ISBN: 978-2-38236-185-6

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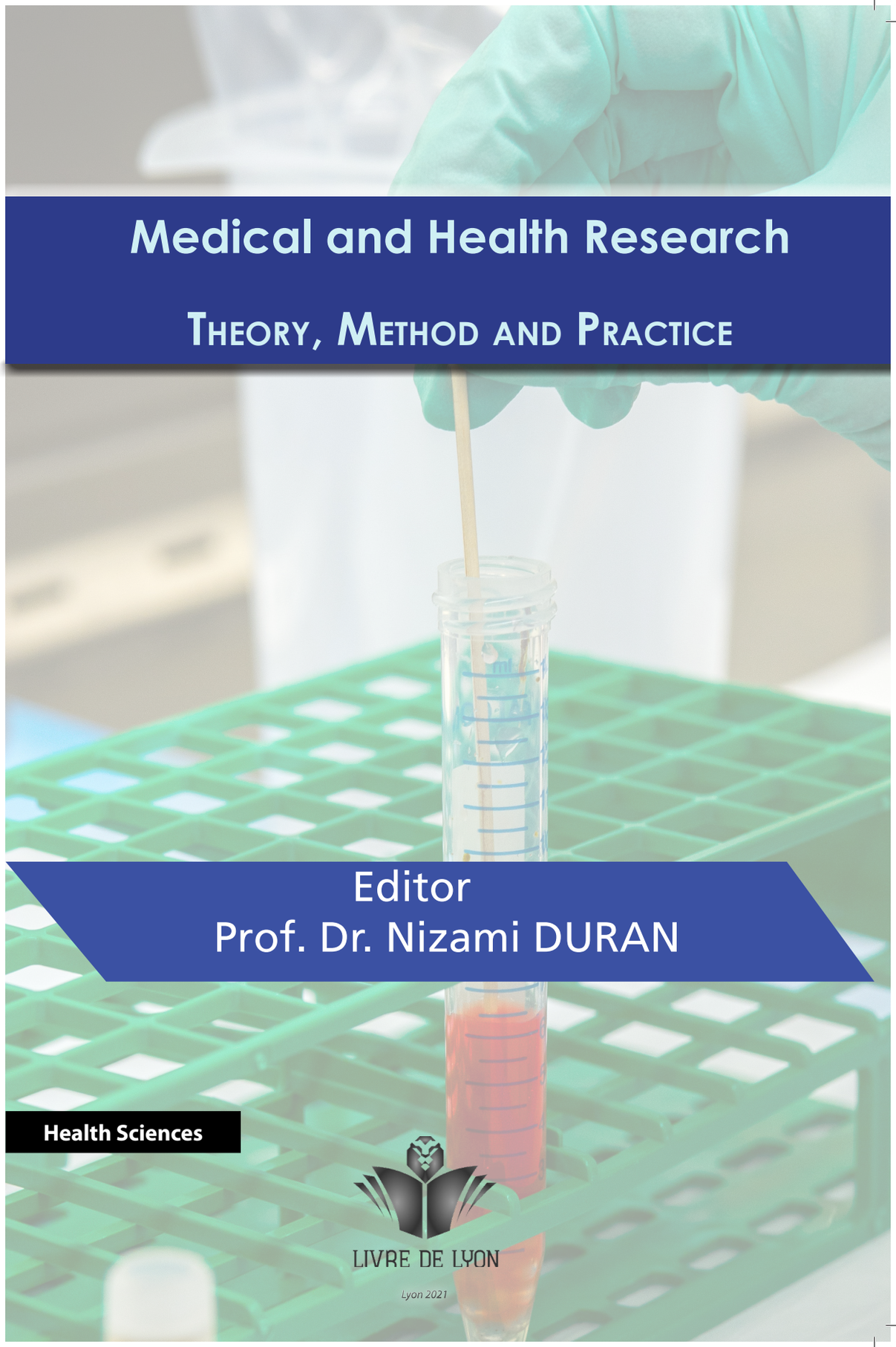

Medical and Health Research

Theory, Method and Practice

Editor
Prof. Dr. Nizami DURAN

Health Sciences

LIVRE DE LYON

Lyon 2021

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LIVRE DE LYON

Lyon 2021

Editor • Prof. Dr. Nizami Duran • Orcid: 0000-0002-2766-3491
Cover Design • Clarica Consulting
Book Layout • Mirajul Kayal
First Published • September 2021, Lyon

ISBN: 978-2-38236-185-6

Publisher • Livre de Lyon
Address • 37 rue marietton, 69009, Lyon France
website • http://www.livredelyon.com
e-mail • livredelyon@gmail.com

PREFACE

Dear reader,

This book contains preclinical and clinical studies in many fields of medicine, and I think that it will make important contributions to your personal development in your field. This book aims to assist health scientists in planning and conducting research. The topics covered in this book are current issues and will contribute to your perspective. It will be seen that important experiences are presented in a wide variety of issues in the field of health, and it also includes innovations in diagnosis and treatment approaches. It will be an important reference that I think can be a very useful guide or reference book that both health students and health professionals, as well as health educators, can benefit from.

I would like to express my deepest gratitude to all the authors who contributed to the preparation of this book.

Prof. Dr.Nizami DURAN
Hatay Mustafa Kemal University, Medical Faculty
Department of Mirobiology
Hatay/TURKEY

CONTENTS

CHAPTER I

CARDIAC TUMORS

Ufuk Turan Kürşat Korkmaz
(M.D. Assistant Professor Doctor.) Department of Cardiovascular Surgery,
Bolu Abant İzzet Baysal University Medical Faculty,Turkey
e-mail: ufuktkk@yahoo.com
Orcid: 0000-0002-6107-2943

1. Introduction

Cardiac tumors are differentiated into primary and secondary (metastatic). In autopsy series, the prevalence of primary cardiac tumors has been reported between 0.001% and 0.03% (1). Of all primary tumors, 75% are benign origin. The most common benign cardiac tumors are myxomas and the most common malignant cardiac tumors are cardiac sarcomas about 40% of these are angiosarcomas. Metastatic (secondary) tumors are 20 to 40 folds more common than primary tumors. Fifteen percent of patients with any form of cancer eventually develop metastases to the heart. This chapter describes characteristics, clinical presentation, diagnosis and treatment of primary benign and malignant tumors and secondary (metastatic) tumors of the heart.When the localization of the tumors is evaluated, the left atrium is in the first place 84.6% (2).

2. Primary Cardiac Tumors

A general classification of benign and malignant primary tumors is given in Figure 1.

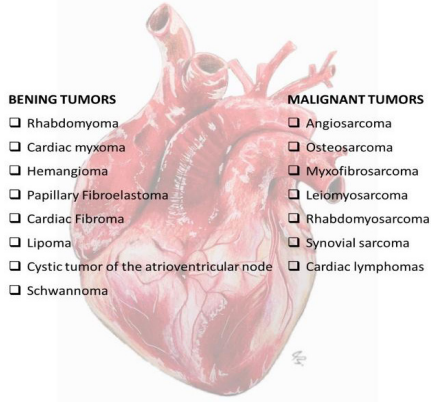

Figure 1. Classification of primary heart tumors

2.1. Benign Primary Cardiac Tumors

Although most cardiac tumors are benign, their malignant potential and the risks due to impaired cardiac functions make evaluation and definitive treatment of these tumors mandatory.

2.1.1. Rhabdomyoma

Rhabdomyoma is the most commonly diagnosed cardiac tumor in fetuses and infants (3). These tumors are identified more commonly in fetal series compared to postnatal series. It is considered a congenital hamartoma. Between 80-90% of rhabdomyomas are associated with tuberous sclerosis (4). These tumors may be detected incidentally or may cause symptoms by obstructing blood flow. The most commonly involved site is ventricular myocardium. The other involved sites include the atria, cavoatrial junction and epicardial surface (5). Rhabdomyomas are characterized by homogeneous echogenicity on imaging. In the histologic examination, nodules of rounded myocytes with large vacuoles are observed. The presentation is in the form of heart blocks or pericardial effusion (6). Cardiac rhabdomyoma is spontaneously regressed and therefore, surgical resection is considered only in case of severe outflow obstruction. Arrhythmias can also occur with rhabdomyomas ranging from bradycardia to atrial/ventricular tachycardia.

2.1.2. Myxoma

Myxoma is the most common benign cardiac tumor in adults. It accounts for about 25% of all cardiac tumors and more than 50% of all benign cardiac tumors. On the other hand, in our study, myxoma was 76.9%(2). In a study from Ireland, myxomas have been reported to occur at a rate of 0.5 million cases per million population annually (7). Myxoma is more common in women than in men with women:men rate is reported as 1.5 to 2. It is usually seen between 4th and 6th decades of life (8). The mean age at presentation has been reported as 53 years (3). Patients with permanent or temporary neurological disorders are at a higher risk of developing myxomas (9). Myxomas are thought to originate from undifferentiated mesenchymal stem cells (10). Tumor cells are believed to exhibit the same characteristics with endocardial-mesenchymal transformation of the endocardial cushion (3). Of all myxomas, 75% are located in the left atrium, 20% in the right atrium and 5% in the ventricles. The composition of these tumors is heterogeneous and includes areas of hemorrhage, cyst formation, fibrosis, calcification and necrosis. Histopathologically myxomas appear as yellowish, white or brownish masses that are often covered by thrombus. Obstructive, embolic and constitutional symptoms are the most common manifestations of myxomas (11). Unusually presenting symptoms often leads to a delayed

diagnosis, resulting in poor prognosis (12). Of all patients with myxomas, 22% develop embolization, resulting in symptoms of stroke or peripheral ischemia. Myxomas have a recurrence rate of 1% after resection. In 5% to 7% of the patients, these tumors are associated with the Carney complex. This syndrome is an autosomal inherited disease. In the case of the Carney complex, myxomas may be accompanied by extracardial myxomas, abnormal skin pigmentation, schwannomas or endocrine tumors (13). Myxomas enhance with the use of echocardiography contrast. The other modalities used for the diagnosis include CT and cardiac magnetic resonance imaging. Myxoma treatment is surgery. The surgeries can be performed with a sternotomy with a bicaval cannulation or with a right anterior axillary torokotomy through the femoral vein cannulation and cannulation of the superior Vena cava. It is resected from a healthy heart tissue together with a quantity. In this case, atrial septal defect may rarely occur, which may require repair. If valve-related, valve repair may require valve replacement. Figure 2 shows the myxoma excisions we made.

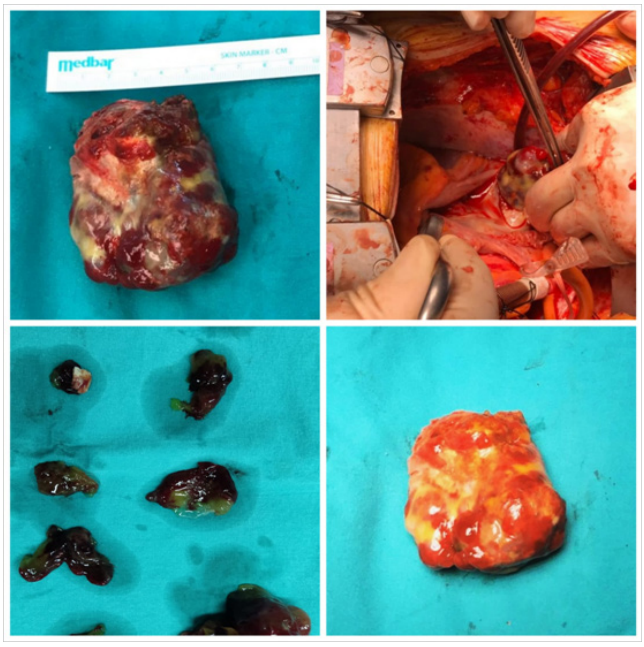

Figure 2. Excision of Myxoma lesions we performed.

2.1.3. Hemangioma

Hemangiomas are vascular malformations of the heart and more commonly seen in adults. These tumors account for 2% of primary cardiac neoplasms (14). The most frequently involved sites are the right atrium in children and left ventricle and cardiac valves in adults. Whereas a large number of these tumors

are found incidentally in adults, hemangiomas may cause pericardial effusions in infants. In the histopathologic examination, hemangiomas are heterogeneous, mostly resembling cavernous and capillary hemangiomas. Sometimes, hemangiomas are misdiagnosed as myxomas. Cardiac hemangiomas of the right atrium may be in the form of papillary structures, making distinguishing them from angiosarcomas challenging. After resection, hemangiomas usually do not recur. These tumors may be occasionally associated with extracardiac hemangiomas such as liver and gastrointestinal tract (15).

2.1.4. Papillary Fibroelastoma

Papillary fibroelastoma is the most common tumor of the cardiac valves. It accounts for 75% of all valvular neoplasms. Also, it is a potential cause of transient ischemic attacks, myocardial infarction and sudden death (16). It more commonly affects elderly people (17). The mean age is 70-80 years old with no gender predilection (18). Papillary fibroelastoma is generally <1 cm in size. The most commonly involved sites include the aortic and mitral valves. Tricuspid and pulmonary valves may also be involved. Histologically, these tumors resemble excrescences. These tumors can be easily seen by echocardiography and are readily diagnosed. Papillary fibroelastoma is currently the most commonly resected cardiac mass (4). Surgical treatment of papillary fibroelastoma involves complete resection of the mass with sparing the underlying valvular tissue.

2.1.5. Fibroma

Cardiac fibromas are the second most common primary cardiac tumors in children following rhabdomyomas. These tumors can be symptomatic with inflow and outflow obstruction, thromboembolic events, conduction defects and coronary artery compromise (19). Cardiac fibromas may be associated with genetic conditions such as nevoid basal cell carcinoma syndrome, also known as Gorlin syndrome (20). Most fibromas are detected in utero. Cardiac fibromas primarily present in infancy, but in the case of Gorlin syndrome, they can be seen up to 60 years old (21). The most commonly involved site is the left ventricle followed by the left ventricle and interventricular septum. These tumors are often mistaken for hypertrophic cardiomyopathy. Fibromas rarely regress spontaneously and never resolve completely. Patients with fibromas mainly typically present with ventricular tachycardia, ventricular fibrillation and murmur. Histopathologically fibromas are composed of monomorphic fibroblasts. Calcifications are common. Symptomatic tumors are treated with resection. Cardiac fibromas in infants that are not resectable have a poor diagnosis and may require cardiac transplantation (22).

2.1.6. Lipoma

Cardiac lipomas are rare, benign primary tumors of the heart. These tumors account for only 3% of all benign primary cardiac tumors. Lipomas are associated with female gender, older age and increased body mass index (8). Lipomas consist of mature adipocytes that are enclosed by a collagenous capsule (23). These tumors are often asymptomatic, but depending on the size and location of the tumor, they may cause symptoms such as chest pain, arrhythmias, obstruction of blood flow and dyspnea, especially in case where they reach a large size (24). The most commonly involved sites include subepicardium, left ventricle, right atrium and interatrial septum. Histopathologically lipomas usually appear as single and well-encapsulated masses. However, multiple lesions can also occur. Cardiac lipomas composed of mature fat cells and vacuolated brown fat. Degeneration and calcification areas may be present. Transthoracic echocardiogram, MRI and CT are used for the diagnosis. The treatment of choice is surgical excision. Lipomas should be differentiated from liposarcomas as well as lipomatous hypertrophy (25).

2.1.7. Cystic Tumor of the Atrioventricular Node

Cystic tumor of the atrioventricular node is a rarely seen benign lesion. It is also known as tawarioma or celothelioma (26). These tumors are composed of ectopic glands that are formed in the atrioventricular node area and in the atrial septum. Congenital heart block is a typical manifestation due to the location of these neoplasms. In the histopathological examination, these lesions appear as cysts lined by flattened epithelium. Cystic tumors of the atrioventricular node are more common in women at a ratio of 3:1. The diagnosis is most commonly established in the 4th decade of life. Patients with these tumors are mostly (75%) present with complete AV block. Sudden death is the first clinical manifestation in 10% of the patients (27). Most of these tumors are incidentally detected at autopsy.

2.1.8. Schwannoma

Schwannoma of the heart is a very rare entity thought to be originated from the cardiac branches arising from the vagus nerve or cardiac plexus (28). Schwannoma, also called neurilemoma, is a nerve sheath tumor consisting of Schwann cells. These tumors show close proximity to the interatrial septum. Timely recognition of these tumors are of paramount importance, because they have the potential for malignant transformation. The growth of these tumors causes clinical signs and symptoms depending on the tumor size and compression of surrounding structures such as cardiac chambers, coronary arteries and mediastinal structures (29). Diagnosis of Schwannomas can only be

made by histopathological examination. In histochemical examination, positive staining for S-100 protein supports the Schwann cell origin of the tumour (30). Cardiac MRI and angiogram can be helpful in determining extent of the lesion. The main treatment of Schwannoma is surgical resection.

2.2. MMalignant Primary Cardiac Tumors

Malignant cardiac tumors are relatively rare, accounting for 25% of primary cardiac tumors. They are usually seen in the 3th to 5th decades of life. Malignant cardiac tumors have a poor prognosis. These tumors infiltrate the myocardium, obstructing intracardiac flow and producing metastases.

2.2.1 Angiosarcoma

Angiosarcomas are malignancies of endothelial cells, accounting for about 40% of cardiac sarcoma. In our study, the rate of angiosarcoma resected from the myocardium of the right ventricle was 7.7% (2). The most commonly involved sites include pericardium and right atrial wall, but angiosarcomas may also occur in any chamber (31). The main symptoms of angiosarcomas include arrhythmias, pericardial effusions, emboli and systemic symptoms such as fever and weakness. Histologically, angiosarcomas appear as anastomosing channels covered by atypical cells. There are usually areas of hemorrhage and necrosis within the tumor (32). Diagnosis of these tumors is made through TTE, CT, MRI, histopathology and immunohistochemistry. Distinguishing these tumors from hemangiomas may be challenging. Prognosis of patients with angiosarcomas is poor due to early blood metastasis and resistance to chemotherapy (33). The mean survival has been reported as 1 year (4).

2.2.2. Osteosarcoma

Cardiac osteosarcomas are extremely rare, accounting for only 3-9% of all cardiac sarcomas. They have an aggressive biology and poor prognosis. Cardiac osteosarcomas may lead to arrhythmias, valve dysfunction and heart failure. Local invasion to the vital structures may also cause symptoms. Primary cardiac osteosarcoma can be seen in any age without sex predilection (34). The most commonly involved site is the left atrium. Cardiac osteosarcomas are often mistaken for atrial myxomas in radiological studies. Macroscopically, osteosarcomas appear similar to myxomas and sarcomas Hence, the diagnosis can be proven only with tumor excision and pathological confirmation (35). The main treatment option of cardiac osteosarcomas is tumor excision. However, close proximity to the vital structures limits benefits from surgery. Of patients with high-grade tumors, 33.3% die within five days following the initial surgery (35).

2.2.3. Myxofibrosarcoma

According to the WHO classification of cardiac tumors, myxofibrosarcoma is defined as a malignant tumor consisting of fibroblasts, intercellular collagen and myxoid stroma (36). The most commonly involved sites are the left atrium, right atrium, right ventricle, left ventricle and pulmonary artery. Patients with myxofibrosarcoma are mostly asymptomatic. Symptoms manifest when local diffusion or distant metastasis occur. The presenting symptoms include chest pain, dyspnea and palpitation (37). Pathophysiologic changes cause hypotension, tachycardia and syncope, while hemodynamic changes lead to cardiac murmurs and signs of heart failure (38). Histologically, myxofibrosarcoma appears whitish, yellowish or grayish in color. Echocardiography can show the location, attachment and diameter of the tumor. Patients undergoing tumor resection have a longer survival compared to patients with unresectable myxofibrosarcoma. In patients with high-grade cardiac myxofibrosarcoma, complete resection is inevitable. Myxofibrosarcomas have a poor prognosis.

2.2.4. Leiomyosarcoma

Leiomyosarcomas are malignant tumors originating from the smooth muscle cell tissue. Leiomyosacromas account for less than 1% of malignant cardiac tumors. The most commonly involved sites include the left atrium and pulmonary veins. Presenting symptoms of cardiac leiomyosarcomas are dyspnea, chest pain, arrhythmias, peripheral embolism and heart failure. Leiomyosacromas grow rapidly and show a high rate of distant metastases. The rate of local recurrence is also high after removal of the tumor. The prognosis is poor. The mean survival is 6 months once the diagnosis is established (39). Since the differential diagnosis of cardiac leiomyosarcoma is challenging, histopathological and immunohistochemical examinations are necessary. Radical surgery is the best option to achieve a good outcome, but complete surgical resection may be difficult due to distant metastases. Palliative chemotherapy may be helpful; however, its effectiveness is still unclear (40).

