetes Epidemiology research, the prevalence of diabetes in adults over the age of 20 is 13.7% and the rate of those who have not been diagnosed before is 7.5% (Özdoğan et al., 2015). Measuring HbA1c values has proven to be a reliable approach in diabetes control. HbA1c, also known as glycohemoglobin (GHb), is the red blood cell pigment (hemoglobin) to which glucose binds and provides information on blood sugar levels for the previous eight weeks.

Poorly controlled diabetes has a negative effect on osseointegration. In the literature, it has been shown that dental implant treatment has similar success rates in diabetic patients with good metabolic control compared to healthy patients. However, impaired implant integration associated with hyperglycemic conditions has been reported in diabetic patients and animal models. In addition, it has been reported that an HbA1c value of more than 8% has a negative effect on implant stability in the early postoperative period and the length of the overall healing time (Oates et al., 2009).

Diabetes negatively affects implant osseointegration in patients with uncontrolled diabetes (Schlegel et al., 2013). This may be due to the known effects of hyperglycemia on impaired immunity, microvascular complications and osteoporosis in individuals. However, there is insufficient clinical information to correlate glycemic control with implant failure. Diabetes constitutes a contraindication for dental implants. In addition, glycemic control is recommended before and after dental implant treatment in patients with a history of diabetes. Antimicrobial antibiotics (penicillin, amoxicillin, clindamycin or metronidazole) should

be used for prophylaxis during surgery, and postoperative antibiotics (amoxicillin and clindamycin) should be used to prevent infection during the primary recovery phase. If there is smoking in patients with a history of diabetes, they should quit. Oral care should be optimized and antiseptic mouthwashes should be used for the prevention of periodontal and peri-implant infections (Diz et al., 2013).

Dementia and Neuropsychiatric Diseases

There are few data in the literature regarding implant therapy in people with dementia and neuropsychiatric diseases. However, there will be an increase in the number of patients with dementia in the future. At this point, a good history should be taken from the patient. The selection of patients with dementia should be discussed with their relatives and the patient's condition should be consulted with the neurologist.

Many psychotropic drugs used in the treatment of neuro-psychiatric diseases, especially when used in combination with other drugs, can increase the effect of adrenaline during surgery and cause severe dry mouth. Therefore, the degree of hyposalivation before surgery should be evaluated in terms of determining the sensitivity of the mucosa. In a study, patients who took serotonin reuptake inhibitors for depression reported higher implant failure rates compared to those who did not (Wu et al., 2014; Schimmel et al., 2018). Studies conducted on patients with Parkinson's disease have fewer as well as few such studies, and the survival rate has been reported as 82%, and this rate is lower than the implant success rate in patients without Parkinson's disease (Packer et al., 2009).

Bleeding Disorders

Although bleeding is a common contraindication in dental implant placement in dentistry, there is no definitive evidence in the literature that bleeding disorders constitute a contraindication to dental implant treatment. Besides, even hemophilia patients can be treated successfully. On the other hand, oral surgical interventions may cause bleeding in patients with bleeding problems. To perform implant surgery without changing the anticoagulation agent, provided that the INR value is less than 3 or 3.5 in patients with bleeding disorders and receiving anticoagulant therapy. Patients with an INR value between 2 and 4 and receiving anticoagulant therapy may not pose a risk for postoperative bleeding, and topical hemostatic agents may be effective in bleeding control in these patients.

Although dental implant treatment is not contraindicated in patients with bleeding problems who use anticoagulant medication, these patients constitute a group at risk in terms of postoperative hemorrhage. In this regard, medical consultation is required as in all other oral surgical procedures.

Hyposalivation and Sjogren's Syndrome

Dental implant treatment can contribute to the retention of prostheses made in patients with dry mouth. There are no systematic studies evaluating the results of dental implant treatment in these patients. However, before recommending dental implant treatment, the amount of saliva flow and the degree of dry mouth should be evaluated.