2.2.5. Rhabdomyosarcoma

Cardiac rhabdomyosarcoma is an aggressive malignant tumor of the heart with a high rate of mortality. Rhabdomyosarcomas are the second most common malignant primary tumors of the heart following angiosarcomas (41), accounting for 20% of all primary malignant cardiac tumors. These tumors are most commonly seen in children with a mean age of 14 years. The most commonly involved sites are the left and ventricles and the left and right atria. The tıumor usually metastasizes to other organs. Differential diagnosis of these tumors is difficult due to lack of specific clinical symptoms. In these tumors,

surgical resection is performed to confirm the diagnosis, relieve symptoms and improve short-term survival. Rhabdomyosarcomas have a poor prognosis. In most patients with rhabdomyosarcomas, survival is <1 year following radical surgical resection (42).

2.2.6. Synovial Sarcoma

Primary cardiac synovial sarcomas are extremely rare malignant tumors of the heart. The most commonly involved sites are the pericardium and the chambers. These tumors mostly occur in the first decades of life with male predilection (43). Presenting symptoms are non-specific and include chest pain, dyspnea and pericardial effusion. Histologically, synovial sarcomas appear with a monophasic or biphasic spindle cell pattern and glandular structures. The treatment of choice is radical resection of the tumor. However, since the disease is extremely rare, it is difficult to establish an efficient treatment option. In addition, a complete resection is often challenging because of the infiltration and extension of the tumor. Since these tumors show frequent relapses and metastases, the prognosis is poor (44). Patients with synovial sarcomas are also at risk of developing pulmonary metastasis. Heart transplantation has been advocated for young patients with small-size and less aggressive synovial sarcomas (45).

2.2.7. Cardiac Lymphomas

Primary cardiac lymphomas are malignant tumors of the heart and/or pericardium. Cardiac lymphomas account for 1% to 2% of all resected cardiac tumors. Most patients with cardiac lymphomas are > 60 years old with male predominance (14). The most commonly involved site is the right atrium, but these tumors may also develop in any cardiac chamber. Right sided involvement is predominant in cardiac lymphomas. Clinical manifestations depend on the sites of involvement in the heart. Presenting symptoms include congestive heart failure, pericardial effusion and arrhythmias. Diffuse large B-cell lymphoma, follicular B-cell lymphoma and Burkitt's lymphoma are the most common histologic types of cardiac lymphomas (46). According to the WHO classification of cardiac tumors, diffuse B-cell lymphoma is the most common subtype of these tumors (4). Histologic/cytologic examination is mandatory for definitive diagnosis and determining an appropriate treatment. If cytology is not available, biopsy of cardiac tissue during thoracotomy is the primary method to establish definitive diagnosis. Echocardiography, CT, PET/CT and MRI are used to help the diagnosis and/or staging. The optimal treatment choice is aggressive resection of the tumor with reconstruction as palliative therapy. Survival of patients with cardiac lymphomas is up to 15 months with surgical resection. High doses of chemotherapy are also used for long term remission (47).

2. Secondary (Metastatic) Cardiac Tumors

Metastatic cardiac tumors are 30 to 40 folds more common than primary cardiac tumors (48). Fifteen percent of patients with any type of cancer eventually develop metastases in the heart. The most commonly involved cardiac site is the pericardium. The most common metastases in the heart are caused by pleural mesothelioma, pulmonary adenocarcinoma, undifferentiated carcinomas, pulmonary squamous cell carcinomas, breast carcinomas, ovarian carcinomas, gastric carcinomas, renal carcinomas and pancreatic carcinomas (49).

Metastases to the heart usually occur as a result of blood dissemination of cancerous cells or direct extension through the adjacent tissues. Pericardial metastasis occurs due to lymphatic spread and myocardial metastasis due to hematogenous spread (50). Cardiac metastasis is often unrecognized and detected only after death by autopsy (32). Clinical manifestations depend on the most commonly affected site. Typical presenting symptoms include arrhythmias, complete atrioventricular blocks and conduction disturbances (51).

The diagnostic evaluation is based on echocardiography, CMR, CT and PET/CT as in primary cardiac tumors. However, echocardiography is preferred to determine metastases in the heart and their complications. The morphology of cardiac metastasis is related to the type and localization of the tumor and spreading capacity. Whereas myocardial metastasis may involve any cardiac chamber, pericardial metastasis may show focal, diffuse or massive infiltration.

There is no standard treatment approach established for the management of secondary cardiac tumors. The treatment usually includes management of the primary tumor and palliative care. Although the prognosis is poor with cardiac metastases, in the case of significant obstruction of inflow and/or outflow, surgical treatment should be considered despite mortality risk (50).

3. Conclusioon

Cardiac tumors are classified as primary and secondary neoplasms. Although rare, these tumors are difficult to manage because of anatomic proximity to the vital sites of the heart. It can be seen as myxomas and other heart tumors in surgeries that evaluate left atrial thrombus on echocardiography. Another difficulty is that there are only case reports or case series published in the literature. In most cardiac tumors, there is no definitive treatment method for complete remission. Aggressive or complete surgical resection is the only preferred treatment method for most primary cardiac tumors, while chemotherapy and/or radiotherapy is added to the treatment for some malignant cardiac masses. The diagnosis is also challenging, mostly requiring histologic/cytologic examination for the differentiation of the tumor. Imaging modalities are helpful for staging. In secondary or metastatic cardiac tumors, the management is primarily focused

on the treatment of the primary tumor and palliative care. In parallel with the advancements in the field of oncology, including surgical methods, radiotherapy and pharmaceutics, as the number of publications increase in the literature, definitive diagnosis and management of cardiac tumors will be more clarified.

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CHAPTER II

RESISTANT PARANASAL SOLITARY FIBROUS TUMOR

Nurten Kucuk[1] & Ali Cemal Yumusakhuylu[2]

1.Department of Otorhinolaryngology Head and Neck Surgery, Memorial Hospital, Istanbul, Turkey

2.Department of Otorhinolaryngology Head and Neck Surgery Marmara University Hospital, Istanbul, Turkey

1. Introduction

Solitary fibrous tumors(SFTs) is described by Klemperer and Rabin in 1931 as mesenchymal bleeding tumors originating from pleura and peritonea. All of SFT, 12-15% of them occur in the head and neck area. SFTs of the head and neck area are mostly seen in oral cavity, rarely it can be also seen in paranasal sinuses. The average age is 40-70 decades. Nasal obstruction, epistaxis, exopthalmos, headache are main complications. As the tumor size bigger, vision problems , diplopia can also be seen(1). SFTs can be misdiagnosed with other soft tissue tumors such as synovial sarcoma, neurofibroma, histiocytoma, fibroma, schwannoma. The immunohistochemical findings are important for differentiating the SFTs from other soft tissue tumors(2). Up to now, only 35 SFTs has been reported in literature. In this case, we presented a solitary fibrous tumor that showed benign histopathologichal findings, but behaved clinically as malign.

2. Case Report

A 33- year-old female patient visited our hospital with an 1 year history of left-sided nasal obstruction. In physical examination, the left nasal cavity is expanded and filled with a smooth reddish mass. Afferent pupil defect was detected in left side. A mass with a size of 4x3 cm in the left nasal cavity with a high contrast showing a uniform soft tissue density that expanded to the nasal septum and eroded nasal septum and invaded to right nasal cavity was discovered on paranasal CT scan. On orbital MR scan, it was seen that the mass destructed the ethmoid lamina and pressured the optic canal and straightened the optic nerve (figure 1). The pathologic result of biopsy was significant of SFT. With an endonasal endoscopic surgery approach, the mass extended into

left sphenoid sinus was removed totally with subsequent elevation and resection of the periosteum of the bones which tumor contacted with. The nasal packing pleased for homeostasis was removed after 48 h.

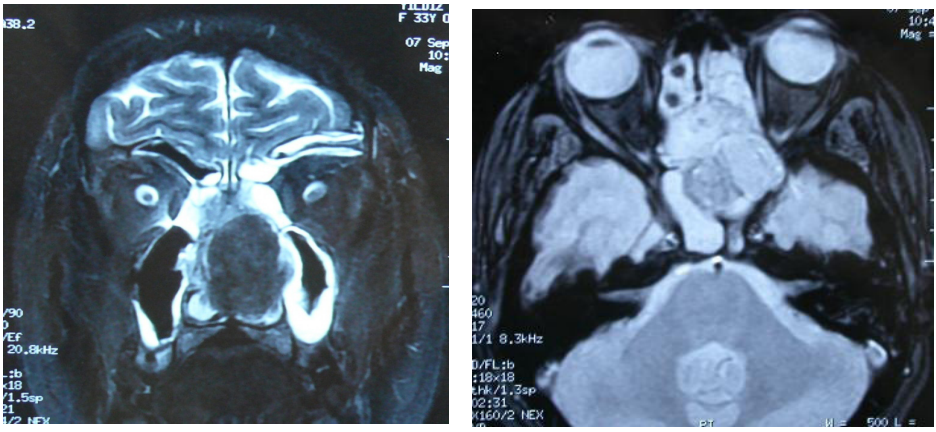

Figure 1. Preoperative MR scan

The cut surface of the mass presented a grayish fish-flesh appearance (herringborne pattern). Final pathology revealed a uniform spindle cells proliferation haphazardly and interspersed thick collagen bundles with a different sizes vessels (figure 2a). A positive immunohistochemical staining for cluster of differentiation (CD)34, B cell lymphoma 2 (BCL2) and vimentin were used to differentiate the tumor from other soft tissue tumors (figure 2b) and negative immunohistochemical staining for cytokeratin, S100-protein, desmin, actin, CD 68, CD 3. The proliferation index, mitotic activity and necroses was minimal. There was no evidence of malignancy in any of the specimen. A diagnosis of SFT was established according to histopathological findings.

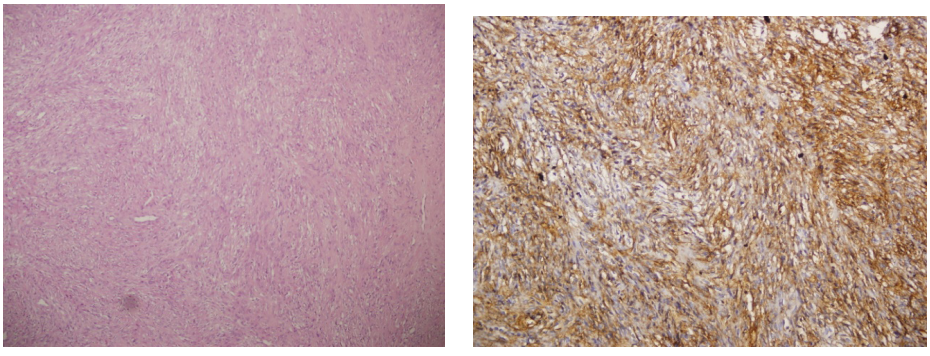

Figure 2a. Hematoxylin ve eosin stained section at original magnification x10. Şekil 2b. CD 34 staining 20x

Up to the 6th month after the surgery, the recurrence was not detected in outpatient follow up. In the 9th month, a pulsatile mass originating from left lateral nasal

wall was seen in endoscopic examination. A high contrast showing mass in size 6x5 cm discovered in paranasal sinus CT scan was totally obliterating the left nasal cavity with extension into the nasopharynx, covered the left spheoid sinus, extended through sella turcica, pituitary gland, optic chiasm, cavernous sinus and and left temporal lobe; eroded the inferiomedial wall of orbita (figure 3). In the physical examination, the patient had an exopthalmos and a limited ability to move the left eye inward, upward and inner side. The afferent pupil defect increased, and she lost color vision on the left eye.

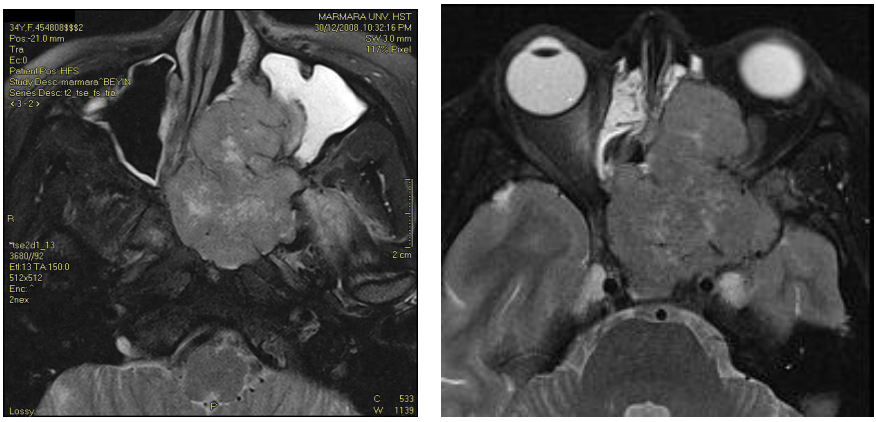

Figure 3. Radiological images before the second operation

The left middle meningeal artery and maxillary artery supplying the tumor was embolized and the blood supply of the tumor was reduced by up to 90% before the second operation (figure 4). The tumor in size 6x5 cm was removed by lateral rhinotomy approach. Because of the invasion of orbital medial wall and extension of the tumor into the durra beyond the temporal lob (figure 5), the tumor couldn't be discovered completely. The patient was planned to undergo external beam radiation therapy for retained tissue and she was consulted to a radiotherapist .

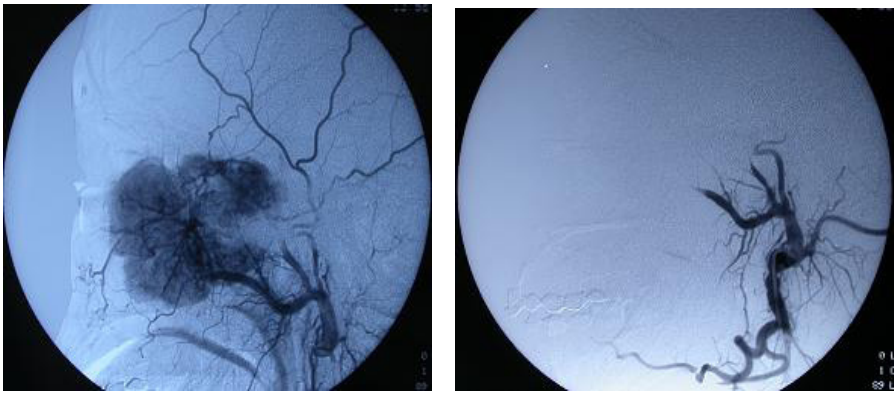

Figure 4. Angiographic embolization images before the second operation.

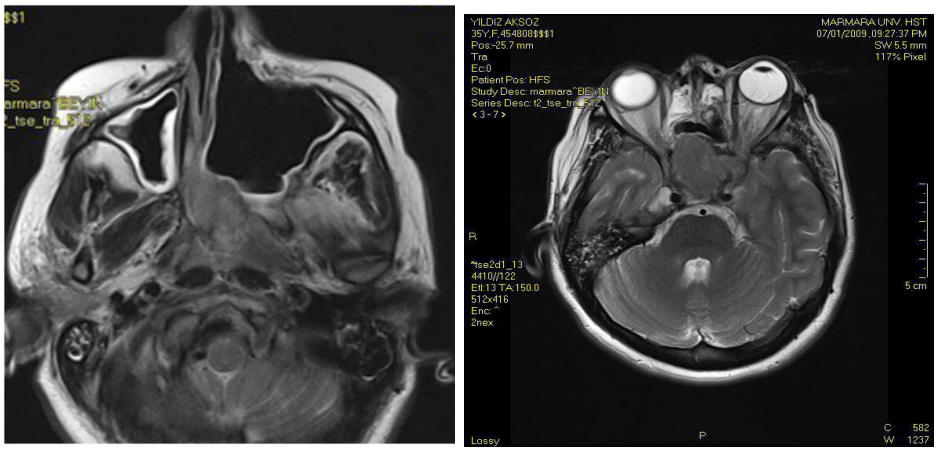

Figure 5. Postoperative radiologic images

DISCUSSION

SFTs mostly originate from the mesothelial cells exclusively within the thoracic cavity. It has been reported also SFT can develop in the peritoneum, liver ,pancreas, thyroid gland, larynx and salivary gland. SFTs of nasal cavity and paranasal sinuses is seen very rare(3). The histoligical and radiological findings of nasal cavity and paranasal sinus SFTs can be mixed with the other soft tissue tumors such as hemangiopericytomas, schwannomas, fibrous histiocytomas, fibrosarcomas and nasopharyngeal anjiofibromas. Immunohistochemistry is the key to differentiate the SFTs from the other soft tissue tumors. Especially CD34 ımmunostaining is very useful for differential diagnosis. Also, bcl-2, CD 99 and vimentin are important markers for SFTs. (3,4)

Although SFTs are reported 80% as benign tumors histologically and clinically, 12-20% can behave in a malignant manner(3). A high mitotic rate and cellularity, tumor larger than 10 cm in size, pleomorphism, bleeding, necrotic change and infiltration are considered an indication of malignancy in SFTs, where as tumors supported with pedicle and tumors not invading adjacent tissues is considered as benign. SFTs does not make metastases, but there is high risk of local nukes and invasion (5). In our case, the histopathologic features of tumor showed benign characters such as minimal proliferation index, minimal mitotic activity and necroses and the resection of the mass totally and and a negative surgical margins, where as the clinical progress of the tumor showed malign character that an aggressive local recurrence and was infiltration into the cavernous sinus, pituitary gland and the orbit was seen.

The main treatment strategy for SFTs is complete surgical excision. The tumor can be removed using a variety of surgical techniques including endoscopic

approach, lateral rhinotomy, medial maxillectomy, external etmoidectomy (3,5). Bleeding control is important for removing the tumor completely. Resectability is the most important prognostic factor and complete removal of the mass is the key point for the treatment of SFTs (3,4). If there is a positive surgical margins or malignant behavior, radiotherapy and chemotherapy can be beneficial after operation (6,7). In our case, in the first operation the tumor was removed completely with nasal endoscopic surgery. In the recurrence, the tumor infiltrated the neighbor areas and this time lateral rhinotomy approach was performed to resect the mass. Unfortunately, because of the expanded of the tumor through cavernous sinus, pituitary gland , optic chiasm, temporal lobe and orbit, the tumor could not be removed totally. So we consulted the patient to the radiotherapist to take radiotherapy for the remain tumor tissue.

It has been only 35 nasal cavity and paranasal sinuses SFTs reported in the literature(8). Generally SFTs are limited in the region originated, and the extansion to other areas is rare. Sometimes, nasal septum is deviated and bony structures damaged due to mass. Rarely the tumor can extend to the orbit (5,7) and intracranial through the cribriform plate and ethmoid roof (7) or can occupy infratemporal area (5). In one case, the mass originating from left side nasal cavity extended to the left orbit and intracranium, the left internal maxillary artery was embolized preoperation, then an anterior craniofacial resection via a midfacial degloving approach was performed to remove the mass an bloc which of histopathological features showed a high mitotic rate. Postoperatif , the patient was planned to undergo external beam radiation therapy(7). In our case, although the histopathologic features demonstrated benign character, it showed an aggressive clinical behavior including local recurrence and distance metastasis that extending into the left orbit, pituitary gland, sella turcica, optic chiasm and cavernous sinus. In our case, only lateral rhinotomy approach is applied to remove the tumor, but it couldn't be resected totally en block and the patient was planned to undergo radiotherapy for the remaining tissue. Perhaps, It could be done transcranial resection with lateral rhinotomy, and after it could be planned to undergo radiation therapy. There is only one case reported in the literature that SFT in nasal cavity and paranasal sinuses invaded to cavernous sinuses (9). Our case is the second reported case that SFT invasion into cavernous sinus. There has not been any reported SFTs extension into the pituitary gland or temporal lobe and this is the first reported case extended into the pituitary gland. This case has been one of the most aggressive and invasive SFT reported in literature up to now.

In conclusion, SFTs are generally benign tumors. Rarely, it can behave as malign. The complete en bloc removal of the tumors are the key point of treatment of SFTs through mostly ESS. But, if it is needed, it can be done through lateral rhinotomy, midfacial degloving or through an external approach. Transcranial

approach with endoscopic approach can be done to be able to remove the tumor en bloc totally. And, if the tumor shows malign character, the radiotherapy can be given to the patient.

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CHAPTER III

MAGNETIC RESONANCE IMAGING FINDINGS OF IDIOPATHIC GRANULOMATOUS MASTITIS

Burçin Ağridağ Üçpinar[1] & Betül Duran[2*]
[1](M.D.) Memorial Sloan Kettering Cancer Center, New York, United States,
e-mail: drburcinagridag@gmail.com
Orcid: 0000-0001-5406-9116
[2](M.D.) University of Health Sciences, İstanbul Şişli *Hamidiye Etfal Training and Research Hospital, Radiology Clinic*
**Corresponding author: Betül DURAN*
e-mail: betulduran_@outlook.com
Orcid: 0000-0001-8598-6814

Introduction

Idiopathic granulomatous mastitis (IGM) is a benign inflammatory disease of breast tissue with unknown etiology that causes non necrotizing granulomas and micro-abscess formation (1,2). The disease was first described by Kessler and Wolloch et al in 1972 (3). Disease is mostly encountered in parous women and the clinical presentation is mostly a palpable breast mass which can mimic malignancy (4). Even there are several treatment options for IGM which includes antibiotics, surgical drainage of the abscess, methotrexate, steroids and partial or total mastectomy, treatment success is low and recurrence rates are high (5).

1. Imaging in IGM

1.1 Mammographic and Sonographic Features of IGM

The mammography and ultrasonography findings of IGM have been described in the literature. The most common mammographic finding of IGM is increased focal density (Figure 1). Parenchymal distortion or microcalcification are not seen with distortion. Second most common mammographic finding is a well-defined mass.

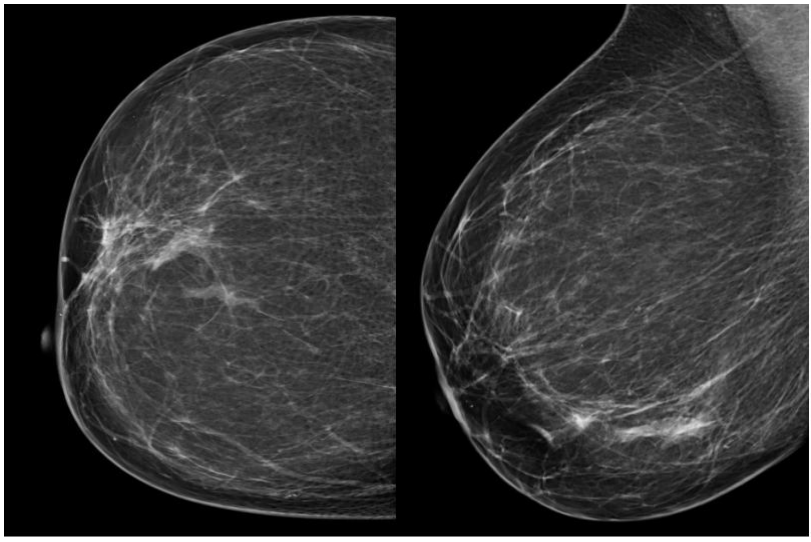

Figure 1. Mammography of a 47-year old woman with complaints of erythema and pain in the right breast. Craniocaudal (right) and mediolateral oblique (left) images show asymmetrical density in inferior medial quadrant. Note that there is no mass effect and accompanying calcification. (Betül Duran, 2021)

The most common sonographic feature of IGM is heterogeneous hypoechoic masses with tubular extensions that may cause posterior shadowing (Figure 2) (5). MRI findings of IGM have been reported in few studies (5-8). In this chapter, we aimed to review magnetic resonance imaging findings of IGM.

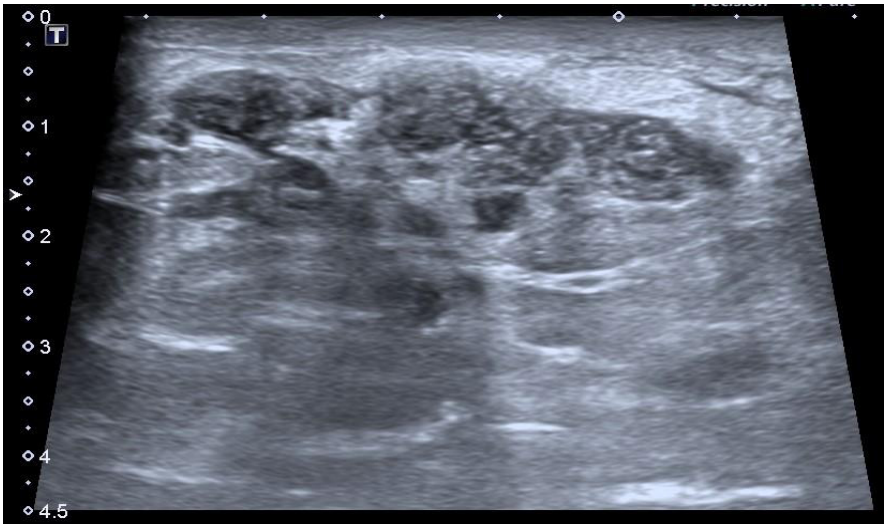

Figure 2. Sonography of 55-year old women with biopsy proven idiopathic granulomatous mastitis. Hypoechoic ill-defined infiltrative and heterogeneous mass is seen (arrow). (Betül Duran, 2019)

1.2 Magnetic Resonance Imaging Features of IGM

IGM has wide spectrum of imaging findings on MRI. Even these findings are not specific for IGM, they may give a clue to radiologists and clinicians (2,5,6). The most common MRI finding of IGM was described as non-mass enhancing lesions. IGM may also show ring enhancement, homogeneous enhancing mass or heterogeneous avidly enhancing mass. In the literature, clustered ring like enhancement pattern was described mostly for non-mass enhancement (Figure 3). This pattern could be the result of periductal inflammation and micro-abscess formations. As an important fact, ductal ectasia and periductal inflammation are seen much more commonly in IGM than breast cancer (9). As a consequence of inflammatory nature of the disease, abscess, redness, edema, skin thickness, retraction, distortion and fistula formation can accompany (2).

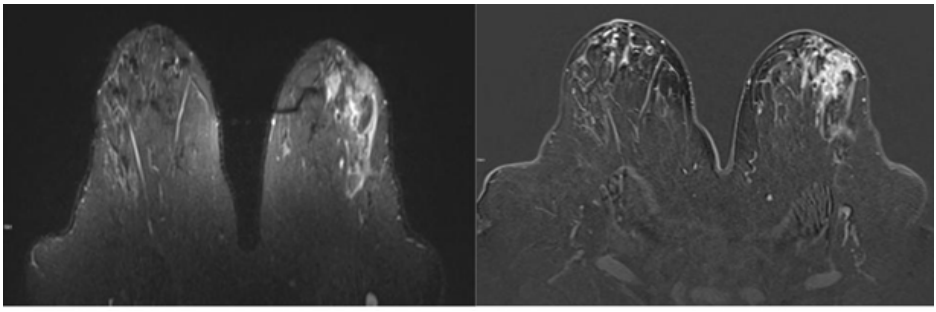

Figure 3. Axial T2 weighted (right) and post contrast subtraction (left) MRI of a 53-year old female with a biopsy proven IGM. On T2 weighted imaging, linear hyperintense areas correspond to ductal ectasia and periductal inflammation. In axial subtraction image on the left, clustered non-mass enhancement is being observed. (Betül Duran, 2021)

After contrast administration, IGM commonly shows benign signal-intensity curve, in which Type 1 is the most encountered However, there are cases in the literature that showed Type 3 enhancement pattern (5,7,10).

With the routine use of diffusion weighted imaging, studies investigating the apparent diffusion coefficient (ADC) measurement in IGM have been reported so far (11-13). These revealed that ADC values of malignant lesions are statistically low when compared with IGM involvement, as expected. In a study which compared the IGM and contralateral normal breast tissue ADC values, granulomatous mastitis showed lower mean ADC values ($0.98 \pm 0.18 \times 10{-}3$) than contralateral normal breast parenchyma ($1.30 \pm 0.341 \times 10{-}3$ mm2 /sn) (9).

CONCLUSION

Non-mass like- enhancement of the breast tissue with clustered enhancement pattern is the most common MRI feature of IGM. Even the ADC value of IGM is higher than the malignant lesions, as expected, it has lower ADC value when compared with the normal breast tissue is. Even the MRI features and ADC measurement give valuable information the final diagnosis of IGM is achieved by biopsy.

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CHAPTER IV

CURRENT APPLICATIONS OF ULTRASOUND ELASTOGRAPHY IN CHILDREN

Dilek Sağlam[1]
[1](MD), *Malatya Education and Research Hospital, Department of Pediatric Radiology,*
e-mail:dilekuzman@hotmail.com
Orcid: *0000-0002-5778-6847*

1. Introduction

Ultrasound elastography is an imaging method evaluating the elasticity and stiffness of tissues. Studies in this subject is mainly performed in adults and publications of ultrasound elastography in children is limited. Ultrasound elastography lacks ionizing radiation, which makes it a suitable and preferable imaging method in children. This review summarizes the applications of ultrasound elastography in pediatric population.

2. Liver

Liver is the most common visceral organ examined by US elastography in children, because of non-invasiveness. The measurements of liver elasticity are performed when the patient is in supine or lateral decubitus position. The region of interest should be at least 2 cm away from liver capsule and perpendicular to the capsule. Postprandial and deep inspiration during measurements should be avoided because they cause falsely increased stiffness [1]. The mean shear wave velocity of children was reported between 1.07-1.19 m/s (Table).

Evaluation of liver fibrosis is the most common indication of ultrasound elastography in children. Ultrasound elastography was found to be useful in determining liver stiffness and the presence/absence of liver fibrosis [2]. Hanquinet et al. reported the cut off value as 1.34 m/s for detecting liver stiffness in children, which has a sensitivity of 0.85 [3].

Biliary atresia is the progressive fibro inflammatory disease of the extrahepatic bile ducts and the main challenging in the newborn period is

differentiating biliary atresia from the other reasons of neonatal jaundice and leads the patients to the surgery, Kasai operation. Previous studies showed a significant increase in liver stiffness in biliary atresia than the other reasons of jaundice [4, 5]. As mentioned above the examining time is important because of the progressive nature of the disease. Although the study of Hanquinet et al. detected high liver stiffness as early as 8-day-old newborn, a slower progression of fibrosis may lead a false negative result [6]. Hanquinet et al. also reported high liver stiffness in 2 patients with giant cell hepatitis and a patient with Allagille syndrome, so false positivity can be present and care should be taken in the evaluation of results[5]. The follow up of biliary atresia patients is also an important issue because of high prevalence of cirrhosis although Kasai operation and the need for liver transplantation. The study of Hanquinet et al. showed patients with higher shear wave velocity than 2 m/s in two consecutive measurements in liver were candidates for transplantation [6].

The only study with US elastography in liver mass in children was performed by Özmen et al. and showed the higher stiffness in malign liver mass in children can be differentiated from the softer hepatic hemangiomas when 23.6 kPa is set as the cutoff value (AUROC: 0.77, sensitivity: 72.7 %, spesificity: 66.7%) [7].

3. Pancreas

The median normal value of pancreas stiffness in children is reported to be between 8.75 kPa and 9.3 kPa in children between 3-10 years old age, and between 1.69 kPa and 1.76 kPa between 11-18 years old age 8 [8]. Pancreas stiffness by elastography measurements were reported to have positive correlations with age, body mass index, height and weight [8]. Qui et al. also reported the median pancreas SWV as 1.31 m/s in their study evaluating 120 healthy children[9]. They reported increased SWV of pancreas in girls compared to boys. There are some difficulties in measurements of pancreas elastography are present, pancreas atrophy, fatty replacement of pancreas, abdominal gaseous and obesity. The depth of SWV measurements also effects SWV values, SWV increases with increasing depth of measurement [9].

Friedrich-Rust et al. reported positive correlation with the ARFI of pancreas and pancreatic enzymes, serum lipase in their study evaluating children with cystic fibrosis. Pancreatic insufficient patients had lower ARFI values in pancreas compared to pancreas sufficient patients**.** Pişkin et al. reported decreased 2D-SWE values in children with CF, compared to healthy controls[10]. Pancreatic 2D-SWE measurements were also reported to be affected by B-mode sonographic changes in CF patients.

Öztürk et al. reported type 1 DM patients had a higher strain ratio than controls, when cut off was 2.245 predicting pathology with 0.98 sensitivity.

According to their study, strain ratio of pancreas in type 1 DM patients was correleated with age and DM duration[11]. However, Sağlam et al. reported no significant difference between pancreas elasticity of healthy children and children with type 1 DM [12].

4. Spleen

The mean spleen stiffness values in healthy children are reported between 2.15 and 2.25 m/s [13-15]. The SWV decreases below the age of five years old whereas the sex and BMI does not affect the SWV[14]. In portal hypertension, congestion and structural changes lead fibrosis and increased tissue stiffness in spleen as well as in liver. Studies showed spleen stiffness could show the degree of portal hypertension [16, 17]. Also the development of the collateral vasculature is more correlated with spleen stiffness when compared to liver [17]. The presence and the degree of varices, presence of variceal bleeding, and splenomegaly is correlated.

5. Kidney

There is a wide range of stiffness values in the healthy children in literature. Various diseases of congenital, metabolic, and genetic origin affect the kidney in children. These diseases induce a pathological process in kidney, which results in fibrosis. Fibrosis improves and leads chronic kidney disease. Chronic kidney disease has five different stages according to the renal function. The first four stages are managed with medical treatment whereas the last stage (stage 5) needs dialysis. The study of Bilgici et al. showed that US elastography was able to differentiate renal fibrosis from chronic kidney disease (cut off= 1.81 m/s, sensitivity= 76.5%, specificity= 92.1%) and also the first four stages from the last stage which needs dialysis (cut off = 1.32 m/s, sensitivity= 76.9 %, specificity= 88.2 %) [18]. In contrast to the increased tissue stiffness in liver with the increasing stage of fibrosis, kidney showed a decrease in stiffness with improving fibrosis. Also, Göya et al. evaluated the renal damage in children with US elastography and their study revealed that US elastography is able to differentiate the focal damaged areas detected in dimercaptosuccinic acid (DMSA) from the non- damaged area [19]. Also, they showed a decrease in kidney stiffness with the increasing DMSA damage score and decreasing differential function of the kidney.

6. Thyroid

The normal values of SWV in healthy children are stated as 1.22±0.20 m/s in the study of Bilgici et al which evaluated 145 children with a mean age of 10.5±3.14 years [20]. Habibi et al. reported the mean elasticity value of thyroid gland in children as 14.6±3.3 kPa in 127 healthy children with a mean age of 10.3±3.9

years [21]. Yurttutan et al. found the mean elasticity value for thyroid gland as 0.54±0.38 in their study with healthy children [22]. Palabıyık et al. found that the mean elasticity value of thyroid gland in healthy children was 1.82±0.3 m/s[23]. Uysal et al. reported the median elasticity values of right thyroid gland as 6.38±1.97 kPa and 1.45±0.21 m/s, the median elasticity value for left thyroid gland as 8.81±3. kPa and 1.69±0.26 m/s[24]. In most of these studies except Palabıyık et al. and Uysal et al., there wasn't correlation between age, gender, thyroid volume or body mass index and the elasticity values of the thyroid gland. Palabıyık et al. reported significant positive correlation with age and SWV of thyroid gland[23]. Uysal et al. found positive correlation between right thyroid lobe elasticity and age, BMI and volume, however, reported no correlation of these parameters with left thyroid lobe elasticity[24].

Hashimoto is the most common autoimmune thyroiditis and the prevelance is 1–2% in children[25]. The diagnosis of Hashimoto's thyroiditis depends on the positivity of anti-tissue peroxidase (anti-TPO) and anti-thyroglobulin anticores in serum. In the study of Çekiç et al., strain index was found to be increased in patients with Hashimoto's thyroiditis compared to controls [26]. Strain index value of 0.98 as cutoff revealed 83% sensitivity and 93% specificity was. Cepeha et al. reported increased thyroid elasticity values in children with chronic autoimmune thyroiditis (15.51± 4.76 kPa) compared healthy controls (10.41± 2.01 kPa)[27]. They also reported lower thyroid elasticity values in children with chronic autoimmune thyroiditis compared adults with chronic autoimmune thyroiditis. The study of Yücel et al. also showed the increased thyroid gland stiffness in Hashimoto thyroiditis with a cut off SWV of 1.41 m/s (73% sensitivity, 80.8 % specificity) [28]. Palabıyık et al. found increased thyroid elasticity in patients with autoimmune thyroiditis with a mean value of 3.7±1.2 compared healthy children[23]. Yücel et al. and Çekiç et al. showed a positive correlation with the increasing anti-TPO levels and thyroid stiffness, however, Palabıyık et al. reported no correlation between thyroid gland elasticity and anti-TPO levels.

The prevelance of thyroid nodules in children is lower than adults with a level of 0.2-5.1% [29]. However, the potential of malignity is higher. The thyroid nodules in children should be strictly evaluated in terms of malignancy. The study of Borysewicz-Sanczyk et al. showed all of the nodules were benign with a strain index lower than 2 in their study evaluating the thyroid nodules in children [30]. They paid attention to the potential false negative results in follicular carcinoma and the false positive results causing from the calcification and fibrosis in various lesions.

Type 1 diabetes also was found to affect the thyroid gland stiffness although in patients with negative anti-thyroid anti-cores and normal B mode US findings. Sağlam et al. revealed the decreased stiffness in the thyroid gland in patients with type 1 diabetes [31].

Habibi et al. reported the mean elasticity of submandibular gland as 11.8±2.2 kPa and parotid glands as 11.8±2.6 kPa in their study evaluating healthy children[21].

7. Muscle

Berko et al. reported a decrease in muscle stiffness in rectus femoris below the age of 10 years old [31]. In their study the stiffness of biceps brachii after the exercise showed negative correlation with the BMI, whereas rectus femoris in the resting state showed positive correlation with the BMI. Their overall results showed increased muscle stiffness after the exercise. Although these stated relationships, Brandenburg et al. found no correlation between the muscle stiffness and age, BMI, wrist movement in their study evaluating passive muscle stiffness in children [32].

Congenital muscular torticollis is a disorder in which, one sided sternocleidomastoid muscle is shortened and may have various US findings. The sternocleidomastoid muscle can be heterogenous or show diffuse thickening in B-mode sonography. The muscle can be hypoechoic, isoechoic, or hyperechoic. Bilgici et al. found increased SCM stiffness, even in cases with isoechoic SCM on gray scale ultrasound.

Cerebral palsy is a disorder with the involvement of the muscle control and 85% are of spastic type. The studies showed increased muscle stiffness in spastic type cerebral palsy. The spastic type cerebral palsy treatment includes the injection of Botulinum toxin A and the efficiency of treatment is determined by Ashwort scale, which is a subjective scale in which the physiotherapist or the doctor evaluates the muscle. In the study of Bilgici et al. the muscle stiffness showed a decrease after Botulinum toxin injection in spastic type cerebral palsy patients and this decrease showed correlation with Ashwort scale[33]. Ultrasound elastography is a quantitative method in determining the muscle stiffness and can be used in the follow up of patients with spastic type cerebral palsy.

Pichiecchio et al. reported increased elasticity of lower limb muscles in preschool children with Duchenne muscular dystrophy compared healthy children[34].

8. Testes

Hattapoğlu et al. reported the mean testes stiffness as 0.82 m/s in children[35]. Undescended testes are a challenging problem in newborn and infancy. Ağladıoğlu et al. showed the stiffer undescended testes than the inguinal lymph nodes in their study by real time elastography [36]. The histopathological changes start as early as 6-9 months of age. The germ cell number decreases, seminiferous tubules get smaller and peritubular fibrosis develops. Some studies

showed higher testes stiffness in both preoperative and postoperative evaluation of the undescended testes compared with healthy controls[35, 37]. However, Shin et al. reported no significant difference between undescended testes and normal testes in their study evaluating children younger than 60 months old[38]. They also reported decreasing elasticity values in healthy children with increasing age. Ultrasound elastography may be useful in evaluating the undescended testes.

Aslan et al. reported increased testes elasticity in children with testicular microlithiasis compared healthy children[39].

9. Brain

The study on ultrasound elastography in imaging neonatal brain was performed by El-Ali et al., who evaluated the feasibility of the technique and also elasticity values in newborns[40]. They reported higher SWV values in deep gray matter compared periventricular white matter in their study evaluating preterm and term newborns. They also presented increasing elasticity in deep gray matter with the increasing age, when preterm and term newborns were compared.

10. Thymus

Thymus is a lymphatic organ that grows in childhood and then resolves in puberty. Thymus has a characteristic B-mode sonographic finding; bright echogenities with a hypoechoic background, called starry sky. Shear wave velocity of thymus is reported as 6.76±1.04 kPa in healthy children[41]. The SWV value decreases with increasing age, height, and weight.

In the study of Stasiak et al. strain elastography of intrathyroidal ectopic thymus was presented between 0.95-1.09[42]. Sağlam et al. reported a 4-year-old boy with intrathyroidal ectopic thymus, with a lower elasticity value than the thyroid gland. Ultrasound elastography may be a useful additional method in diagnosis of the ectopic nodular thymus.

11. Conclusion

Ultrasound elastography in children has promising results in various organs reported in the literature. Further studies with larger populations and different patient group will strengthen the usefulness of ultrasound elastography in children.