Sjögren's syndrome is an autoimmune disease that affects the functions of all exocrine glands, including the salivary glands. As a result of this disease, xerostomia, difficulty swallowing and taste disturbance can be seen in people. For this reason, it should be taken into serious consideration by physicians for all kinds of dental treatments besides dental implant treatment. Although implant-supported prostheses can be considered as an alternative treatment option due to the difficulties experienced in the use of removable prostheses in these patients, the seriousness of the medical condition should be carefully considered.

Immunosuppressive Therapy

In dental treatments, good immune system is important for wound healing. There is not enough data in the literature regarding the success of dental implant treatment in patients under immunosuppressive drug use or immunosuppressive therapy. In addition, there is no evidence that there is a contraindication to dental implant treatment.

Patients who receive immunosuppressive therapy should be evaluated medically, and antibiotic prophylaxis and the use of topical antiseptics should be considered in order to reduce the risk of a possible infection.

Mucosal Diseases

In the literature, there are many case reports documenting the success of dental implant treatment in patients with mucosal disorders including ectodermal dysplasia, epidermolysis bullosa and lichen planus. Dental implant treatment is frequently encountered as a treatment option in patients with ectodermal dysplasia and severe oligodontics and hypodontics. The most common clinical finding in these patients is low bone density, especially in the upper jaw. While implant treatment is largely successful in adult patients with ectodermal dysplasia, implants placed in the maxilla, especially in children and young patients, are less promising. Therefore, the success of dental implant treatment in pediatric patients is still unclear.

Implant treatment is not a good treatment option in patients with oral lichen planus. Because, there is not enough epithelium to adhere to the titanium surface. Periimplantitis and periimplant mucositis are more common in patients with oral lichen planus. In conclusion, clinical follow-

up of both the disease and dental implants is recommended in these patients.

Smoking

In 2000, 4.83 million people worldwide died due to nicotine addiction. Today, the number of smokers in the world is >1.1 billion and more than 8 million people die annually due to smoking (Jiang et al., 2020). Nicotine, which is responsible for tobacco addiction, is the main chemical component in the pathogenesis of many diseases. Nicotine increases the heart rate, blood pressure, and respiratory rate. Smoking influence general and dental health. Smoking has adverse effects, especially on oral health. Smoking affects bone metabolism systemically and locally by disrupting blood flow. Smoking is a risk factor for periodontal disease, oral precancerous and cancerous lesions, root caries and peri-implantitis (Mumcu, 2018).

Periodontitis is an inflammatory disease that affects the supporting tissues of the tooth called periodontium. There are four tissues in the structure of the periodontium: gingival, cementum, alveolar bone and periodontal ligaments. The mildest form of periodontal disease is gingivitis. Gingivitis occurs as a result of the accumulation of dental plaque on the teeth adjacent to the gum. Symptoms of gingivitis are red, bleeding-prone gums. Gingivitis is reversible and inflammation is limited to the gingiva. If gingivitis is not treated, it turns into periodontitis. Periodontitis is irreversible. It causes loss of connective tissue and bone support. Furthermore, ıt is the major cause of tooth loss in adult patients. In addition to pathogenic microorganisms in dental plaque, genetic and environmental factors are also effective in the formation of periodontal disease (Borojevic, 2012). Smoking has been recognized as an important risk factor in the progression of periodontitis and influencing treatment response. According to the results of epidemiological studies, smokers have a higher risk of periodontal disease than non-smokers, and the risk is related to the duration and rate of smoking.It has been demonstrated that the prevalence of moderate to severe periodontitis is higher in smokers than in nonsmokers. The prevalence of attachment loss and gingival recession is also higher. Periodontal health in non-smokers is better than smokers. Moreover, smokers had a higher number of missing teeth than non-smokers.