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CHAPTER V

CURRENT ADVANCES IN GLIOBLASTOMA THERAPY

Fatih Senturk
(RA. Dr.), Düzce University, Faculty of Medicine,
Department of Biophysics,
e-mail: senturkfatih52@gmail.com
Orcid: 0000-0002-2436-3362

1. Introduction

Glioblastoma multiforme (GBM) is the most common, highly invasive, and aggressive malignant glioma (1). The current standard treatment of newly diagnosed GBM consists of maximal surgical resection, then concomitantly radiotherapy (RT) and temozolomide (TMZ) followed by adjuvant TMZ (2). TMZ was approved by the Food and Drug Administration (FDA) for newly diagnosed GBM in 2005 and became a standard part of therapy in a clinical trial by Stupp et al. (3). According to Stupp protocol, after surgical resection, the treatment of RT+TMZ improved 2.5 months in median survival of GBM prognosis compared to RT alone (3, 4). Despite TMZ improves GBM survival, recurrences are inevitable, and most patients do not survive more than a year after diagnosis (5). Although potential therapeutic strategies provide essential contributions to understanding tumor pathogenesis, there is no FDA-approved agent that effective than TMZ in treating newly diagnostic GBM (6). In addition, GBM is the most lethal primary malignant brain tumor and remains an incurable disease (7).

However, some promising attempts have been developed on the biological effects of radiofrequency (RF) electromagnetic fields (EMF) in treating GBM. It is known that EMF has potential anti-tumor and anti-mitotic activity on clinical cancer therapy. These activities can be defined as -direct or -mediated biological effects of EMF. As a direct effect, tumor treating fields (TTFields), based on the anti-mitotic effect of alternating electric fields on GBM cells, have been included in the 'standard treatment protocol' by the FDA to treat recurrent and newly diagnosed GBM in 2011 and 2015, respectively (8, 9). Also, TTFields were approved by FDA for patients with unresectable malignant pleural

mesothelioma in 2019, and clinical trials are currently being investigated for several types of cancer (10).

Magnetic nano hyperthermia (MNH), which combines an alternating magnetic field with magnetic nanoparticles, is a new promising approach for GBM therapy (11). Also, MNH utilizes mediators, typically superparamagnetic nanoparticles (SPIONs), activated by EMF (12). This treatment approach, which is based on heat generation at the tumor site, is utilized as an adjuvant therapy in recurrent GBM cases in Europe (13). Although substantial advances in GBM treatment, especially using EMF, GBM remains an incurable disease with a poor prognosis. Therefore, there is an urgent need to improve survival and even quality of life for patients. This chapter summarizes the latest advances in currently available GBM treatments and improvements of treatment approaches.

2. Glioblastoma multiforme

World Health Organization (WHO) has classified glioblastoma multiforme as a grade IV diffuse glioma, characterized by an atypical nucleus, increased mitotic activity, microvascular proliferation, and necrosis (14, 15). Glioblastoma is the deadliest primary malignant brain tumor, and it constitutes 57.3% of gliomas, 48.3% of malignant brain tumors, and 14.6% of all brain tumors (16). After diagnosis, the 5-year overall survival of GBM patients is 6.8%, with overall survival ranging from 12 to 15 months (17).

2.1. Standard treatments of GBM

Glioblastoma treatment decisions were evaluated by a multidisciplinary team, including neurosurgery, radiation oncology, and medical oncology. Also, treatment strategies and clinical outcomes depend on tumor location, potential malignancy, grading, and the patient's age and physical conditions (18). Since 2005 standard treatment protocol in newly diagnosed GBM with good KPS (KPS: patient's general status scale, KPS $\geq$ 60) includes maximal safe surgical resection, followed by radiotherapy plus concomitant and adjuvant TMZ therapy (3, 15).

2.1.1. Surgery

The first step in treating GBM is surgical resection, which aims to remove the maximal tumor mass without neurological damage to functional brain regions (19). Also, surgical resection is performed to obtain tumor tissue for definite histopathological diagnosis, provide information underlying biology of disease, and alleviate the patient's symptoms by reducing the tumor volume (mass effect) (20). Many researchers stated that the size of the resected tumor volume was positively correlated with overall and progression free survival (PFS) of the

GBM patients (21, 22). Although complete resection is usually attempted, the benefit of resection has not been investigated in a prospective, randomized controlled study (20). In addition, complete surgical resection of the tumor with sharp borders is more suitable for superficial and small tumors, and it is almost impossible for deep and midline tumors (20). However, the tumor is most likely to recur in the resected cavity despite attempts for complete resection. Therefore, due to the invasive nature of GBM and to prevent recurrence after surgery, postsurgical treatment is usually recommended for patients (7).

2.1.2. Radiation Therapy

Radiotherapy (RT) can be used after surgery or primarily for GBM treatments (18, 23). After surgical resection, the total radiation dose is 60 Gray (Gy), given in 2 Gy fractions over 6 weeks (3). External beam radiotherapy is the most widely used form of radiotherapy. However, other radiation approaches such as brachytherapy, fractional stereotactic radiotherapy, and radiosurgery are more successful in treating recurrent GBM (18, 23).

2.1.3. Chemotherapy

Temozolomide (TMZ) is the first and only choice chemotherapeutic agent for the prognosis of patients with newly diagnosed GBM (24). TMZ has been used in GBM patients after surgery, as concomitantly TMZ (75 mg/m^2/day) and adjuvant TMZ (150-200 mg/m^2/day, every 28 days for 6 cycles) (20). After surgical resection, patients who received daily TMZ concomitantly with RT and adjuvant TMZ achieved OS of 14.6 months compared with an OS of 12.1 months in patients receiving post-surgical RT alone (3). However, in a retrospective analysis of four randomized studies, it was reported that adjuvant TMZ (6 cycles) exposed after RT+TMZ treatment had not prolonged the OS, on the contrary, exposure to ongoing treatment had more toxic effects on patients (25).

Nitrosoureas, such as carmustine, lomustine, nimustine, and fotemustine are alkylating agents that cause DNA cross-linking (26). In addition, these agents cause the inhibition of cell cycle progression resulting in cell death (27). Before the FDA-approval of TMZ in 2005, carmustine has been used as an FDA-approved alkylating agent in systemic injection or polymeric wafer forms in the treatment of GBM (28). As an alternative therapy, carmustine wafer (Gliadel®) implanted into the tumor resection cavity is the local treatment to kill the remaining tumor cells (29, 30). It is reported that concomitantly carmustine wafers with radiotherapy increase OS in young patients with high-grade glioma (18). However, carmustine is used mostly in second-line treatments due to limited clinical utility and severe side effects in GBM patients (15). Lomustine

is another alkylating agent approved by FDA in newly diagnosed and recurrence GBM (31). Recently, a phase III study of newly diagnosed GBM patients with methylated MGMT promoters reported that the combined lomustine-TMZ therapy increased OS compared to TMZ alone (32).

In general, nitrosoureas have been classified as highly toxic agents compared to TMZ (27) and cause easily develop drug resistance (23). Also, these alkylating agents show serious side effects such as pulmonary fibrosis, myelosuppression, and hepatotoxicity, and they are considered as second-line treatment agents (33, 34). Bevacizumab was also approved by the FDA for the treatment of recurrent GBM in 2009. However, the combination of bevacizumab and TMZ therapy did not significantly prolong the overall survival (35, 36).

Even though GBM is susceptible to TMZ therapy, tumors inevitably recur in all patients (37, 38), and higher doses of TMZ to recurrent GBM have not shown any clinical success (39). In addition, the median survival of GBM patients after recurrent tumors has been reported to be only 6 months (15).

2.1.4. Tumor Treating Fields (TTFields)

Tumor treating fields (TTFields) were approved by the FDA as a treatment protocol for recurrent and newly diagnosed GBM in 2011 and 2015, respectively (8), and clinical trials are currently ongoing in many types of cancer (40). TTFields have been defined as a method that creates an anti-mitotic effect on tumor cells by applying a low intensity (1–3V/cm) and intermediate frequency (100–400 kHz) alternating electric fields to the GBM region (41). Alternating electric fields can disrupt mitosis by biophysical driving force on polarizable and charged molecules in tumor cells (10). Kirson et al. showed that TTFields have a strong inhibitory effect on many proliferating tumor cells, whereas no effect on the nonproliferating cells (42). The rapid dividing character of tumor cells underlies their specific susceptibility to TTFields (43).

Preclinical studies have suggested that TTFields affect mitotic progression of the cell cycle interfering with chromosomal segregation (8). During chromosomal segregation in mitosis, chromosomes align at the spindle equator in metaphase, and mitotic spindle drives chromosome segregation, pulling each cluster of sister chromatids to the opposite poles of the cell (44). The mitotic spindle is formed by the microtubules that composed of tubulin polymers, and each subunit of tubulin has a high dipole moment (45). These highly polarized tubulin dipoles align with the direction of the applied TTFields, in which microtubules undergo constant cycles of polymerization and depolymerization process (46). This situation causes abnormal spindle formation, leading to cellular arrest and eventually mitotic cell death (47). In a phase III clinical trial of patients with newly diagnostic GBM, it was shown that adjuvant TTFields

combined with TMZ therapy prolonged overall survival by 5 months and increased the 5-year survival rate from 5% to 13%, compared to adjuvant TMZ alone (48).

TTFields can affect a wide spectrum of neurological processes, including autophagy, DNA repair, antitumor immunity, transient impact on blood-brain barrier integrity and permeability, and enhanced tumor cell membrane permeability (49). Therefore, TTFields may also represent promising strategies for the treatment of various neurological diseases.

2.2. Potential drugs in GBM therapy

In this section, potential agents that inhibit growth factors in the treatment of GBM, platinum-based agents, and natural compounds that sensitize GBM cells to chemotherapeutic drugs are briefly summarized. Although the current treatment approaches for GBM have improved, the OS of patients has not yet prolonged to levels that achieved for other solid tumors (50). In this sense, clinical studies are carried out by integrating FDA-approved agents, which are used to treat other cancer types, into GBM standard treatment protocol. Also, the effects of natural or synthetic agents on GBM cells are examined by *in vitro/ in vivo* studies.

The various genetic and epigenetic alterations present in GBM lead to modification of many signaling pathways that affect disease prognosis and patients' response to therapy (19, 51). For example, the increase and mutation of epidermal growth factor receptor (EGFR) are commonly detected in 40–60% of GBM cases (52). Overexpressed EGFR leads to tumor growth and inhibition of apoptosis that associated with poor prognosis in GBM (53). Therefore, numerous studies on GBM treatment that targeting EGFR have been reported in recent years (54). There are several FDA-approved EGFR inhibitors, such as erlotinib, gefitinib and afatinib (55), however, the prognosis of GBM patients has not significantly improved by using these agents (56).

The vascular endothelial growth factor receptor (VEGFR) is the main receptor regulating tumor angiogenesis (57). VEGF produced by tumor cells can activate tyrosine kinase receptors and subsequently stimulates survival, cell proliferation, migration, and invasion, leading to tumor angiogenesis (58). Therefore, using VEGFR inhibitors to treat GBM can block tumor angiogenesis and indirectly reduce tumor volume and metastasis (59). Therapeutic anti-VEGFR agents, such as VEGFR tyrosine kinase inhibitors (e.g., cediranib, sunitinib and sorafenib) or anti-VEGFR monoclonal antibodies (e.g., bevacizumab) have been approved by FDA (60). Also, bevacizumab is an FDA-approved monoclonal antibody against VEGF for recurrent GBM tumors (61). However, there is much controversy regarding the side effects of bevacizumab (hypertension, arterial

thromboembolic effects, etc.) (62). In a phase II study, it was reported that the combination of bevacizumab and TMZ did not significantly alter the OS compared to bevacizumab alone (63). In addition, bevacizumab has not shown survival benefit with or without standard TMZ therapy in different clinical trials for newly diagnostic GBM (64).

Platinum-based anticancer drugs have also been used in clinical trials to evaluate their effectiveness in treating GBM. FDA-approved platinum-based drugs, such as cisplatin, carboplatin and oxaliplatin are transported into the cell by copper transporter protein-1 (CTR1) (65, 66). Following getting into the cell, platinum-based agents induce cell death through several mechanisms (67). In a phase III study, treatment of cisplatin and carmustine followed by radiation did not improve the OS of GBM patients, also this protocol showed more severe toxicity compared to standard therapy (68).

Many natural components have been considered for the treatment of cancer (69). Resveratrol is a natural polyphenolic compound with potent antioxidant and anti-inflammatory effects, and it can cause cancer cells more susceptible to chemotherapeutic agents (70, 71). Also, *in vitro/in vivo* studies have shown that resveratrol increases TMZ activity by various mechanisms (70). For example, combining resveratrol and TMZ increased the anti-tumor activity in GBM cells by activating the ROS-dependent 5' adenosine monophosphate-activated protein kinase (AMPK) signaling pathway. Furthermore, activation of the AMPK pathway induced cell apoptosis by changing the expression level of the apoptosis-related B-cell lymphoma-2 (Bcl-2) protein (72).

Curcumin is another natural agent that has become one of the therapeutic potential candidates for GBM therapy (73, 74). Recent studies showed that curcumin has antimicrobial, anti-inflammatory, antioxidant, and especially anticarcinogenic effects (75). Also, curcumin treatment before TMZ therapy may sensitize GBM cells to standard therapy (76). The lipophilic nature of curcumin may allow it to cross the blood-brain barrier (BBB), accumulate in the hippocampus, and be appropriately absorbed into the central nervous system (77). However, curcumin's therapeutic application is limited, due to its poor bioavailability, low aqueous solubility, and rapid degradation (78). In order to overcome such limitations, nanocarriers have been studied extensively by many researchers. Nanocarriers improve the aqueous solubility of hydrophobic drugs, overcome physiological barriers, increase permeability, offer controlled release systems, and enhance circulation (79, 80). However, an effective nanocarrier system that can carry the agents used in GBM therapy has not yet been developed for the clinical application. On the other hand, a new promising approach for GBM therapy has been developed in which magnetic nanoparticles are physically stimulated.

2.3. Magnetic nano hyperthermia

Magnetic nano hyperthermia (MNH) relies on local heat generation at the tumor site via magnetic nanoparticles under the applied alternating magnetic fields (AMF) (81). This non-invasive method leads to physiological alteration and induce apoptosis in tumor cells (82). The efficacy of MNH depends on the physical properties of exposure system, such as magnetic field strength, frequency, and the heat distribution pattern in the tumor region (83, 84). Furthermore, the heat generation under the applied AMF depends on the size, shape, size distribution, magnetic character, homogeneity, and Curie temperature of magnetic nanoparticles. (85).

Recently, the superior magnetic properties of superparamagnetic iron oxide nanoparticles (SPIONs) have revealed many new biomedical applications related to the central nervous system (CNS) (86). For example, SPIONs have been used for targeted drug delivery, magnetic nano hyperthermia, magneto-thermal stimulation, magnetic resonance imaging (MRI), increasing the blood-brain barrier permeability, and enhancing the accumulation of chemotherapeutic drugs in the tumor regions (87, 88). Moreover, SPIONs are the most preferred thermal agents in hyperthermia applications due to their size-dependent magnetic properties, biocompatibility, minimal toxicity, high accumulation targeted region, and ease of excretion out of the body (89).

The application of magnetic hyperthermia using SPIONs was approved in Europe as an adjuvant therapy for recurrent GBM tumors (90). In the related therapy, SPIONs are applied locally under applied AMF, leading to enhanced overall survival of seven months compared to standard treatments of GBM (91). The MNH system manufactured by MagForce Nanotechnologies (Berlin, Germany) that consists of three main components: NanoTherm® aminosilane coated magnetite solution (12–20 nm), NanoActivator® alternating magnetic field system (100 kHz, 18 kA/m) and NanoPlan® temperature simulation software program (92).

Magnetic nanoparticle-mediated hyperthermia has advantages in two ways. First, magnetic nanoparticles accumulate in the tumor area due to the physiology of the tumor (enhanced permeation and retention effect, EPR), thus, the damage of nanoparticles on healthy cells is limited. Secondly, magnetic hyperthermia treatment has non-invasive penetration of magnetic field into biological systems. Therefore, it has become one of the leading approaches for difficult-to-reach areas, especially brain.

3. Conclusion

Glioblastoma is regarded as an incurable disease due to its poor prognosis. Unfortunately, success criteria of clinical outcomes for improving GBM therapy

are based on modest increases in patient overall survival. Furthermore, heavy treatment burdens significantly reduce the neurological functions, quality of life, and life expectancy of GBM patients. Therefore, it is urgent to develop new strategies to improve therapeutic responses of GBM.

The well-designed treatment protocol of electromagnetic fields promises a non-invasive technique to fight all types of cancer. TTFields, the fourth treatment modality of GBM, utilize the -direct biological effects of RF that interfere with dividing GBM cells. MNH uses the -mediated biological effects of RF is considered as an alternative method for GBM therapy. These methods have become the leading treatment methodologies, with the advantages of non-invasive penetration of RF field into biological systems. Considering all these, electromagnetic field-based cancer treatments will enable a new generation of cancer therapy strategies in the near future.

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CHAPTER VI

SPERM SELECTION TECHNIQUES USED IN ASSISTED REPRODUCTIVE TECHNOLOGIES AND ARTIFICIAL INSEMINATION

Fatih Mehmet Gür[1]
[1](Asst. Prof.), Nigde Omer Halisdemir University, Faculty of Medicine, Department of Histology-Embryology, 51240 Nigde, Turkey,
e-mail: histolog44@gmail.com
Orcid:0000-0001-7748-3272

1. Introduction

Infertility, which threatens the future of the human generation, is one of the biggest problems of our time. The absence of clinical pregnancy despite regular and unprotected sexual intercourse for one year is defined as infertility by the World Health Organization (WHO) (1). This can be caused by problems in the reproductive system of one or both partners (2). About half of the infertility cases are male-borne (3). In general, assisted reproductive technologies (ART) are procedures performed in vitro on human oocytes, sperm, or embryos to create pregnancy. Concepts such as in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI), embryo biopsy, preimplantation genetic testing, embryo transfer, gamete, and embryo cryopreservation are within the scope of ART. Artificial insemination using sperm from a woman's partner or a sperm donor is not covered by ART (3, 4). ART has revolutionized the treatment of patients who were previously thought to be infertile. It is estimated that more than 8 million babies were born with ART from 1978 to 2018 since Louise Brown, the first baby to be born using IVF (5, 6).

Semen analysis is indispensable in the evaluation of infertile pairs (7). Semen analysis is performed to assess whether there are enough living, motile, and morphologically normal spermatozoa in the sample to perform fertilization (8). Accurate semen analysis is very important in the evaluation of infertile males (7). The criteria for how to perform human semen analysis are described by the

WHO laboratory manual for the examination and processing of human semen (1). As a result of the analysis made according to these criteria, the diagnosis is made and the most appropriate sperm selection technique is applied to the patient.

Normally, only a few hundred of the sperm that are emptied into the vagina with coitus reach the ampulla, where fertilization takes shape. Probably only sperm with the highest fertilization ability and the best characteristics to support embryo development have a chance of fertilization by subjecting them to natural selection along the oviduct (3, 9-12). Especially in ART such as IVF and ICSI, the importance of sperm selection techniques has increased due to the elimination of the natural selection stage, which allows the determination of quality sperm to be used for fertilization. While under normal conditions seminal plasma helps penetrate sperm into the cervical mucus, it prevents pregnancy from occurring because natural barriers are omitted in intrauterine insemination (IUI) and ART (1, 3, 5, 8). Sperm should have separated from the seminal plasma within 30 minutes of ejaculation, otherwise, IVF capacity will be permanently reduced (13). WHO recommends separating the sperm from the seminal plasma within 1 hour after the sperm sample is taken to prevent the harmful effects of other cells in the semen (1). The sperm selection techniques aim to eliminate toxic or bioactive substances such as seminal plasma, cell debris, non-germ cells, dead sperm, and bacteria, as well as reactive oxygen species (ROS) and to obtain high percentages of morphologically normal and motile sperm (1, 3, 5, 8). Sperm selection techniques should be simple and economical, but also distinguish high-quality spermatozoa in the sample as soon as possible (3, 5).