Smoking has a series of direct and indirect systemic and local effects on bone metabolism (Chrcanovic et al., 2015). Implant failure rate in smokers increases with local exposure of the peri-implant tissues to tobacco products in smokers. There is a higher risk for dental implant failure in smokers. In a meta-analysis conducted in 2021, it was reported that the survival rate of dental implants amongst nonsmokers was significantly better than

smokers (Hadadi & Mezied, 2021). Accoarding to the results of another study, while smoking did not have an effect on the primary stabilization of dental implants, secondary stabilization decreased (Badenes-Catalán & Pallarés-Sabater, 2021).

The number of the periodontal pathogens also increased with smoking. Smoking impairs many immune responses such as neutrophil function and fibroblast activity. In smokers, alveolar bone destruction and tooth loss are more than non-smokers.

Conclusion

In the literature, there is insufficient evidence regarding the effect of systemic diseases on the success of dental implant treatment. This situation should not be considered as systemic factors do not affect the success of dental implants. However, more prospective studies are needed in order to support what is known about the subject and to obtain more data.

The number of situations that constitute a definite contraindication for dental implant treatment is very few. On the other hand, there should not be a perception that implant treatment is a risk-free procedure. For this reason, dentists should consider the advantages and disadvantages of surgical procedures and the treatment method to be applied and a medical consultation should be requested if deemed necessary. Systemic diseases that can reduce the results of dental implant treatment and the recovery potential of patients should always be considered. In patients with smoking habit and poor oral hygiene, which are known risk factors for implant success, follow-up is important during the treatment process.

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Chapter 16

THE EFFECT OF CONVALESCENT PLASMA THERAPY ADDED TO STANDART TREATMENT ON CLINICAL IMPROVEMENT IN PATIENTS WITH SEVERE OR LIFE-THREATENING COVID-19

Filiz KIZILATEŞ

Introduction

The current pandemic, Coronavirus disease 19 (COVID-19), originated at Wuhan city of China in early December 2019 has rapidly widespread with confirmed cases in almost every country across the world. The etiological agent which was designated as Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is a positive-sense, single-stranded ribonucleic acid (RNA) virus (1). The clinical features of infection is ranging from asymptomatic or self-limited respiratory disease to acute respiratory distress syndrome (ARDS), severe progressive pneumonia, multiple organ failure and death (2, 3).

During the last year, many treatment strategies were discussed against to COVID-19; like antiviral drugs (remdesivir, lopinavir and ritonovir, favipiravir), antimalarial drugs (chloroquine and hydroxychloroquine), convalescent plasma (CP) treatment, immunoglobulins, inflammatory modulators (tocilizumab) and also corticosteroids.

CP was used in viral epidemics such as measles, mumps, poliomyelitis and influenza in the beginning of twentieth century (4). There were studies evaluated the effectiveness of CP in treatment during the 1918 H1N1 influenza virus pandemic, 2009-2010 H1N1 influenza virüs pandemic and 2013 Ebola virüs epidemic (5, 6, 7).

With a declaration published on January 28, 2020, World Health Organization (WHO) stated that immune plasma or immunoglobulin concentrates can also be used for SARS-CoV-2 when vaccines and/or effective antiviral drugs cannot be reached, as previously practiced in the MERS-CoV epidemic. American Food and Drug Administration (FDA), issued an emergency statement for the use of CP for SARS-CoV-2 on March 24,2020 (8). The FDA recommends the use of CP in severe and life-threatening COVID-19 patients, preferably in first 7-14 days and before the onset of cytokine storm (9, 10).

CP in treatment is a passive antibody therapy. Antibodies are collected from recovered patients and developed humoral immunity. By this way, administration of antibodies against a certain pathogen can be used for preventing or treating an infectious disease (11, 12).

The aim of this study was to evaluate the efficacy of CP added to standart treatment, compared with standart treatment alone, for patients with severe or life-threatening COVID-19.

Material and Methods

This retrospective matched-control study was conducted in Health Sciences University Antalya Training and Research Hospital between May

01, 2020- September 30, 2020. This study was approved by the Ethics Committee of Health Sciences University Antalya Training and Research Hospital (Date May 04, 2021; Number 1/14).

Patients

A total of 439 patients (201 patients who received CP and 238 patients as controls) were enrolled to the study. Controls were matched by gender, age±5 years, comorbidities and disease severity scale.

Inclusion Criteria for both group was; aged at least 18 years; COVID-19 diagnosis based on polymerase chain reaction (PCR) testing; bilateral typical findings on chest computerized tomography; clinical symptoms meeting the definitions of severe or life-threatening COVID-19 and hospitalization.

Exclusion Criteria for both group was; pregnancy; Ig A deficiency; breastfeeding; death in the first three days; disseminated intravascular coagulation; severe septic shock; severe congestive heart failure and receiving remdesivir treatment (Remdesivir has not yet been licensed and commercially available by the Ministry of Health in Turkey and negotiations are ongoing with the Turkish Medicines and Medical Devices Agency for its high priorty evaluation and licensing.)

Definitons:

A respiratory distress (≥ 30 breaths/min) or oxygen saturation ≤ 93% on room air or arterial partial pressure of oxygen (PaO_2)/ fraction of inspired oxygen (FiO_2) ≤ 300 was referred as ***Severe COVID-19*** (13).

A respiratory failure requiring mechanical ventilation; shock; or other organ failure requiring intensive care unit (ICU) monitoring was defined as ***Life-threatening COVID-19*** (13).

The disease severity scale

1. Hospital discharge,
2. Hospitalization with no supplemental oxygen,
3. Hospitalization plus supplemental oxygen (not high-flow or non-invasive ventilation),
4. Hospitalization plus non-invaziv ventilation or high-flow supplemental oxygen),
5. Hospitalization plus extracorporeal membrane oxygenation (ECMO) or invasive mechanical ventilation,
6. Death.

Convalescent Plasma Administration Time: Duration between the onset of symptoms and plasma administration

Standart Treatment was consisted of symptomatic and supportive care for COVID-19, based on Turkish National COVID-19 treatment guidelines and hospital practice; included antiviral medications (favipiravir), corticosteroids (dexamethasone, methylprednisolone), immunomodulators (anakinra, tocilizumab), vitamin support and anti-thrombotic therapy.

Clinical improvement was defined as patient discharge or a reduction of 2 points on a 6-point disease severity scale (14).

Procedure

COVID-19 convalescent plasma donors were patients that were infected with COVID-19 and at least 28 days have passed since recovery and disappearrance of the symptoms. Plasma donors were qualified according to the Turkish National guidelines for the preparation, use and quality assurance of blood and blood components which is compliant the European ones (15). The plasma was collected and obtained from local blood collection centers and national blood suppliers. Patients were transfused two units (approximately 400 ml total volume) of ABO-compatible CP in accordance with institutional guidelines. At the time of this study, neutralizing antibody levels could only be measured qualitatively.

Statistical Analysis

The descriptive findings were presented with mean±standart deviation (SD) or median (0.25-0.75 percentile) for the continuous data, and with frequency and percentage for the categorical data. Pearson chi-square test and Fisher's Exact test performed for comparison of categorical data. The normality assumptions were controlled by the Shapiro-Wilk test. Mann-Whitney U test and Student's test were used for analysis of non-normally and normally distributed numerical data, respectively. Kruskal Wallis test was used for comparison of nonparametric variables among groups and Bonferroni-Dunn test was used as a pos-hoc test for significant cases. McNemar's test and McNemar-Bowker test were used to compare paired categorical data. Wilcoxon Signed Ranks test was used for nonparametric comparison of repeated measurements. Statistical analysis was made using IBM SPSS Statistics for Windows, Version 23.0 (IBM Corp., Armonk, NY). Two-sided p values <0.05 were considered statistically significant.