Sperm selection techniques can be examined in two categories: conventional and advanced sperm selection techniques. Conventional sperm selection methods such as simple washing, swim-up (SU), discontinuous density gradients, and glass wool filtration are often the first step in semen processing for IUI and ART (8). Glass wool filtration from these simple, fast, reliable, and inexpensive techniques is a more expensive technique than others (5, 8). The sperm selection technique is determined according to the characteristics of the semen sample (1). The simple washing technique is often used to obtain normozoospermic samples for IUI. However, SU and density gradient centrifugation (DGC) techniques can also be used (14, 15). In samples with abnormal one or more of the semen parameters, DGC or direct-SU method is generally preferred. The SU technique is often considered to be used when the semen sample is largely normal. However, since a greater number of motile sperm are obtained in cases of severe oligozoospermia, teratozoospermia, or asthenozoospermia, the technique of DGC is generally preferred. It has been reported that glass wool filtration is as effective in separating sperm from semen with suboptimal properties as it is for the DGC (1). SU and DGC are successful methods of selecting motile sperm with normal morphology in the sample. It was

determined that the sperm selected by both techniques had the longest telomeres (16). However, conventional semen analysis methods are not always sufficient to determine sperm quality or fertility potential. Although spermiogram results are normal in 20% of infertile men, pregnancy does not occur (17, 18). It has been pointed out that both SU and DGC are not effective methods for spermatozoa selection in terms of apoptosis, DNA integrity, membrane maturation, and sperm ultrastructure (3, 19). In addition, centrifuge steps inherent in both techniques produce ROS, which causes harmful effects on sperm quality (5, 20).

Unsuccessful IUI and ART applications that occur despite the use of sperm with normal morphological criteria, as well as the use of ART in immotile but healthy sperm, thanks to the ICSI technique, has increased the need for more advanced sperm selection techniques. Research has shown that determining sperm vitality, structural properties of the sperm cell membrane, sperm DNA fragmentation rate, morphological structures of sperm organelles, and mimicking physiological sperm selection mechanisms exposed to sperm in the female genital tract can be used to select quality sperm and determine the etiology of infertility (3, 8, 21). Especially in sperms that are obtained by testicular sperm extraction methods, the selection of living sperm for use in the ICSI process has gained great importance in samples with immotile or very little moving sperm. In such cases, it is not appropriate to use methods such as SU and DGC. Therefore, many methods aimed at distinguishing between dead sperm and living ones have been developed (3).

The sperm cell membrane is very important for the functionality of sperm, as it is involved in many basic processes such as metabolism, capacitation, attachment to the egg, acrosome reaction. Since the structural and functional properties of this organelle give important clues about sperm vitality and quality, it is one of the target structures in the development of quality sperm selection methods for ART (3, 8).

Successful pregnancy is associated with the DNA fragmentation index (DFI), an indicator of sperm chromatin integrity. The high level of DFI adversely affects both fertilization and pregnancy rate. For this reason, clinicians acknowledge that DFI is important in evaluating male infertility in couples with recurrent pregnancy failure and choosing an ART procedure (21).

Morphometric analyzes are used to evaluate sperm quality because it is related to fertilization ability (3, 22). With computer-aided advanced systems, sperm can be enlarged 6000-12500 times and quality sperm can be selected by examining structures such as acrosomes, nuclei, neck, tails, and mitochondria (8, 23).

It has been reported that the female genital tract has physiological mechanisms that enable the selection of sperm of quality that can fertilize the egg and provide a normal embryo development (10, 11). In natural fertilization,

mammalian sperm travels a long way through the female genital tract to reach the egg cell or oocyte (24). Although the mechanisms that direct sperm towards the egg are not fully known, three routing mechanisms called rheotaxis, thermotaxis and chemotaxis have been discovered. Rheotaxis is when sperm are aligned from the egg canal towards the uterus and swim against the flow of fluid. Thermotaxis is when sperm swims against temperature gradient in the oviduct, while chemotaxis is the withdrawal of sperm by the chemical attractants secreted by oocyte and surrounding cumulus cells and swims against this chemical gradient (24-29). By mimicking these physiological mechanisms that sperm are exposed to in the female genital tract, some new sperm selection techniques have been developed and continue to be developed.

Advanced sperm selection methods in many individuals whose spermiogram results are not good can ensure the selection of the highest quality sperm for ICSI. In the following sections, conventional and advanced sperm selection methods, which are most widely used in IUI and ART, will be tried to be explained.

2. Conventional Sperm Selection Methods

The culture media to be used in these methods includes a balanced salt solution with a protein (e.g. human serum albumin (HSA)) additive and a buffer solution suitable for the ambient conditions in which sperm will be processed. Any routine sperm preparation medium can be used for preparation. If the incubator contains only atmospheric air, and if its internal temperature is 37°C, it must be buffered with HEPES medium or a similar buffer solution, and the tubes must be tightly closed. The medium in an incubator environment with 5% CO2 and 37°C temperature should be buffered with a buffer solution similar to sodium bicarbonate, and the tube caps should be left open to ensure gas exchange. It is important to pre-incubate before use to ensure optimum pH of the medium (For a medium with HEPES, one to two hours may be sufficient; a longer pre-incubation may be necessary without HEPES). Following these guidelines will optimize the PH of the culture medium for sperm survival. In the DGC method, isotonic density gradient medium (commercially available, used according to the manufacturer's protocol) is used either ready-to-use or diluted with an iso-osmotic medium according to the manufacturer's recommendations. In addition, consumables and equipment such as incubators, sterile test tubes (6- or 13-ml volume), centrifuge, sterile pipettes (variable volumes), microscope slides, coverslip, and microscope are also used in these methods (1, 30).

2.1. Simple Washing

It is a simple, fast, and effortless technique that should be used when the semen sample has normal. This technique is generally used for sperm selection for

IUI purposes (1). The number of centrifugation steps and centrifugation power (less than 500Xg) should be reduced to reduce the generation of ROS from dead sperm and leukocytes. Increased ROS levels cause DNA damage, decreased motility, increased apoptotic sperm count, and adverse changes in the sperm plasma membrane (13, 31).

2.1.1. Procedure (1, 30)

1. Stir the semen well (a glass rod can be used for mixing).
2. Dilute the entire semen sample with a culture medium at a ratio of 1+1 (1:2).
3. Fill the diluted semen into centrifuge tubes with a maximum of 3 ml per tube.
4. Centrifuge for 10 minutes (300-500×g)
5. Gently aspirate the supernatant
6. Combine the pellets and add 1 ml of culture medium and pipette gently
7. Centrifuge for 3-5 minutes (300-500×g)
8. Gently aspirate the supernatant
9. Suspend the pellet with a freshly balanced medium to a final volume of 0.5 to 0.7 ml (for IUI the amount should not be higher than 0.5 ml; for other purposes, the volume may be higher)
10. Check sperm concentration, motility and, morphology

2.2. Swim-Up (SU)

SU is one of the simplest, fastest, and most widely used sperm selection techniques with excellent results in normozoospermic and moderately oligoasthenoteratzoospermic (OAT) samples (8, 30). The main principle of this technique, which was first developed by Mahadevan and Baker (1984), is to select motile sperm from the sperm sample, which migrates to the cell-free culture medium placed on sample (32). When the SU technique, which reduces ROS production along with the DNA fragmentation rate, is used, it is most likely that the selected fraction consists of motile spermatozoa with normal morphology (13, 15, 33). Before applying the technique, dilution and centrifugation of the sperm sample are not recommended. Otherwise, oxidative damage to sperm membranes may result (20). The direct SU technique is a preferred method for distinguishing motile sperm. After liquefaction, either the culture medium is slowly added to the semen, or the culture medium is added to the test tube first and then the semen is carefully placed under the culture medium. After this process, the sperm with the highest motility swim into the culture medium. Although the sperm count selected in this method is lower than the washing method, it is suitable for ART procedures such as IVF and ICSI, where few but high-quality sperms are required (1). This method also

has some disadvantages. One of them is that only a small amount (5-10%) of morphologically normal and motile sperm in the sample can be selected with this method (13). Another disadvantage is that high volume and concentration samples require precision and additional workload. Since increasing the contact surface with the culture medium will increase sperm retrieval, more than one test tube should be preferred for semen samples with a high volume. Again, in samples with high concentrations, it may be recommended to dilute the samples and divide them into several tubes to prevent the sperm from getting stuck in the middle of the pellet and not migrating (8).

2.2.1. Procedure (Direct-SU) (1, 30)

1. Stir the semen well (a glass stick can be used for mixing)
2. Transfer 1 ml sample to a test tube (if the sample is high volume, divide it into several test tubes)
3. Pipette 1.0 to 1.2 ml of culture medium into the semen sample (it is easier to put on the top than on the bottom)
4. To increase the semen-culture medium interface area, position the tube at a 45° angle to the horizontal and incubate at 37°C for 1 hour. Take care that the incubation period does not exceed 1 hour so that zinc and other seminal plasma components do not diffuse into the medium and adversely affect the sperm
5. Gently turn the tube upright and take the 1 ml aliquot from the top of the tube. The part taken contains the selected sperm
6. Dilute the part containing the selected sperm with culture medium (1.5-2 ml)
7. Centrifuge for five minutes (300–500xg) and remove the supernatant
8. Suspend the pellet with culture medium (0.5 ml) to check sperm concentration, motility, and morphology (according to WHO)
9. Unprocessed samples can be used directly for therapeutic or research purposes

2.3. Density Gradient Centrifugation (DGC)

It is a technique used to select the high number of motile sperm from moderate/severe OAT sperm samples and epididymal sperm samples for application in ART (IUI, IVF, or ICSI) (1, 14, 30). This technique, which enables the selection of quality spermatozoa by distinguishing them from dead sperm, other cells, and debris, gives consistent results compared to SU (1). Oshio et al. 1987 reported that one hour after ejaculation, the densities of motile human sperm with normal morphology were above 1.10 g/ml, and the densities of immotile and/or immature sperm were between 1.06-1.09 g/ml. (34). The basic principle of this

method is to separate the cells in the semen according to their density through density gradients containing colloidal silica-coated silane (1, 8, 30). Also, motile sperm that swim through the gradient material form pellets at the bottom of tube (1). There are different gradient variants of the method, continuous and discontinuous. In a continuous gradient, the intensity gradually increases from the top of the gradient to the bottom. There are clear boundaries between discontinuous gradient layers (5, 8, 13). The most commonly used simple two-stage discontinuous-DGC method typically consists of an upper layer of 40-45% (v/v) density and a lower layer of 80-90% (v/v) density (1, 8). Semen is placed on the low-density top layer and sperm forms a soft pellet at the bottom of the tube by centrifugation and swimming along the gradient (1, 30). Sperm selected using the DGC method are generally highly motile sperm that are free of cell debris, contaminating leukocytes, non-germ cells, and degenerative germ cells (1). Sperm with genetic anomalies such as aneuploidy and diploidy are largely eliminated by this selection method (35).

In the DGC technique, the lowest centrifugation time and force that can be applied (usually 300 g and 20 minutes) should be used to keep the oxidative damage caused by the centrifugation step to a minimum. Reagents to be used to protect sperm from "cold shock" must be at 37 °C (36). Overloading the gradient should be avoided as this can cause 'rafting' which means the collection of sperm and other unwanted components on the sample after centrifugation (13). After the gradient is prepared, it should be used within 1 hour to avoid mixing of the layers (30).

Although fewer sperm are obtained, the use of the SU technique after DGC can further improve sperm quality. Both techniques are successful in selecting viable and morphologically normal sperm with intact chromatin and DNA when used alone or in combination (37-40).

2.3.1. Discontinuous-DGC Procedure (1, 30)

1. Prepare the gradient medium: fill 1 ml gradient medium (80%) in a test tube; then add 1 ml medium (40%) on top of the first layer
2. Stir the semen sample (a glass stick can be used for mixing)
3. Gently transfer 1 ml of semen to the pre-layered gradient medium (divide into several test tubes if the sample is high volume)
4. Carefully place the test tube in the centrifuge and centrifuge for 15 to 30 minutes (300-400×g)
5. Discard most of the supernatant from the sperm pellet
6. Wash the sperm pellet by adding 5 ml of equilibrated medium and resuspend by gently pipetting
7. Centrifuge for 4 to 10 minutes (200×g)
8. Repeat steps 5, 6 and 7

9. Remove most of the supernatant from the pellet
10. Suspend the pellet in 0.5 to 0.7 ml medium
11. Check sperm concentration, motility, and morphology

2.4. Glass Wool Filtration

It was first developed by Paulson & Polakoski in 1977 (41). With this method, motile sperm are separated from immotile ones, leukocytes, and debris by densely packed glass wool fibers and their active motility. After using this technique, a centrifugation step is required to separate the selected sperm from the seminal plasma. In this method, since immotile sperm, leukocytes, and debris in the sperm sample are largely eliminated before centrifugation, cellular damage caused by ROS originating from these cells will be greatly reduced. Eliminating up to 90% of non-motile sperm as well as leukocytes, this technique significantly contributes to the reduction of free radicals in the ejaculate (5). It was also stated that this method selected motile sperm with normal chromatin condensation and sperm containing highly intact acrosomes better than DGC and simple washing technique. It has been reported that sperm selected by this method has a higher fertilization rate after IVF compared to those selected by SU (8). The disadvantages of the method are that it is a bit expensive compared to other conventional sperm selection techniques and that there is a small amount of debris at the end of the procedure (5, 13).

3. Advanced Sperm Selection Methods

3.1. Annexin V Magnetic Activated Cell Sorting (AV-MACS)

It is a technique that enables cells to be selected according to their surface antigens (42). After the magnetic nanoparticles coated with a molecule with affinity for these antigens are incubated with the desired cell population for a while, exposing the mixture to a magnetic field ensures that the cells carrying the target antigen in the cell membranes are held by the magnetic field, separating them from the cells that do not carry the target antigen (3, 8, 13). Phosphatidylserine, a negatively charged phospholipid and normally found on the inner surface of the plasmalemma of both spermatozoa and somatic cells, translocate to the outer surface of the membrane in the early stages of apoptosis (8, 43, 44). In the AV-MACS method, magnetic nanoparticles coated with Annexin V, a molecule with a high affinity for phosphatidylserine, are used. These nanoparticles coated with Annexin V binding to the phosphatidylserine found on the outer surface of the apoptotic spermatozoa plasmalemma, separating them from living ones (3, 45). It is recommended that this method be associated with a standard sperm washing technique such as DGC, as there are other components in semen that may reduce the effectiveness of this technique. In many studies, it has been

reported that high-quality sperm with a low DNA fragmentation rate can be selected with the AV-MACS technique, and the combination of this technique with SU or DGC methods further increases the quality of the selected sperm (3, 8, 19, 46-49).

Studies have shown that the combination of DGC and AV-MACS may be beneficial for clinical pregnancy in patients with male-borne factors undergoing ICSI. However, it is not clear exactly how much it increases the live birth rate. More studies are needed to establish that AV-MACS is useful in human clinical practice (3).

In this method, a 100 µL sperm sample is mixed with 100 µL of MACS magnetic microparticles and incubated for 15 minutes at room temperature. The mixture is loaded into the separation apparatus placed in the magnetic field. The apparatus is washed with buffer. All unlabeled (Annexin V-negative) non-apoptotic sperm pass through the separation apparatus. The Annexin V-positive (apoptotic) fraction is retained in the apparatus. Non-apoptotic sperm that pass through the sorting apparatus can be used in ART (8, 13).

3.2. Hyaluronic acid-mediated sperm selection

The basic principle of this technique is to select mature and quality sperm with hyaluronic acid receptors on the cell membrane and acrosome membrane through hyaluronic acid. Hyaluronic acid (hyaluronan), a glycosaminoglycan, is densely located around the cumulus-oocyte complex (50). Some receptors bind to hyaluronic acid on the cell membrane and acrosome membrane of mature spermatozoa (51-53). Two sperm selection techniques have been developed, based on the sperm-hyaluronic acid interaction, namely physiological intracytoplasmic sperm injection (PICSI) and collection of slow-moving sperm while swimming in an environment containing hyaluronic acid (3, 8). In the PICSI technique, a PICSI dish with hyaluronic acid-coated areas at the base is used. After washing, the sperm sample placed in the PICSI dish is kept there for a while. At the end of the waiting period, the sperm attached to the hyaluronic acid-coated areas are used in the ICSI procedure. In the other technique, slow-moving sperm are collected in a solution rich in hyaluronan and used in the ICSI procedure (54).

The effect of selection techniques using hyaluronan on sperm DNA fragmentation rates is controversial. Some studies have reported that sperm selected using these techniques have a lower DNA fragmentation rate (3, 55-57). No difference was reported in other studies (58). Again, in some of the studies comparing the results of the ICSI procedure performed after the application of normal ICSI and the hyaluronic acid-mediated sperm selection technique, it was reported that this technique increased pregnancy and live birth rates with

embryos with normal chromosomes (59-62). Others have been reported to have no effect (3, 17, 56, 63, 64).

When the results of the studies are evaluated, the selective effect of this method on quality sperm has not been fully determined yet. More studies are needed to prove the success of this method for routine clinical use.

3.3. The Zeta Method

It is a sperm selection method developed based on the electrical charge of the mature sperm membrane. The mature sperm membrane has a negative electrical charge of –16 to –20 mV, called the zeta potential (13, 65). Chan et al. (2006) developed a method for selecting negatively charged mature sperm via positively charged tubes, while Ainsworth et al. (2005) developed methods that can select through an electrophoretic device (17, 65). Since there is no relationship between zeta potential and total motility, it is recommended to apply this method after the DGC step (66). In the method using positively charged tubes, washed sperm (0.1 mL) is diluted by pipetting into a positively charged tube containing 5 mL serum-free HEPES–HTF (Human Tubal Fluid) medium. A positive charge is obtained by placing new centrifuge tubes (glass tube preferred) in a latex glove and rotating several times. Electrostatic charge can be verified using electrostatic voltmeters. To allow mature spermatozoa to adhere to the wall, the tube is kept at room temperature for 1 minute and then centrifuged (300Xg for 5 minutes). After centrifugation, the tubes are slowly turned upside down, their caps are opened, and the sperm and medium that do not adhere to the tube wall are emptied. To separate the negatively charged sperm adhering to the tube wall, serum-supported HEPES-HTF medium (0.2 mL) is pipetted into the tube so that the medium reaches the entire inner surface of the tube so that at the end of the procedure, the selected sperm is collected at the bottom of the tube (8, 13).

In the method in which the electrophoretic device is used, sperm selection is made from the semen placed in a chamber with the help of an electric current. The selected sperm are collected in the adjacent chamber (8).

Studies have shown that both selection methods increase the rate of quality sperm and the rate of sperm with high integrity DNA (3, 48, 58, 67). Esfahani et al. (2016) reported that the application of the zeta method after DGC significantly increased the rate of pregnancy and best quality embryos compared to the application of only DGC (68). However, the usefulness of the zeta method is limited, especially in oligozoospermic patients, since the number of sperm obtained as a result of selection using this method is low. Also, this method is not useful for testicular or epididymal sperm aspirates that do not have sufficient net electrical charge on the sperm membrane surface (13). Despite these positive results, more studies are needed to determine the efficacy of the zeta method in clinical practice.

3.4. Selection Techniques Based on Physiological Sperm Guidance Mechanisms

This technique, which is particularly suitable for ICSI applications, has been developed by imitating rheotaxis, thermotaxis, and chemotaxis, which are physiological selection mechanisms that sperm are exposed to in the female genital tract (3, 8). DeMartin et al. (2017) reported in their study that sperms obtained from normozoospermic samples selected based on rheotaxis had higher chromatin integrity than those selected by DGC technique (69). Also, Nagata et al. (2018) in their study in which they used a microfluidic device to select frozen bull spermatozoa, reported that sperms selected by rheotaxis had a lower DNA fragmentation rate compared to unselected ones (70). As a result of sperm selection based on chemotaxis, it was determined that the sperm selected using this method had three times more capacity than other sperms, had less DNA fragmentation rate and oxidative stress (71, 72). In a study conducted by Pérez-Cerezales et al. (2018) in mice and normozoospermic patients, it was reported that sperms selected by the thermotaxis method had a lower DNA fragmentation rate than those selected by the SU method. In this study, it was also determined that the cleavage, blastocyst development, implantation, and live birth rates formed after ICSI with sperms selected by thermotaxis method were higher than those not selected by this method (10). Studies have shown that only capacitated sperm can be selected with selection methods based on chemotaxis and thermotaxis (3, 73). These methods are still in the testing and development stage. There are not enough studies on the positive effects of these methods on ART and their clinical use.

3.5. Microfluidic Sperm Selection

Microfluidic devices were developed to select sperm based on motility, rheotaxis, chemotaxis, thermotaxis, electrophoresis, and optical forces (3, 74). In this title, the use of the microfluidic device for sperm selection according to progressive motility is mentioned. One of the biggest advantages of this method is that it eliminates the damage caused by the centrifugation steps in conventional techniques. Moreover, this technique is considered advantageous for difficult-to-process samples such as severe cases of oligoasthenozoospermia (3). Nosrati et al. (2014) reported that they developed a microfluidic device and an easy-to-apply technique based on this method. They also reported that the sperm selected by their technique showed an improvement of more than 80% in DNA integrity and that with this technique, a subpopulation of sperm with nearly intact chromatin and DNA integrity could be selected (74). In many subsequent studies, it has been reported that the use of this selection method significantly reduces DNA fragmentation rates in sperm compared to not using it or SU and

DGC methods (70, 75, 76). In another study, it was determined that the DNA fragmentation rate in the sperm selected with microfluidic devices (Fertil Plus) was lower than those selected by the DGC method, and the fertilization rate obtained with these sperms was higher in patients with recurrent IVF failure (77). In another study, in which sperm from 122 infertile patients of unknown etiology were selected by microfluidic device, and SU methods, fertilization, pregnancy and live birth rates were found to be similar between the two sperm selection methods after ICSI (78). However, in this study, it was reported that the embryos selected with the microfluidic device were of higher quality(78). As microfluidic systems are still in development, it is not yet clear how they affect ART outcomes and in which patients they are beneficial. More experimental and clinical studies are needed on this subject.