Results

Totally 439 patients with laboratory-confirmed SARS-CoV-2 infection were enrolled to the study in which 201 patients received CP and 238 patients as controls. All patients were treated with favipiravir as standart

treatment. Intravenous corticosteroid was applied to the 64% (128/201) of CP patients and to the 50.2% (119/238) of control patients (p=0.004) at enrollment. Antimicrobial treatment was applied to the 79.6% (160/201) of CP patients and to the 77.6% (184/238) of control patients (p=0.618). Duration of hospitalization (median, IQR) was significantly high in CP group [15 days (11-24) versus 9 days (7-13)] (p<0.001). Mortality rate was 49.8% (100/201) patients in CP group and was significantly higher than control group (37.8%; 90/238) (p=0.012). Median time between symptom onset and mortality was 21 days (16-29.5) in CP group and 17 days (12-23) in control group.

Baseline characteristics, clinical features and laboratory parameters of patients were summarized in Table 1. Demographic characteristics as age, gender, underlying diseases, initial symptoms and disease severity scale were similar in both group. Only O_2 saturation at enrollment was lower in CP group (p<0.001) (Table 1). And also in CP group, blood albumin level (p=0.024) and lymphocyte count (p=0.013) was lower; lactate dehydrogenase (p<0.001), c-reactive protein (p=0.003) and interleukin-6 level (p<0.001) was higher than the control group (Table 1).

The complication rates in CP group and control group were similar; acute respiratory distress syndrome (24.4% versus 18.9%: p=0.164), pulmonary thromboembolism (10% versus 8.8%: p=0.674) and myocardial infarction (5% versus 3.4%: p=0.383).

Patients were grouped according to the CP administration time. First; the patients who were administered CP in the first 5 days and patients who were administered CP after the first 5 days were compared with the control group (Table 2). Mortality was significantly higher (p=0.007) and clinical improvement was significantly lower (p=0.010) in patients who were administered CP more than 5 days after symptom onset, compared to those who were administered in the first 5 days and in the control group (Table 2). Clinical improvement was 65.6% (21/32) in CP administered group in the first 5 days and was higher than control group (62.2%; 148/238) but the difference was not significant statistically (Table 2).

Then; the patients who were administered CP in the first 7 days and patients who were administered CP after the first 7 days were compared with the control group (Table 3). Mortality was significantly higher (p=0.003) and clinical improvent was significantly lower (p=0.03) in patients who were administered CP more than 7 days after symptom onset, compared to those who were administered in the first 7 days and in the control group (Table 3).

Demopraphic and clinical features of CP patients were compared according to mortality (Table 4). Although the type of supplemental O_2 support at enrollment was similar between two groups (p=0.262), the type of supplemental O_2 support while CP administration was significantly higher in the mortality group (p<0.001) (Table 4). And also laboratory parameters of mortality group was significantly different than the survived group, statistically (Table 4).

Discussion

Our study was retrospective, matched-control study. The demographic characteristics, underlying diseases and clinical features at hospital admission of all patients were similar. We believe that, the close similarity in the features of groups, will increase the value of our work.

As a result of our study; the use of convalescent plasma therapy added to standart treatment, compared with standart treatment alone, did not result a significant benefit to clinical improvement in severe or life-threatening COVID-19. In a randomized clinical trial conducted with 103 laboratory-confirmed with severe or life-threatening COVID-19 patients (52 of them received CP), there was no significant difference in 28-day mortality (13). And also in another randomized clinical trial (228 CP patients and 105 control), no significant differences were observed in clinical status or overall mortality between patients treated with CP and those who received plasebo (16).

Early in the COVID-19 pandemic, CP therapy was found to be effective in COVID-19 in studies conducted with a small number of patients (17). Meta-analysis results of randomized controlled trials showed that, there were no statistically significant differences between CP transfusion and the control group in terms of reducing mortality and improving clinical symptoms (18). In the same meta-analysis, the results of controlled non-randomized studies of interventions showed that CP therapy may reduce mortality in COVID-19 patients (18). In a meta-analysis including 10 randomized clinical trials, 20 matched control studies, 96 case reports or case series; random effects analysis of randomized clinical trials and matched control data demonstrated that patients with COVID-19 transfused with CP exhibited a lower mortality rate compared with patients receiving standart treatments and early transfusion (within 3 days of hospital admission) of higher titer plasma is associated with lower patient mortality (19).