3.6. Intracytoplasmic Morphologically Selected Sperm Injection (IMSI)

Since the morphological structure of spermatozoa is related to fertilization, the examination of the morphological structure of the sperm is a widely used method for determining sperm quality (3). Some spermatozoa that appear normal on routine microscopic examination may have ultrastructural defects that adversely affect the outcome of ART (8). In 2002, with the discovery of computer-assisted digital microscopy, which allows examining the morphology of motile sperm, a sperm examination technique called motile sperm organelle morphology examination (MSOME) was developed (79). With the help of this technique, sperms can be magnified 6000-12500 times and structures such as acrosome, post-acrosome lamina, nucleus, neck, tail, and mitochondria can be examined in detail. One of the most important structures in determining sperm quality is the nucleus. The presence of nuclear vacuoles in sperm indicates problems in sperm chromatin packaging (8, 23). Sperm selection for ICSI using the MSOME technique is called IMSI. For this technique, an inverted microscope should be equipped with Nomarski DIC optical digital imaging and manipulator system, and a glass petri dish should be used instead of a plastic petri dish. IMSI is a time-consuming and experience-based technique. In addition, the devices used in this technique are quite expensive (3, 8, 23). In some studies using the IMSI technique, it has been reported that the presence of large vacuoles in the nucleus increases the sperm DNA fragmentation rate (80, 81). In another study, it was reported that vacuoles in the head region were not associated with sperm DNA fragmentation and live birth rates (82). When the results of the studies investigating the effectiveness of the IMSI technique in ART are evaluated as a whole, it is possible to say that the use of this technique may increase the chance of treatment in recurrent implantation failures after ICSI and in severe infertility cases due to male factor (oligoasthenoteratozoospermia and teratozoospermia) (3, 8).

3.7. Direct Selection of Immotile Sperm

More advanced techniques are used in addition to routine morphological methods (selection of sperm with normal heads and tails) in sperm selection for ICSI, especially in cases where the spermatozoa are immotile, such as in sperm samples obtained by TESE. These are the hypo-osmotic swelling (HOS) method, the laser-assisted immotile sperm selection (LAISS) method, and the polarization microscopy (3).

3.7.1. The HOS Method

The HOS test was mainly developed to assess the functional integrity of the plasma membrane of human sperm (38, 83). This test is based on the principle that cells with intact membranes, which are left in the hypo-osmotic environment, swell with fluid until the osmotic pressure in the intracellular and extracellular environment is balanced. The HOS test can be used to distinguish immotile but live sperm from dead ones (1). Tails of living sperm swell and curl in 5 minutes in a hypo-osmotic environment (1, 3, 8). Using the HOS method as the sperm selection method for the ICSI procedure for the first time, Desmet et al. (1994) reported that they achieved a fertilization rate of 30% (84). In this method, sperm cells are incubated at 37ºC in a hypo-osmotic environment. After incubation, selected HOST-positive spermatozoa are washed and used in ICSI (85). In many studies investigating the effectiveness of this technique, it has been shown that it is possible to obtain clinical pregnancy from immotile but viable spermatozoa by using this technique (8).

3.7.2. Polarisation Microscopy

With the discovery of the ICSI method, fertilization and even healthy delivery with a single healthy and high-quality spermatozoa became possible, increasing the importance of sperm selection methods in male factor infertility cases. Under the polarized light microscope, the head of living spermatozoa is birefringent. It has been reported that birefringence analysis in sperm cells is an indicator of structural normality. The presence of birefringence was also confirmed by transmission electron microscopy as an indicator of quality sperm (3, 17, 23, 86). Based on this fact, a new sperm selection technique has been developed by applying polarization microscopy to the ICSI technique. In a study, it was reported that successful results were obtained with sperms selected using this method from severe OAT sperm samples and samples obtained by TESE (87). Ghosh et al. (2012) reported that pregnancy rates with sperm selected with this technique were higher than those with the HOS testing (45% vs. 11%, respectively) (88). Despite the successful results, more studies are needed to prove the effectiveness of the method.

3.7.3. LAISS

The laser stimulation technique, which was used for the first time for the immobilization of motile spermatozoa (89), was later tested in the selection of live but immotile sperm (90). Aktan et al. (2004) discovered that when the tail end of immotile but viable sperm is stimulated with a single laser pulse, it curls up, similar to the HOS test. There was no reaction in dead sperm exposed to the same procedure. The same researchers reported that ICSI with sperm selected by this method increased fertilization and cleavage rates (90). It was stated by Nordhoff et al. (2013) that this method is a risk-free and safe method that can be used to separate live spermatozoa from dead ones in immotile sperm samples (91). Ozkavukcu et al. (2018) reported that triplets, one boy and two girls, were born after ICSI with sperm selected by the LAISS method from a patient with Kartagener's syndrome (92). Chen et al. (2017) reported that successful pregnancy was achieved after the ICSI procedure using live sperm selected by this method from immotile spermatozoa after being thawed which were obtained by TESE and frozen (93). The possible advantages of this selection technique are that there is no need for a separate environment, processing, and extra time as in the HOS test, while the disadvantages are that it is somewhat complex and expensive (3, 93).

4. Conclusion

One of the basic life goals of all living things in nature is to continue their lineage. Man, who is a part of nature, has a very strong desire to continue his lineage. This desire pushes infertile individuals to seek treatment for their problems. This search increases the interest in scientific studies on the etiology and treatment of infertility. Especially the birth of Louise Brown in 1978 broke new ground in the treatment of infertility and increased the motivation on this issue. This was followed by ICSI and other developments, and many couples who were previously deemed impossible to have children have had children thanks to these methods developed. Studies show that infertility tends to increase gradually around the world. It has been reported that electronic devices such as mobile phones used in daily life, varicocele, and many other factors adversely affect the male reproductive system by increasing oxidative stress (94, 95). This trend towards increasing infertility is motivating the studies on sperm selection techniques and other infertility treatments.

Today, this problem has been solved to a great extent by using the sperms selected by the conventional methods described above in cases of mild and moderate infertility with male-borne factors. As in the sperm obtained by TESE, some success has been achieved by using advanced sperm selection techniques in more severe infertility cases where immotile, undeveloped, and high DNA fragmentation rate sperm are responsible. However, there is still a long way

to go. With the discovery of the ICSI method, fertilization and even healthy delivery with a single healthy and high-quality spermatozoa became possible, increasing the importance of sperm selection methods in male factor infertility cases. As information about the structural and physiological properties of spermatozoa and technological possibilities increase, existing methods will be further developed and even newer and more advanced methods will be discovered. It is believed that the developments in microfluidic sperm selection techniques and the development of more advanced methods that will make it possible to examine sperm DNA without damaging spermatozoa will increase the success rate in ART.

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CHAPTER VII

MICROBIOTA AND SOME DISEASES

Berrak Zeynel[1] & Görkem Kısmalı[2]
[1](PhD Student), Ankara University, Ankara, Turkey
e-mail: berrakzeynel8@gmail.com
Orcid: 0000-0001-9547-5719
[2](Associate Proffesor, Dr.), Ankara University, Ankara, Turkey
e-mail: gorkemkismali@yahoo.com
Orcid: 0000-0003-3414-4697

1. Introduction

Microbiota is defined as an ecological community of various microorganisms located in a specific microenvironment in our body. The system formed by the genetic loads of this specific microorganism community consisting of commensal symbiotic and pathogenic species is expressed as the microbiome (1).

Our bodies are colonized by numerous microorganisms, including areas such as the placenta and lungs that were thought to be sterile until recently. This community of microorganisms, which also varies in different segments of the same organ, consists not only of bacteria, but also of archaea, protozoa, fungi and nematodes (2). Signals from the microbiota, which vary according to the organ and the part of the organ, lead to different responses. For example, stimuli from the stomach microbiota, including species such as *streptococcus, lactobacillus, prevotella*, cause protease and gastric acid secretion, while the small intestinal microbiota, which includes species such as *bacteroides clostridium, enterococcus,* ensures the absorption of nutrients. (3).

Gastrointestinal system microorganisms constitute the intestinal microbiota (4). The intestinal microbiota, which consists of host-specific species, regulates the physiology and metabolism of the host by producing various metabolites and immunomodulatory substances (5,6).

The intestinal microbiota, which begins to form in the intrauterine period, matures under the influence of various factors and reaches an adult-like shape around the age of two, remaining stable until the old age, except for temporary

minor changes (7, 8). Studies show that there is a common microbiota that constitutes 50% of the gut microbiota in all humans and is largely composed of *Bacteroidetes, Firmicutes* and *Proteobacteria* species. The remaining part is shaped by many factors such as diet, delivery type, genetic structure, breast milk, oral antibiotic use in the early period, and is host specific (9, 10).

The healthy microbiota is called eubiosis. Disruption in the intestinal microbiota balance is defined as dysbiosis (11). Dysbiosis caused by intrinsic and extrinsic factors is associated with intestinal infections, metabolic and autoimmune diseases, associated with an increase in intestinal permeability, changes in the production of short-chain fatty acids, formation of autoantibodies and a decrease in colon resistance (12).

A healthy gut microbiota has many functions such as production of some vitamins, detoxification, barrier resistance against pathogenic species, production of short-chain fatty acids that epithelial cells use as energy source, regulation of gene expression (13). Metabolic substances produced by the intestinal microbiota increase the function of T-lymphocytes and ensure the regular functioning of the immune system (14).

Advanced methods developed to analyze the gut microbiota have shown that dysbiosis plays a role in various diseases (15). Dysbiosis has been associated with many diseases such as allergy at an early age, inflammatory bowel disease, type 2 diabetes, metabolic syndrome, chronic inflammation, cancer and autism, in connection with the negative impact of the immune system balance (14). While diseases that have been clearly shown to be associated with dysbiosis are inflammatory bowel diseases such as Crohn's disease and ulcerative colitis, there are strong indications that autoimmune diseases such as atopic eczema, asthma, celiac, are also associated with abnormal microbiota (15).

2. Dysbiosis-Related Diseases

2.1. Inflammatory Bowel Disease

Inflammatory bowel disease (IBD), defined as chronic inflammation of the intestinal mucosa with successive periods of remission and acute disease, consists of ulcerative colitis and crohn's diseases (16).

Although invasive *E. coli* infection (AIEC), some viruses and mycobacteria are associated with inflammatory bowel disease (17), further analysis reveals that IBD is caused by disruption of the intestinal microbiota balance, which interacts with genetic, immunological and environmental factors (18). Metagenomic analyzes show that Crohn's patients have a different microbiota composition from healthy individuals (18). The production of reactive oxygen species (ROS) and nitric oxide (NO) by inducible nitric oxide synthase (iNOS) during intestinal inflammation in Crohn's disease creates a favorable environment for

the proliferation of the facultative anaerobe *Enterobactericea*. The narrowing of the intestinal lumen due to inflammation creates a favorable environment for the proliferation of facultative anaerobes and oxygen-tolerant bacteria, while strict anaerobes such as *Clostridium group IV* and *XIVa* cannot compete under these conditions and are reduced in number (19). Nitric oxide produced by iNOS is used by *Enterobacteriaceae* for anaerobic respiration, resulting in decreased colonization resistance and epithelial inflammation (20). It has also been determined that there is a significant increase in *E. coli* levels in Crohn Disease (21).

Some studies have observed a decrease in *Bacteroidetes* and *Hemophilus* species in Crohn disease and an increase in *Proteobacteria* in IBD (22). Decreased levels of *Faecalibacterium prausnitzii* are associated with increased recurrence rate of Crohn disease. Further analysis revealed that *Faecalibacterium prausnitzii* plays an important role in decreasing the levels of proinflammatory cytokines such as NF-κB, IL-2 and INF gamma and increasing the level of an anti-inflammatory cytokine IL-10 (23). It has been determined that there is a change in intestinal fungal composition in Crohn disease. Increased levels of *C. Albicans, Aspergillus clavatus* and *Cryptococcus neoformans* are observed compared to healthy subjects (24).

In genetically susceptible individuals, chronic epithelial inflammation as a result of defect in anti-inflammatory signals causes the development of dysbiosis. When dysbiosis develops, the inability of the intestinal microbiota to produce short-chain fatty acids and anti-inflammatory signals makes the intestinal mucosa sensitive to inflammatory substances (25).

Investigations to understand the pathogenesis of IBD reveal a change in the immune system. According to one study, abnormal Paneth cells support microorganisms that cause an increase in proinflammatory substances (25). Norovirus infection has been found to cause abnormalities in Paneth cells of mice carrying the hypomorphic Atg16L1 gene (26).

Diversity of gut microbiota is decreased in inflammatory bowel disease. It has been demonstrated in various studies that there is a decrease in *Firmicutes* species and an increase in *Enterobacteriaceae* species in ulcerative colitis patients compared to healthy controls (27). At the same time, decreased expression of T-bet, a transcription factor involved in the regulation of levels of proinflammatory substances, triggered ulcerative colitis in mice as a result of loss of tumor necrosis factor alpha (TNF-α) (28).

2.2. Diabetes Mellitus

Type 1 diabetes is an autoimmune disorder that occurs when autoreactive T-lymphocytes attack β-cells in the pancreas, resulting in the inability

to produce insulin in the required amount (29). Studies show that the composition of the gut microbiota plays a role in the development of type 1 diabetes. In studies conducted to elucidate the relationship between intestinal microbiota and type 1 diabetes, it has been determined that species such as *Faecalibacterium* and *Roseburia*, which produce butyrate, the energy source of colonic epithelial cells, are decreased in type 1 diabetes patients (30). The genus *Bacteroides*, which also produces glutamate decarboxylase (GAD), has been shown to be associated with type 1 diabetes (29, 30, 31 32, 33). *Bacteroides* enterotype triggers inflammation via TLR4 via lipopolysaccharides (34). It has been determined that a diet rich in protein and animal fat causes an increase in *Bacteroides* levels (35) and a decrease in the enterotype of *Prevotella*, which is associated with an increase in the prevalence of type 1 diabetes (36).

In a study investigating the relationship between intestinal microbiota and type 1 diabetes, it was determined that segmented filamentous bacteria triggered the transformation of CD4+ T cells into Th17 cells, which is thought to have a protective effect against type 1 diabetes. According to this report, intestinal microbiota controls the development of antigen-specific T cells and microbial peptides produced by commensal bacteria can trigger antigen-specific T cells (37). Segmented filamentous bacteria are thought to inhibit the development of type 1 diabetes in a correlation with other members of the microbiota (38).

In studies conducted to clarify the relationship between type 2 diabetes and dysbiosis, it was observed that secondary bile acids were produced less in type 2 diabetes patients than in healthy controls. It is stated that this situation may be related to the decrease in *Firmicutes* bacteria and the deterioration of the *Bacteroides/Firmicutes* balance in favor of *Bacteroides* (39). It has also been reported that the decrease of *Akkermansia muciniphila* enterotype in type 2 diabetes patients results in an increase in intestinal permeability. Increased intestinal permeability triggers metabolic endotoxemia (40). In a study evaluating the microbiota composition of healthy controls, prediabetes and type 2 diabetes patients, *Akkermansia muciniphila* and *Verrucomicrobia* enterotype were found to be decreased in prediabetes and type 2 diabetes patients compared to healthy individuals (41).

The accumulation of inflammatory substances such as flagellin and peptidoglycan as a result of the disruption of the microbiota balance increases inflammation in type 2 diabetes patients (42). Studies have shown that *Bifidobacterium* enterotype is decreased in type 2 diabetes patients and *bifidobacteria* have a protective role against diabetes (43, 44). It has been determined that *Bifidobacterium* species increase the secretion of glucagon-like peptide-1 (GLP-1), which reduces insulin resistance (45).

2.3. Cancer

In studies investigating the relationship between the dysbiosis process and cancer development, it has been found that disruption of microbiota homeostasis triggers chronic inflammation, suppresses apoptosis, and promotes carcinogenesis through cytokines and chemokines (46). Disruption in the intestinal microbiota balance causes disruption of epithelial tissue integrity in many tumor types, increasing the infiltration of potentially pathogenic bacteria and promoting cell proliferation. Dysbiosis, which causes a decrease in the production of short-chain fatty acids, especially butyrate, triggers carcinogenesis by increasing histone deacetylase expression. Increased HDAC enzymes in many tumor types are important for gene expression (47). In Table 1, HDAC enzyme types with increased expression according to tumor type are schematized.

Table 1. HDAC enzymes increasing by tumor type (48)

TUMOR	HDAC
LUNG	HDAC 1, 2, 3, 5, 10
STOMACH	HDAC 1, 2, 3, 4, 10
LIVER	HDAC 1, 2, 3, 5, 6
COLORECTAL	HDAC 1, 2, 3, 5,7
PANCREAS	HDAC 2, 6, 7
BREAST	HDAC 1, 2, 3, 6

2.3.1. Colorectal Cancer

It has been shown in various studies that colorectal cancers, which are the third rank among adult cancers and are formed by the gradual accumulation of epigenetic changes in the colon and rectal epithelium, are associated with dysbiosis (49). The most common finding in studies investigating the relationship between colorectal cancer and microbiota is that the concentration of short-chain fatty acids (SCFA) is significantly lower than in healthy controls. Acetate and propionate, which are short-chain fatty acids, which are the end products of bacterial fermentation, are produced by the *Bacteroidetes* phylum, while butyrate is produced by *Firmicutes*. The decrease in fecal SCFA level in colorectal cancer patients is attributed to the decrease in the number of SCFA-producing bacteria (50).

Butyrate, which is metabolized by oxidative phosphorylation, binds to G-protein-dependent receptors, triggering the expression of IL-18, which strengthens epithelial barrier integrity and increases bacterial diversity (51). Various studies have shown that intestinal epithelial cells prefer butyrate rather than glucose as an energy source (52). Butyrate production suppresses malignant

colonocytes and promotes proliferation of normal colonocytes (53). Butyrate, which increases MUC-2 expression as well as IL-18 expression, protects intestinal epithelial integrity (54). Reduction of butyrate to acetyl-CoA by beta oxidation suppresses histone deacetylase (HDAC) expression and increases histone acetylation, which prevents tumor development (55).

Obesity-induced dysbiosis, which is a risk factor for colorectal cancer, increases NF-kB expression (56). It has been reported that the NF-kB pathway contributes to the progression of colorectal cancer by preventing apoptosis and increasing proliferation in tumor tissue (57).

Various bacteria such as *Escherichia coli, Bacteroides fragilis, Streptococcus gallocticus, Enterococcus spp.* are implicated in the development of colorectal cancer (58). It has been shown in various studies that these bacteria produce toxins that cause inflammation and disruption of epithelial barrier integrity by promoting hyperplasia. It has been reported that disruption of epithelial tissue integrity causes these microorganisms to trigger chronic inflammatory reactions and the cytokines and chemokines they produce to have protumorogenic effects (59).

It has been shown in various studies that commensal bacteria stimulate IL-6, IL-17, IL-22 and IL-23 signaling in colon adenoma (60). High levels of IL-22 and IL-17 are accepted as an indicator of unfavorable prognosis in colorectal cancer patients (61). It has been reported that IL-6 is a proinflammatory cytokine that increases angiogenesis and progression by affecting STAT 3 activator. High levels of IL-6 are associated with poor prognosis and metastasis in colorectal cancer patients (62).

Studies have shown that the virulence factor F. Nucleatum Adhesin A (Fad A), which regulates the mucosal adhesion and invasion ability of *Fusobacterium nucleatum* bacteria, is increased in colon adenocarcinoma compared to normal tissues (63). Fad A binds to cells expressing E-cadherin. E-cadherin has been found to promote cell growth and proliferation in colorectal cancers by contributing to the activation of β-catenin (64).

Fragilysin enterotoxin produced by *Bacteroides fragilis*, another bacterium known to be closely associated with colorectal cancer, has been shown to activate the c-myc oncogene by promoting IL-8 production (65). Increased amounts of phenylacetic acid, phenol, indole, p-cresol, ammonia and polyamines produced by this bacterium have been associated with DNA damage.

2.3.2. Pancreas Cancer

The five-year survival rate of pancreatic ductal adenocarcinoma (PDAC), which is shown among the most aggressive and deadly diseases, is less than 7% (66). In 2015, 367,000 new cases of pancreatic cancer were detected worldwide (67). This number increased significantly in 2018, reaching 458,918 new cases and 432,242 deaths. An average of 94% of patients die (68).