In our study, clinical improvement was higher in those who received CP therapy within the first 5 days after symptom onset, but it was not statistically significant. In a retrospective cohort study (341 CP patients and 594 matched controls), mortality was significantly decreased in patients who received CP with an anti-spike proteein receptor binding domain

(RBD)IgG titer of ≥1:1350 within 72 hours of admission (20).

Although the type of supplemental O_2 support at enrollment was similar between two groups, the type of supplemental O_2 support while CP administration was significantly higher in the mortality group in our study. And also laboratory parameters of mortality group was significantly different than the survived group, statistically. These results suggest that CP therapy is more beneficial when given early before the disease worsens.

There are several key questions for the use of CP as a therapeutic agent in COVID-19 treatment. These include dose, administration time and duration of treatment and selection of the patients most likely to benefit. There is a few study about the administration time of the CP therapy.

And also in those studies, the time was defined by hospital admission and mortality was found lower in early transfusion group (within 3 days of hospital admission) (19). If transfusion time is defined more universal, relative to symtom onset, as our study, it will be more easier to compare the results of the studies.

The selection of patients to be given CP therapy is also increasingly important issue. Altough the complication rates of groups were similar in our study, a reason may also be antibody-dependent enhancement (ADE) in patients who do not benefit from CP treatment. ADE is a theoretical concern related to passive or active antibodies, targeting s protein domains other than RBD facilitating IgG-coated virion entry into macrophages, leading to activation of the RNA sensing Toll-like receptors 3, 7 and 8 and finally to elevated production of tumour necrosis factor and interleukin-6 (20, 21). Genetic polymorphism and worsening of underlying COVID-19-specific coagulopathy from clotting factors in transfused plasma can also be causes in patients who do not benefit from CP treatment (21, 22).

In order to mitigate potential ADE risks in CP therapy, plasma donors could be pre-screened for high neutralization titreş and anti-S or anti-RBD antibodies could also be purified from donated CP to enrich for neutralizing antibodies and to avoid the risks of ADE caused by non-neutralizing antibodies against other SARS-CoV-2 antigens (23). The preclinical and clinical data will be helpful to deconvolute the risk profiles for ADE versus other known severe adverse events that can occur with human CP, including transfusion-related acute lung injury.

Experience in the dynamic pandemic process, “COVID-19 Immune (Convalescent) Plasma Supply and Clinical Use Guide” prepared and published by our Ministry, is updated according to development and requirements in October 2020. With this guide, the requirement for ‘Plasma should be given to the patient in the absens of pneumonia findings

and before the need for intensive care develops and the disease has not exceeded 7 days since the onset of symptoms' has been introduced (24).

As conclusion; multicenter randomized controlled trials should be encouraged in order to establish the most effective shedule (timing, dose and patient selection) of CP therapy.

Table 1. Baseline Demographics and Clinical Characteristics of Patients

	Convalescent Plasma group (n=201)	Control group (n=238)	p
Age (years) (mean±SD)	62.25±13.26	62.46±14.01	0.874
Male sex (n,%)	141(70.1)	161(67.6)	0.573
Smoking (n,%)	68(34)	74(31.1)	0.517
Underlying Diseases (n,%)			
Cardiovascular Disease	39(19.5)	34(14.3)	0.145
COLD	20(10)	17(7.1)	0.291
Diabetes Mellitus	74(36.8)	90(37.8)	0.829
Malignancy	11(5.5)	19(8)	0.294
Hypertension	95(47.3)	102(42.9)	0.355
Hyperlipidemia	37(18.4)	43(18.1)	0.927
Initial symptoms (n,%)			
Fever	117(58.2)	119(50)	0.086
Cough	100(49.8)	117(49.2)	0.902
Dispnea	165(82.1)	178(74.8)	0.065
Myalgia	106(53)	113(47.5)	0.250
Gastrointestinal symptoms	55(27.4)	55(23.1)	0.305
Days since symptoms initiation (median, IQR)	5(3-7)	4(2-7)	0.051
Disease severity scale at enrollment (median, IQR)	3(3-3)	3(3-3)	0.833
O_2 saturation at enrollment (median, IQR)	90(88-93)	93(90-94)	**<0.001**
Type of supplemental O_2 support at enrollment (n,%)			
Nasal cannula	85(42.3)	112(47.1)	0.258
Mask	35(17.4)	39(16.4)	
Mask with reservoir	47(23.4)	48(20.2)	
High flow	22(10.9)	19(8)	
Continious positive airway pressure mask	9(4.5)	8(3.4)	
Mechanical ventilation	3(1.5)	12(5)	
Laborotory parameters at enrollment			
Alanine aminotransferase (U/L)	32(21-50)	29(19-48)	0.212
Albumin (g/dL)	3.3(2.9-3.6)	3.5(3-3.19)	**0.024**
Lactate dehydrogenase (U/L)	414.5(313.5-534.5)	334.5(261-460)	**<0.001**
C-reactive protein (mg/L)	127(71-204.5)	100.3(41.5-171.2)	**0.003**
Lymphocyte count (/mm^3)	800(600-1200)	1000(600-1450)	**0.013**
Neutrophil/lymphocyte ratio	6.9(4.3-12.2)	5.01(3.03-12.07)	**0.003**
Troponin (ng/L)	9(3-23)	8(3-26)	0.616
D-dimer (µg/L)	347.5(229.5-704)	312(185-731)	0.173
Ferritin (µg/L)	451(226-757)	288(109-564)	**<0.001**
Interleukin-6 (pg/ml)	61.5(29-170.8)	64.28(26.52-135.3)	0.787

SD: Standart deviation; COLD: Chronical Obstructive Lung Disease; IQR: Inter Quantile Range

Table 2. Comparison of Clinical Features and Outcomes of Patients according to Convalescent Plasma Administration Time (5 days)

	CP administration in the first 5 days (n=32)	CP administration after the first 5 days (n=169)	Control Group (n=238)	p
Clinical improvement	21(65.6)[a]	81(47.9)[b]	148(62.2)[a]	**0.010**
Duration between symptom onset and clinical improvement	16(13-23)[a]	21(17-29)[b]	14(11-16)[c]	**<0.001**
Mortality	11(34.4)[a]	89(52.7)[b]	90(37.8)[a]	**0.007**
Duration between symptom onset and mortality	14(9-20)[a]	22(17-30)[b]	17(12-23)[a]	**<0.001**

Findings were shown with median(IQR= Inter Quantile Range) or n(%) (Kruskal-Wallis test, Pearson chi-square test). Different lowercase exponential letters in a row indicated statistically significant difference between groups.

Table 3. Comparison of Clinical Features and Outcomes of Patients according to Convalescent Plasma Administration Time (7 days)

		CP administration in the first 7 days (n=65)	CP administration after the first 7 days (n=136)	Control Group (n=238)	p
Duration of Hospitalization					
	Ward	9(3-12)	7(4-13)	7(6-10)	0.864
	Intensive care	12(6-15)[a,b]	12(7-21)[a]	9(6-15)[b]	**0.021**
	Total	15(11-23)[a]	16(11.5-24)[a]	9(7-13)[b]	**<0.001**
Duration of Mechanical ventilation					
	Non-invasive	4(2-6)	3(2-6)	3(2-4)	0.167
	Invasive	5(3-9)	7(4-13)	5(2-10)	0.070
Clinical improvement		40(61.5)[a]	61(44.9)[b]	148(62.2)[a]	**0.003**
Time between symptom onset and clinical improvement		17(13-26)[a]	22(18-30)[b]	14(11-16)[c]	**<0.001**
Mortality		25(38.5)[a]	75(55.1)[b]	90(37.8)[a]	**0.003**
Time between symptom onset and mortality		18(14-21)[a]	24(17-31)[b]	17(12-23)[a]	**<0.001**

Findings were shown with median(IQR= Inter Quantile Range) or n(%) (Kruskal-Wallis test, Pearson chi-square test). Different lowercase exponential letters in a row indicated statistically significant difference between groups.