Many factors affect the development of pancreatic cancer. Disruption in homeostasis of the pancreatic microbiota (69), which is thought to be composed of microorganisms from the oral, lower gastrointestinal tract, and mesenteric lymphatic drainage, is thought to be a risk factor for PDAC. It has been shown that PDAC tissues contain dense fungal populations, especially *Malasezzia* species, compared to healthy pancreatic tissue (70).

Studies have shown that *Fusobacterium nucleatum* bacteria, which is associated with colorectal cancer, is also associated with PDAC and indicates an unfavorable prognosis (71, 72). Butyrate, which is detected at low levels due to the decrease in the number of bacteria producing SCFA in many tumor types, has been shown to inhibit metastasis by reducing b4 integrin expression in PDAC. At the same time, acetate, one of the short-chain fatty acids, has been found to inhibit cell proliferation in pancreatic cancer cell lines (Capan-2, ASPC-1, MiaPaCa-2) (73).

Obesity, which is caused by Western-style high-fat and sugar-based diet, triggers chronic inflammation and is also shown as a risk factor for PDAC. In studies conducted to clarify the relationship between obesity and pancreatic cancer, it was found that the change in the e-cadherin gene is associated with PDAC. Obesity increases the expression of KRAS and triggers the change in the e-cadherin gene (74).

2.3.3. Liver Cancer

It is known that genetic and environmental factors are effective in the development of hepatocellular carcinoma (HCC), which is the third leading cause of death from cancer worldwide. Although the relationship of gut microbiota with HCC development has not been clearly defined, it is thought that immune reactions of microorganisms taken with food may contribute to the development of HCC (75). It has been shown that *Helicobacter hepaticus* bacteria increase cell proliferation by triggering cytokine formation in mice, and cause HCC development by activating NF-κB and Wnt signaling pathways (76).

In studies investigating the relationship between intestinal microbiota and hepatocellular carcinoma, a mechanism associated with Toll like receptor 4 (TLR4) and secondary bile acids is mentioned. Accordingly, dysbiosis triggers hepatic inflammation by causing TLR4 activation and cytotoxic bile acid formation. Dysbiosis is associated with hepatocarcinogenesis in association with the suppression of SCFA formation and the development of hepatic inflammation (77).

The inclusion of inulin-type fructans (ITF) in the diet has been shown to reduce tumor size in hepatocellular carcinoma. Fermented by saccharolytic bacteria, ITF increases host immunity by increasing SCFA production (77).

Helicobacter pylory, which is among the most accused bacteria in gastric carcinoma, can reach the liver tissue by migrating backwards from the duodenum via the portal vein. Vag A and Cag A toxins produced by *H. pylory* were found in HCC tissues. At the same time, it has been shown that lipopolysaccharides produced by *H. pylory* increase the expression of IL-8 and TGF-β1 and support growth and metastasis in tumor tissue (78).

2.3.4. Gastric Cancer

Gastric adenocarcinoma is the fifth most diagnosed adult cancer worldwide. In gastric adenocarcinoma, which has a 5-year survival rate of 32%, family history is reported as 10% (79). Many studies have been conducted to elucidate the relationship between this type of cancer, where environmental factors are highly effective, and the microbiota.

The stomach microbiota is largely composed of phyla *proteobacteria, firmicutes, actinobacteria, bacterorides* and *fusobacteria* (80). *Helicobacter pylory*, which has been shown to be associated with gastric cancer, initiates gastric inflammation by disrupting the homeostasis of the stomach microbiota. *H. pylori* infection causes the accumulation of interferon y-containing TNF-a, IL-1, IL1p, IL-6, IL-7, IL-8, IL-10 and IL-18 inflammatory cytokines. (78).

Cag A factor produced by H. pylory triggers carcinogenesis by inhibiting apoptosis in epithelial cells. Cag A and Vac A oncoproteins have been shown to promote gastric carcinoma by activating ERK/MAPK, PI3K/Akt, NF-κB, Wnt/β-catenin, Ras and STAT3 pathways (78).

In studies carried out to understand the pathophysiology of gastric cancer, it has been determined that *E.coli, Lactobacillus, Nitrospirae, Clostridium, Veillonella, Haemophilus* and *Staphylococcus* phyla, which convert nitrogen compounds in gastric juice into carcinogenic N-nitrozo (NOC) compounds, are associated with the development of gastric cancer. Although *Nitrospirae* bacteria was detected in all gastric cancer patients in one study, it could not be demonstrated in patients with chronic gastritis. *Peptostreptococcus stomatis, Slackia exigua, Parvimonas micra, Streptococcus anginosus* and *Dialister pneumosintes* bacteria belonging to the oral mucosa were found to be significantly increased in gastric cancer patients compared to patients with atrophic gastritis (81).

2.3.5. Breast Cancer

In breast cancer, which is the most frequently diagnosed malignancy in women, western diet and lack of physical activity have been shown to significantly increase the risk of the disease. Especially in estrogen-dependent breast cancer, it has been determined that a low-fiber, high-fat and sugar-containing western

diet prevents the excretion of estrogen by increasing bacteria containing β-glucorinidase and promotes carcinogenesis (77).

Obesity caused by Western diet causes a decrease in the production of short-chain fatty acids such as butyrate, which reduces the formation of pro-inflammatory substances. Pro-inflammatory substances cause an increase in insulin-like growth factor 1 (IGF-1) due to a decrease in adiponectin levels. This situation promotes cell proliferation (77).

Mediterranean diet with high fiber content prevents carcinogenesis by increasing the production of alkaline phosphatase and short-chain fatty acids that protect intestinal epithelial integrity. The Mediterranean diet also contributes to the increase of estrobolom bacteria, which use fiber as an energy source. It has been reported that estrobolom bacteria control estrogen metabolism, promote the elimination of estrogen from the body and reduce the risk of estrogen-dependent breast cancer (77).

Studies have shown that an increase in the amount of butyrate formed by fermentation of a diet high in fiber prevents proliferation in breast cancer by stimulating the formation of the cyclin-dependent kinase inhibitor p21. It is stated that when butyrate, which functions as an HDAC inhibitor, is used together with retinoic acid, it inhibits cell growth in MCF-7 cells (82).

3. Conclusion

The dysbiosis process, which we can define as the disruption of microbial balance in our organism, which is a holobiont consisting of 10% human and 90% microbial cells, is associated with many pathological conditions from obesity to neurological diseases, diabetes to cancer. It has been concluded that the increase in research on microbiota will be effective in understanding the origin of diseases and developing new treatment methods.

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CHAPTER VIII

RELATIONSHIP OF BETWEEN OBESITY AND LEPTIN

Mehmet Özdin
(Exp. Dr.), Sakarya Training and Research Hospital,
Department of Clicical Biochemistry, Sakarya, Turkey
E-mail: drmozdin@33mail.com
Orcid:0000 0003 3077 7171

1. Introduction

Obesity is an important public health problem. Leptin-related problems are seen especially in developed countries. It is estimated that 60% of men, 40% of women and 25% of children will be obese in 2050 if the current upward trend continues (1). Obesity usually occurs due to insulin resistance and is accompanied by disorders in lipid metabolism. Recently, studies have been done on insulin. The complex linkage between leptin and insulin was detected (2-4).

Leptin consist of a 167 amino acid. Leptin is produced by the obesity gene. Leptin has been extensively researched after its discovery. It is mainly secreted from adipose tissue. Leptin provides information about fat stores to the brain (5,6). Leptin is a regulator hormone. Leptin is synthesized from adipose tissue and controls body fat mass (7,8). Leptin is produced mainly in white fat tissue. In addition, it has been shown to be secreted in some brown fatty tissue. Leptin is also secreted from the stomach epithelium, skeletal muscle, pituitary gland, mammary glands, placenta and various tissues (9). Leptin release has a diurnal rhythm, peaks at night when peaking at night. This rhythmic oscillation may vary according to eating times (10). Leptin performs its action both by the periphery and by the special receptors in the central nervous system. Leptin receptors are of the cytokine receptor family. Leptin is encoded by the db gene. There are six isoforms of leptin receptor, one long (Ob-Rb) and five short (Ob-Ra, Ob-Rc, Ob-Rd, Ob-Re, Ob-Rf). While long form is effective in signal transmission, it is thought that short forms of leptin have an important role in transporting to central nervous system (11). Short-term hunger leads to reduction in energy

intake. Leptin causes a decrease in weight loss levels (12). Leptin is available in two forms, either free-blood or protein-bound.

The free form of leptin activity is thought to be responsible. Leptin has been reported to be in free form in obese, while most of leptin is weak in weak individuals. Through the blood circulation, the hypothalamus achieves suppression of appetite and increases energy expenditure. Leptin release is basically proportional to body fat mass. Leptin levels were high in obese subjects and leptin levels were found to be low in weak individuals (13). Leptin hormone, which decreases appetite. Leptin increases energy expenditure, theoretically less than in obese, but studies do not confirm this. Leptin levels are quite high in obese patients. This is believed to be due to an insensitivity to leptin versus hypothalamic receptors in obese individuals (14,15). Leptin levels and body mass index is correlated (16,17). Insulin increases leptin production and secretion (18). Besides its effects on appetite and energy metabolism, leptin has important functions in control of metabolism, immunity, reproduction, angiogenesis and lipid oxidation (19). Leptin is a molecule which make a relation with obesity and cancer (20).

The effect of inflammation on the development of cancer is known. It has structural and functional similarity with proinflammatory cytokines. Leptin increases reactive oxygen species from blood mononuclear and endothelial cells, monocyte chemoacrylate protein-1 (MCP-1). Leptin also increases other proinflammatory cytokines such as TNF-α. In addition, leptin causes an increase in vascular smooth muscle cells and stimulates hypertrophy and causes the production of proliferative and profibrotic cytokines. Thus, it contributes to the diversity of proatherogenic factors (21). The role of leptin in neovascularization increases enzyme levels and activity of angiogenesis such as matrix metalloproteinases 2 and 9 (22). In a study, a decrease in leptin levels was observed after exercise. These investigators indicated that aerobic exercises had a significant decrease in serum leptin levels (23).

2. Conclusion

Increased living standards, especially in economically developed countries, high energy levels of consumed foods and decreases in physical activities together with insulin resistance, which is the problem of contemporary life, including metabolic syndrome including DM, hypertension and obesity, cause many diseases to occur in orthopedic problems. Due to these problems, scientists are looking for new ways to get rid of diseases. For this reason, hormones, proteins, diet, exercise type and duration secreted in the body have become the subject of further research. As studies on adipose tissue, obesity and exercise increase, hormones such as leptin are associated with energy balance and insulin resistance. The plasma level of leptin may be influenced by variables such as

body fat tissue ratio, sex, age and lifestyle. Leptin improves fatty acid oxidation in skeletal muscle, increasing both the long-term energy requirement and the use of fats, but many more controlled studies are needed in this area.

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CHAPTER IX

MOLECULAR APPROACHES TO THE COVID 19 IN RESPIRATORY AND IMMUNE SYSTEMS

Nilgün Kuşçulu
(Asst. Prof. Dr.) Department of Chemistry and Chemical Processing Technologies, Mustafa Çıkrıkçıoğlu Vocational School, Kayseri University, TR-38039, Kayseri, Turkey
E-mail: nilgunkusculu@kayseri.edu.tr
Orcid: 0000-0003-3022-4876

1. Introduction

The coronavirus family are single-stranded, positive-polarity, non-segmented, enveloped pleomorphic viruses with the largest RNA genome. The large genome causes the virus to be less dependent on the host cell during replication. Its replication takes place in the cytoplasm of epithelial cells of the respiratory system and gastrointestinal tract. The term "corona" means crown in Latin, and the virus takes its name from the crown-like structures in its structure. In general, the genome size of RNA viruses is usually less than 10 kb, since the mutation rates in the replication of RNA viruses are much higher than that of DNA viruses. However, the coronavirus genome has the largest genome among RNA viruses and is 30 kb in length (1-4).

Coronaviruses belong to the order Nidovirales, family Coronaviridae, subfamily Coronavirinae. The subfamily contains four genera within which they are denoted by Greek letters: Alphacoronavirus, Betacoronavirus, Gamakoronavirus and Deltacoronavirus. Only alpha and beta coronaviruses are known to infect humans. Bats are the natural host of coronaviruses, and their development takes place in bats and it is accepted that most human coronaviruses are derived from bat reservoirs. The genetic similarity of bat betacoronavirus belonging to SARS-CoV-2 and subgenus Sarbecovirus has been confirmed by various research groups. While there was 96.2% similarity between the sequences of bat SARS-associated coronaviruses (SARSrCoV; RaTG13) collected in Yunnan province, China, and the entire sequence of the new virus,

this degree was compared with the genomes of SARS-CoV (approximately 79%) or MERS-CoV (approximately 50%). No similarity was found (5-7). In the genomic sequence analyzes of SARS-CoV-2 viruses isolated from some patients, the fact that the sequence similarity was found over 99.9% is the most important indicator of the transmission of the virus to humans (8-11). However, the difference in the incidence of adults or children among humans makes one think about the sequence difference between them. Is it the lack of growth factors and their gene sequences in adults? What are the gene sequence differences between adults and children, I think some issues need to be investigated. In this regard, genetic sequence analyzes may do more intensively and examined.

2. Immune system related to Corona virus structure

The protein structures that make up the crowned structure of the coronavirus are known as S proteins. S protein; It is in the form of protrusions on the virus surface, on the viral envelope, and provides attachment of the virus to the host cell by binding to the receptor and membrane fusion. It is an important viral protein that determines host cell tropism. S protein has S1 and S2 loops S1 protein is responsible for host cell receptor binding while S2 protein is responsible for membrane fusion. The S2 protein of 2019-nCoV shows 93% similarity with bat-SL-CoVZC45 and bat-SLCoVZXC21. In the S1 protein, this similarity is approximately 68%. Both the N and C terminal portions of the S1 loop can bind to the host cell receptor. Although 2019-nCoV and SARS-CoV are in different tribes, both viruses have 50 conserved proteins in the S1 loop. This result; suggests that the new coronavirus may also use angiotensin 12 COVID-19 converting enzyme 2 (ACE 2) as a receptor, as in SARS-CoV. Apart from the S protein, M, E, N and Hemagglutinin Esterase Proteins are also proteins in the coronavirus structure. Among these proteins, the N protein differs from the others in terms of location and function. N protein; together with the M protein, they are envelope proteins that play a very important role in virus formation and release. It contains two domains that can bind the viral genome through different mechanisms. This protein binds to the nsp3 protein to help the genome bind to RTK and contributes to virion formation. It plays a role in the regulation of replication and transcription of viral RNA. The N protein also acts as an interferon antagonist, thus inhibiting the attempt to destroy the virus by the immune system (12). While our immune system defends against the virus, the N protein of the virus prevents it. In this case, even if our defence system is strong, we can sometimes succumb to the virüs. The way to prevent this is to consume N protein-inhibiting drugs, foods, and to detect such inhibitors from plants. Researchs can do on this subject. N protein gene expression RT-PCR studies can be performed. Does the effect of N protein against the functioning

of our immune system cause it to cause more serious, unavoidable, dangerous and threatening consequences for human health, which distinguishes the coronavirus from others? This may include the answer to why we don't get over the coronavirus like a cold or flu.

3. Immunostimulatory agents in coronavirus

It is known that vaccines are used for the formation of immune response specific to antigens such as viruses in metabolism, that is, for stimulating the immune system (13). Biological agents or synthetic, organic substances are used in vaccines. However, the contribution of phytochemicals in naturally sourced foodstuffs to the immune system has been investigated. Many immunostimulatory plants have been found in the studies. These include many plants such as Actinidia macrosperma, Allium sativium, Aloe vera, Aesculus indica, Andrographis paniculata, Asparagus racemosus, Baliospermum montanum, Curcuma longa. Immunostimulatory effects of different extracts of ethanol, methanol and distilled water from parts of these plants such as roots, leaves, flowers and fruits were determined. It is recommended that they are widely used as drugs in autoimmune diseases, allergic conditions, cancers and virological infections (14-16).

4. Immunomodulatory agents in coronavirus

Immunomodulators are known as biological or non-biological agents that can affect the immune response, which represents the activated reaction sequence that changes the immune response of the host, to protect the organism against infective agents, environmental injury and disease (17-19). There are many herbs that modulate the immune system. There are many studies about the effects of herbal extracts prepared in different solvents such as methanol and water on the immune response. (20-25). Seaweeds are not only rich in polysaccharides, minerals, and certain vitamins but they also contain bioactive substances such as proteins, lipids, and polyphenols. These bioactives are known to possess potent antibacterial, antiviral, and antifungal properties. C. crispus has antiviral properties and is said to be particularly useful for dislodging mucus (26). In the treatment of severe Covid 19, immunomodulatory synthetic drugs such as antimalarials, anti-IL6, antiIL-1, calcineurin and JAK inhibitors, corticosteroids, immunoglobulins, heparins, angiotensin-converting enzyme agonists and statins are used (27). In many international clinical studies, it is also known that vitamin D has positive immunomodulatory effects against covid 19 (28). In the later stages of Covid 19 disease, systemic proinflammatory cytokines and biomarkers are elevated and show worse correlation and reduced survival chances. Here, immune modulators come into play and inhibit cytokines and

treat cytokine storm (29). A rapid and strong increase of IFNa/β is required to inhibit dsRNA replication of the Covid 19 virus. To allow efficient production of IFNa/β, as well as the synthetic drugs used for this, it is necessary not only to alleviate a TGFβ-dependent brake but also to strengthen its production. For this, 1,8-cineole, a small, aromatic and plant-derived molecule, can be used and many similar plant molecules can be discovered (30).

5. Respiratory system in coronavirus

The main cause of various viruses seen in the last 20 years is by members of the Betacoronavirus genus. SARS-CoV and MERS-CoV viruses, which emerged in 2002-2003, cause severe acute respiratory syndrome and viral pneumonia. This syndrome is a respiratory failure characterized by widespread inflammation of the lungs with symptoms such as shortness of breath, rapid breathing, and bluish skin colour (31). In Covid 19, which was seen as of 2019, many and different symptoms were seen compared to SARS and MERS (32). The especially long and severe acute respiratory syndrome may increase the risk of arterial or venous thromboembolism (33). Mucus secretion and the effect of mucociliary clearance play an important role in preventing viruses taken from the nose through the airway epithelium from damaging the epithelial tissue before reaching the lung. Inhaled virus particles probably also infect different epithelial cell types on their way to the distal lung. It is understood that the first viral contact in the nasal mucosa occurs by binding the viral S protein to the ACE2 (angiotensin-converting enzyme-2) receptor and then cleavage of the S protein by TMPRSS2 (transmembrane serine protease 2) (34-36). Many immunostimulating agents are needed to combat so many symptoms. These include currently used vaccines and drugs (37). However, there are studies on the use of natural substances as food and beverage. In addition, 179 plants are recommended to be used in the treatment of Covid 19. The fact that phytochemicals such as β-sitosterol, stigmasterol and quercetin are related to the antiviral signalling pathway indicates that plants containing them can also be used in the treatment of covid 19 symptoms (38-40).

6. Real Time RT-PCR studies in coronavirus

The first test method detected at the time the virus emerged in 2019 was the nasopharyngeal swab test containing RT-PCR. The reliability of the negative result of this test is also low (41). However, a real-time RT-PCR nasopharyngeal swab test is used in the 2019 novel coronavirus detection and laboratory diagnosis study (42, 43). In January 2020, the World Health Organization focused on research on more reliable covid 19 detection tests than real-time RT-PCR. It has been suggested that various methods should be used to evaluate

the proposed tests. Dogs, in particular, have been successfully trained and used to detect diseases in humans. For Covid 19 detection, they used explosive detection dogs and after training them to smell COVID-19 in the sweat of patients, these dogs were able to successfully screen 3249 people who tested negative for SARS-CoV-2 from a cohort of 3290 people. In addition, using Bayesian analysis, the sensitivity of the K9 test was found to be superior to the RT-PCR test performed on nasal swabs from a cohort of 3134 individuals. Given its high sensitivity, short turnaround time, low cost, less infestation, and ease of administration, detection dog testing is emerging as a better alternative to RT-PCR in screening for SARS-CoV-2 in asymptomatic individuals (44-46). In another study, a comparison of the basic analytical and clinical performance of RT-PCR kits produced and released to the market by many different companies at the end of 2019 was included. It was concluded that all RT-PCR kits evaluated in this study can be used by experienced molecular diagnostic laboratories for routine diagnosis of COVID-19 in patients (47). In addition to these, there are applications of RT-PCR test by developing different methods. In one study, four virus inactivation methods were used for the rapid detection results of COVID-19 nucleic acid. The results of the four treatment methods and specimens without inactivated treatment have shown good consistency (48). In another study, a novel antigen-based rapid detection test (RDT) for the diagnosis of SARS-CoV-2 was evaluated in respiratory samples. The RDT assay has the potential to become an important tool for the early diagnosis of SARS-CoV-2, particularly in situations with limited access to molecular methods (49). There is even a study in which it was applied in serological tests for severe acute respiratory syndrome coronavirus 2 (50).