Table 4. Demopraphic and Clinical Features of Convalescent Plasma Patients according to Mortality

	Survived (n=101)	**Dead (n=100)**	**p**
Age (years) (mean±SD)	59.28±13.46	65.26±12.41	**0.001**
Male sex (n,%)	71(70.3)	70(70)	0.963

Blood group (n,%)			
A+	44(43.6)	44(44.0)	0.653
B+	14(13.9)	12(12.0)	
AB+	8(7.9)	12(12.0)	
0+	26(25.7)	23(23.0)	
Others	9(8.9)	9(9.0)	
Underlying diseases			
Cardiovascular Diseases	14(13.9)	25(25.3)	**0.042**
COLD	7(6.9)	13(13)	0.151
Diabetes Mellitus	34(33.7)	40(40)	0.352
Malignancy	7(6.9)	4(4)	0.361
Hypertension	39(38.6)	56(56)	**0.014**
Hyperlipidemia	14(13.9)	23(23)	0.095
Symptoms			
Fever	60(59.4)	57(57)	0.730
Cough	55(54.5)	45(45)	0.180
Dispnea	79(78.2)	86(86)	0.150
Myalgia	56(56)	50(50)	0.395
Gastrointestinal symptoms	27(26.7)	28(28)	0.840
Days since symptoms initiation (median, IQR)	5(3-6)	5(3-7)	0.912
O_2 saturation at enrollment (median, IQR)	91(88-93)	90(86-93)	0.087
Type of supplemental O_2 support at enrollment (n,%)			
Nasal cannula	45(44.6)	40(40)	0.262
Mask	22(21.8)	13(13)	
Mask with reservoir	21(20.8)	26(26)	
High flow	10(9.9)	12(12)	
Continious positive airway pressure mask	2(2)	7(7)	
Mechanical ventilation	1(1)	2(2)	
Time between symptom onset and plasma administration	9(6-11)	9(7.5-11.5)	0.097
Disease severity while plasma administration			
Severe	89(88.1)	63(63)	**<0.001**
Life-threatening	9(8.9)	36(36)	
Type of supplemental O_2 support while plasma administration (n,%)			
Nasal cannula	20(20.4)[a]	3(3)[b]	**<0.001**
Mask	15(15.3)[a]	5(5)[b]	
Mask with reservoir	34(34.7)[a]	15(15)[b]	
High flow	17(17.3)[a]	37(37)[b]	
Continious positive airway pressure mask	3(3.1)[a]	6(6)[b]	
Mechanical ventilation	9(9.2)[a]	34(34)[b]	
Laboratory parameters			
Alanine aminotransferase (U/L)	37(24-67)	31(21-51)	0.058
Albumin (g/dL)	3.1(2.8-3.35)	2.9(2.6-3.2)	**0.001**
Lactate dehydrogenase (U/L)	373(270-459)	463(410-561)	**<0.001**
C-reactive protein (mg/L)	97(43-156)	126.75(86-209.5)	**0.002**
Lymphocyte count (/mm^3)	800(600-1100)	600(400-800)	**<0.001**
Troponin (ng/L)	5(3-21)	16(6-35)	**0.005**

D-dimer (µg/L)	401(240-751.5)	617(323-1239)	**0.007**
Ferritin (µg/L)	458(303-771)	570(310-1111)	0.098
Interleukin-6 (pg/ml)	35.82(17.3-83)	60.95(39.43-173)	0.068

SD: Standart deviation; COLD: Chronical Obstructive Lung Disease. Different lowercase exponential letters in a row indicated statistically significant difference between groups.