7. Conclusion

Human body systems have different metabolic activities. This is why many diseases and COVID-19 affect different people in different ways. Most infected people develop mild to moderate illness and can recover without hospitalization. In other patients who do not recover, two important metabolic systems play a role. These are the immune system and the respiratory system. In this study, evaluations were made about the quantitative molecular changes and the status of RT-PCR studies, the effects of coronavirus on the immune and respiratory system, from the onset of Coronavirus 2019 to the present. Expression of molecules that will provide defence against quantitative molecular increases and changes in these systems will change the course of the disease. There are substances of synthetic and natural origin that must be imported to support the defence. Thanks to these agents' immunomodulatory and immunostimulating effects, molecular changes in metabolism will positively change the course of

covid 19 diseases. And also, in this study, the development studies carried out in the real-time RT-PCR method used in virus detection. During the period from the beginning of the virus in 2019 to the recent times are included. Because, since 2019, there are many studies in the literature on the real-time RT-PCR method and its development, which is used in the detection and laboratory diagnosis of the Coronavirus. However, studies using agarose gel electrophoresis molecular techniques and quantitative gene expression studies by RT-PCR are rare. We hope that scientific studies that will fill the gap on this subject will enlighten the unknowns about coronaviruses.

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CHAPTER X

UREA CYCLE DISORDERS

Özlem Unay Demirel[1] & Olida Çeçen[2]

[1](Asst. Prof. Dr.); Department of Medical Biochemistry, Goztepe Medical Park Hospital, School of Medicine, Bahcesehir University, Istanbul, Turkey,
e-mail: ozlem.unay@med.bau.edu.tr, Orcid: 0000-0002-3059-9398
[2] Bahcesehir University School of Medicine,
e-mail: olidacecen.med@gmail.com,
Orcid: 0000-0001-7969-2481

1. The Urea Cycle

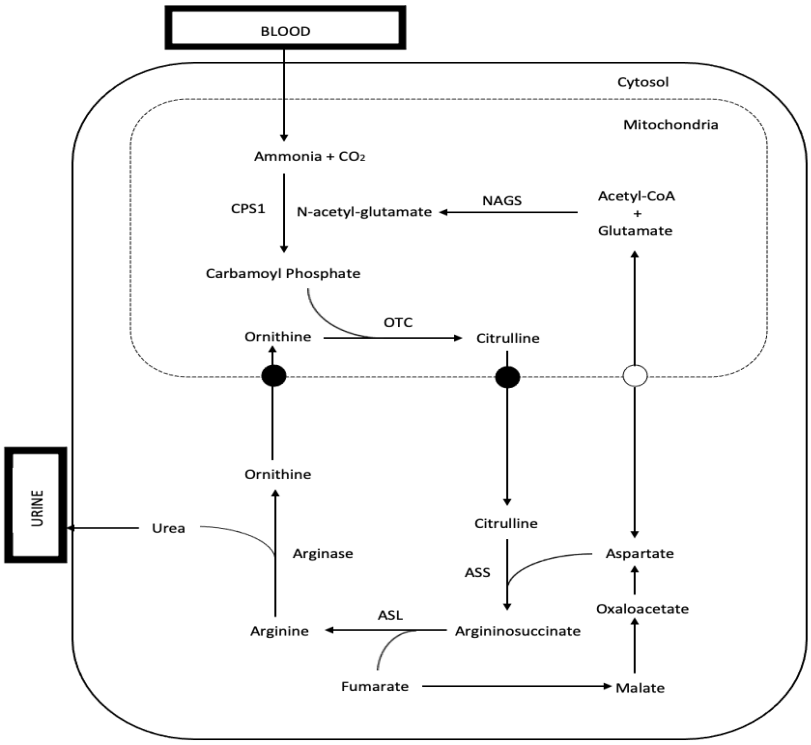


Figure 1: The Urea Cycle

The urea cycle, discovered in 1932 by Krebs-Henseleit, is defined as the conversion of extra nitrogen generated by the catabolism of protein and other nitrogen containing compounds into urea exclusively in the liver. All of the consecutive reactions occur under the control of multiple enzyme complexes, so the process of urea biosynthesis is highly energy dependent, occurring in the hepatocyte's mitochondria and cytoplasm.

Formation of carbamoyl phosphate (CP) from CO_2, ammonia and ATP via the enzyme carbamoyl phosphate synthetase 1 (CPS 1, EC 6.3.4.16) initiates the cycle in the mitochondrial matrix. Aforementioned step of the cycle is rate-limiting and it is only activated in the presence of a molecule called N-acetyl-glutamate (NAG) which is synthesized by NAG synthase (NAGS, EC 2.3.1.1) from glutamate and acetyl-coenzyme A. Apart from that, the activity of the rest of the urea cycle enzymes are determined by their substrate concentrations. At the next step, ornithine transcarbamylase (OTC, EC 2.1.3.3) catalyzes the formation of citrulline from carbamoyl phosphate and ornithine. Citrulline is then carried from the mitochondrial matrix to the hepatocyte's cytosol by a transporter named as ornithine translocase (ORNT1). In the cytosol, citrulline and aspartate combine to form argininosuccinate under the control of argininosuccinate synthetase (ASS, EC 6.3.4.5). The proceeding reaction is cleavage of argininosuccinate with the enzyme argininosuccinate lyase (ASL, EC 4.3.2.1) which yields arginine and fumarate. Addition of water to fumarate builds malate which is essential in the generation of NADH for the reactions of the citric acid (TCA) cycle. Subsequent hydrolysis of arginine via arginase 1 (EC 3.5.3.1) releases urea and ornithine. Formation of urea concludes the stepwise reactions. Reentrance of ornithine in the mitochondria occurs through the same transporter that takes the citrulline out in the previous steps. The resulting activity of the exchange between cytosolic ornithine for mitochondrial citrulline reinitiates the cycle. As the product of the enzyme, increase in the levels of ornithine potentially inhibits arginase. On a side note, the two suppliers of the nitrogens in urea are being derived from ammonia at the first reaction and aspartate afterwards. At the end, the ammonia transported to the liver by the portal vein becomes urea and urea gets expelled from the kidneys in the urine. The majority of the renal excretion of the nitrogen containing compounds consists of urea and ammonia, nearly 99%, in addition to others such as urinary proteins, uric acid, etc. and the regulation of the process maintains the physiological balance between health and disease states (1).

1.1. Ammonia Toxicity

Table 1: Clinical Features of Ammonia Toxicity in the Pediatric Population (5)

Digestive Symptoms
Nausea
Vomiting
Anorexia
Abdominal pain
Failure to thrive
Liver failure
Neuropsychiatric Symptoms
Headache
Ataxia
Dysarthria
Hypotonia
Behavioral changes
Neurodevelopmental delay
Seizures
Loss of consciousness
Central hyperventilation
Multisystemic Symptoms
Multiorgan failure

Ammonia is a colorless, highly disturbing gas with a sharp odor. It arises at the end of the protein catabolism and immediately dissolves in the water and becomes ammonium. Nearly 17 grams of ammonia are produced in a healthy human body daily, some of which is absorbed back from kidneys, the rest is excreted through urine. Even though ammonia is mainly the side product of the human metabolism, it shows some deleterious effects at higher levels of concentration in the blood (2). Ammonia toxicity occurs at the time when the body's elimination capacity is overruled by the blood levels of ammonia. To prevent the toxic effects of ammonia, it is converted to a nontoxic compound, urea, in the tissues, especially in the liver. However, when there is a defect in any point of the detoxifying process of ammonia, increased blood ammonia levels create some physical consequences seen as clinical signs and symptoms of hyperammonemia. The reference range of ammonia in the blood is 170-340 mcg/dL in newborns, 70-135 mcg/dL in children, 15-60 mcg/dL in adults. Human metabolism consists of an overlapping network of different but interconnected reactions. Because of that, the reasons behind the hyperammonemia can be

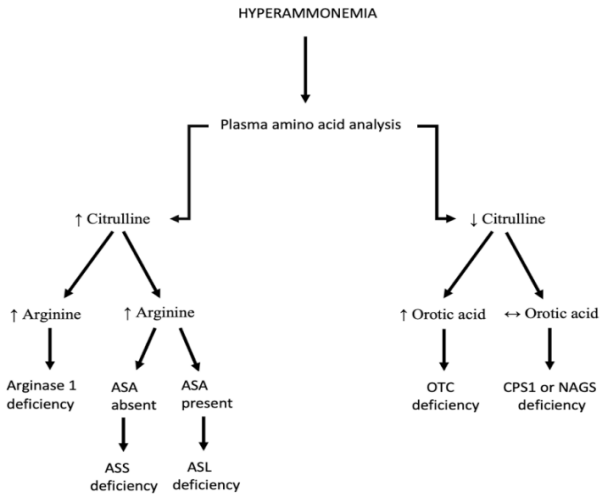
originating from many different sources. Congenital or acquired diseases, different metabolic syndromes affecting the urea cycle, infections, shunt formations causing the blood to bypass the liver and any insult to liver such as an enzyme deficiency or hepatocellular damage which disturbs the metabolization of the ammonia could be the potential reasons of hyperammonemia (3).

Clinical signs and symptoms of the condition are generally neurological in origin, but mainly nonspecific presentations are seen, differing according to age. Loss of appetite, vomiting, lethargy, hyperventilation, irritability complexed by seizures and coma, ataxia, confusion, disorientation, hallucinations are some of the common observed presentations seen in patients aged from infants to adults (4). At the hospital administration, first line tests accompanied with a clinical examination includes following biochemical tests: electrolytes and anion gap measurements, blood gases, ketonuria, glycemia, lactic acid, plasma and urine amino acids and urine organic acids chromatographies, plasma acylcarnitine profile on dried blood, dosage of plasma total, esterified and free carnitine, and urinary orotic acid, liver function tests including aspartate aminotransferase (AST) , alanine aminotransferase (ALT) , bilirubin, gamma-glutamyl transferase (GGT), factor V, prothrombin ratio (PT), international normalized ratio (INR) and lastly renal function tests like blood urea nitrogen (BUN) and creatinine (5). With the suspicion of urea cycle disorders (UCDs), DNA mutation analysis and enzymatic activity detection tests are applied afterwards to confirm the diagnosis. In addition, neuroimaging with CT or MRI is used to see the effects of increased ammonia levels on the brain besides laboratory analysis. It is important to emphasize that the potential risk of brain injury makes the condition more crucial to rapidly evaluate and interfere with the appropriate treatment modalities. The key point in the treatment of the ammonia toxicity is lowering the level of ammonia in the blood and excluding the risks of further complications. As there is a problem with the protein catabolism, all protein intake should be stopped immediately, the calorie intake should be supported with glucose solutions, pharmacological compounds that transform nitrogenous products other than urea to omit the urea cycle should be used to expel those products in the urine and lastly, dialysis methods can be used to lower the ammonia levels (3,4).

2. Urea Cycle Disorders

Urea cycle is a detoxification method of ammonia to urea, regarding the nitrogen clearance in the body. There are six enzymes and two transporters related with the urea cycle: N-acetyl-glutamate synthase (NAGS), carbamoyl phosphate synthetase 1 (CPS1), ornithine transcarbamoylase (OTC), argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL) arginase 1, ornithine transporter and citrin. Inherited disorders affecting the functions of this enzymes resulting

with interference of the cycle continuation are called urea cycle disorders, those defects are known to be the part of inborn errors of metabolism. The incidence of all the types of UCDs are 1 in 35.000 births, upon that OTC deficiency is being most common with an occurrence of 1 in 56.000 births (6). Age of onset and clinical presentations differ from individual to individual and between the disorders also. Abnormal analytes found in blood and urine in addition to other tests light the way for the diagnosis. Disorders of the urea cycle is discussed in detail in the following sections.



↑, increased; ↓, decreased; ↔, unaffected

Figure 2: Biochemical Diagnosis of Urea Cycle Defects

2.1. Carbamoyl Phosphate Synthetase 1 Deficiency

There are two carbamoyl phosphate synthetases in the human body. The first one catalyzes the first and the rate-limiting step of the urea cycle by using ammonia inside the hepatocyte mitochondria and for a lesser extent inside the intestinal mucosa cells, producing carbamoyl phosphate only with the activation of NAG. On the other hand, carbamoyl phosphate synthetase 2 works in the de novo pyrimidine synthesis pathway in the cytosol. The latter one produces CP from glutamine instead of ammonia which is being the rate limiting step of the pyrimidine biosynthesis and does not require NAG to get activated. At the proceeding steps of the synthesis, orotic acid which will be important at the diagnosis of other UCDs too, forms as a by-product of the pathway.

CPS1 deficiency is an autosomal recessive congenital disorder of the urea cycle presenting with hyperammonemia (7). CPS1 gene located at the chromosome 2q35 locus encodes CPS1 enzyme. Since lacking the enzymatic activity creates

an interruption in the urea cycle, ammonia would not get converted to urea thus it starts to accumulate in the blood. Severity of the hyperammonemia gets more intense as the ratio of the deficiency gets higher. Urea cycle is not active in utero therefore, the signs and symptoms of the disease become prominent after birth, at any time from the neonatal period to adulthood (8). Between the neonatal and late-onset presentation, neonatal-onset shows quicker worsening with more severe outcomes starting from 24-72 hours after birth, usually presenting with the first calorie intake. Affected patients show lack of appetite, vomiting, sleepiness, irritability, lethargy but increased levels of ammonia are especially known to be neurotoxic so nervous system manifestations like seizures, altered consciousness, postural disturbances, motor dysfunction can also be seen. Those symptoms repeat after the end of the neonatal period if ammonia continues to accumulate in the blood and growth retardation with intellectual disability may be experienced by the patients (9).

First line parameters of the administered patients show low levels of citrulline, high levels of glutamine along with the high levels of ammonia in the blood; low or normal levels of orotic acid in the urine (10). Furthermore, in addition to physical examination, other biochemical laboratory tests like complete blood count, C-reactive protein, liver and kidney function tests, glucose, lactic acid levels, blood gas analyses are essential parameters to evaluate (10). Detection of hyperammonemia is important for the elimination of sepsis in a child with nonspecific clinical findings. However, the sampling of blood for ammonia detection is tricky. The ammonia levels in the withdrawn blood tents to increase as it stays in the tube. In order to avoid the false results, blood should be separated immediately and plasma should be frozen and sent to the laboratory inside the ice bags. Because of the nonspecific nature of the ammonia increase, clinicians need to behave vigilantly to differentially diagnose the enzyme deficiency with a medical family history including consanguinity, unexpected neonatal deaths, patient's drug intake, psychiatric and neurologic disorders (11). The confirmation of the initial diagnosis is done by the molecular and enzymatic testing. Enzymatic tests diagnose the disease by measuring the enzyme activity of CPS1 using a liver biopsy whereas genetic sequencing reveals the cause of the disorder by detecting the mutations in the related regions. In addition, from the first detection day of CPS1 gene mutation to today, the number of the types and spectrum of the mutations have been expanded which broadens the clinicians' horizon to understand the pathogenesis more clearly (12).

The aim of the therapy is balancing the biochemical pathways in terms of nitrogen formation and destruction by lessening the protein intake, channeling the excess nitrogens to alternative pathways and correcting the nutritional deficiencies (13). The prompt therapy for the hyperammonemic

coma includes immediate lowering of the ammonia levels via hemofiltration methods, scavenger drugs for nitrogen, reversing of the catabolism with glucose and lipid supports and close monitoring of the nervous system. For the long term management, protein restriction with additional support for the essential amino acids, scavenger drugs, proper nutritional support with high calorie diets are recommended. For the patients who experience hyperammonemic coma periodically despite the treatment, liver transplantation can be considered. Aside from that, symptomatic treatment is applied for the systemic manifestations such as epileptic seizures etc.

2.2. N-Acetylglutamate Synthase Deficiency

NAG synthase is not one of the direct enzymes of the urea cycle, but it produces NAG from glutamate and acetyl-CoA in the mitochondrial matrix. Produced NAG is the obligate allosteric activator of the rate limiting enzyme of the urea biosynthesis, CPS1. NAGS gene is located at the chromosome 17q21 locus in the human genome. Like all the other urea cycle disorders, except OTC deficiency, NAGS deficiency has an autosomal recessive transmission. The mutations affecting the region were reported to be mostly missense but also nonsense, insertion, deletion and enhancer region types (14). Considering all UCDs, NAGS deficiency is the rarest one.

Clinically, there is no difference between signs and symptoms of NAGS and CPS1 deficiency. Initial presentation of the complaints which is characterized by hyperammonemia can start at early or late stages of life depending on whether the deficiency is complete or partial. Progression of the symptoms is nearly the same for all UCDs; starting with vomiting, diarrhea, eating refusal, lethargy; proceeding to confusion, seizures and respiratory distress. Life- threatening complications occur in untreated conditions afterwards.

Diagnosis of a rare disease can be challenging for the clinicians. Since NAGS deficiency also shows the typical findings of the other UCDs, it requires special attention. Excessively high levels of ammonia in the blood along with increased levels of glutamine, alanine, asparagine whilst normal levels of arginine and citrulline can be measured with quantitative plasma amino acid analyses when urine parameters do not show any abnormalities. Monitoring after hyperammonemic coma achieved with following serum ammonia, arterial blood gases, serum electrolytes etc. The suspected diagnosis of NAGS deficiency can be confirmed by mutation analysis or enzyme activity analysis. Enzyme analysis is not usually preferred since it requires an invasive procedure, a liver biopsy, but it can be used to rapid detection of the difference between CPS1 and NAGS deficiency (15).

Therapy is the same as the previous UCD.

2.3. Ornithine Transcarbamylase Deficiency

OTC transfers the carbamoyl group of the CP to ornithine to yield citrulline and an inorganic phosphate. The reaction again occurs in the hepatocyte mitochondrial matrix as a part of the urea cycle. Citrulline passes to the cytosol via a carrier called ornithine translocase. OTC gene which is located at the chromosome Xp21.1 locus is responsible for the synthesis of the enzyme. Mutations affecting this region of the genome cause defects of the OTC, making this deficiency the most common in all UCDs. Up to date, there have been more than 500 mutations reported related with OTC gene in which 74-80% being single point, 12% being small fragment deletion and 4% being large fragment deletion mutations (16). Since OTC deficiency is inherited as X-linked recessive fashion, male patients show earlier onset of the disease with poorer prognosis resulting with life-long neurological deficits or even death caused by intolerably high levels of ammonia compared to female patients with late-onset and milder forms of the OTC deficiency (17). People who have the deficiency to a lesser extent may experience partial lack of enzymatic activity meaning more tolerance to the nutritional proteins.

Cyclic vomiting, sleepiness, attacks caused by recurrent hyperammonemia, neurological manifestations such as palsy, epileptic seizures, cerebral atrophy can be listed as the symptoms of the OTC deficiency (18). Potential development of neurological symptoms is quickened if the presented symptoms are left untreated. High serum ammonia, glutamine, alanine; decreased citrulline, arginine and elevated levels of orotic acid in the urine are the typical laboratory presentation. The reason behind the urinary orotic acid increase is alternative metabolization of excess carbamoyl phosphate to orotic acid via the enzymes of the pyrimidine biosynthesis. Genetic tests as well as enzymatic tests with liver biopsy samples are used in detection of OTC deficiency like other UCDs. Sensitivity ratios of the sequencing and deletion-duplication analysis is 80% and 10% respectively supported with approximately 90% clinical sensitivity overall (19).

Logic of the treatment, preventing the neurologic damage caused by excess ammonia while supporting the growth and development, is similar for all the urea cycle defects and it requires a multidisciplinary approach for regulating the drug regimens, diet and supplements and further follow up. In the life-long process, effective nourishment, parameters of the physical growth, liver functions, amino acid and ammonia levels in the blood should be closely checked for the better life quality of the patients (20).

2.4. Argininosuccinate Synthetase Deficiency (Citrullinemia)

ASS links aspartate and citrulline to make argininosuccinate in the cytosolic side of the mitochondrial outer membrane (21). The second nitrogen of the urea

comes from aspartate through this reaction. Argininosuccinate is one of the key precursors for supplying arginine to be used in various reactions including de novo arginine synthesis, urea cycle, nitric oxide (NO) synthesis, polyamine and creatinine synthesis in the body (21). Here, one of the overlaps is between the urea cycle and the nitric oxide synthesis. Primary precursor of NO is arginine which emerges at the end of one of the stepwise reactions of the urea cycle. After the subsequent activity of the ASS and ASL, generated arginine can be converted back to the citrulline by the activity of NO synthase (NOS) or it can be used by arginase to complete the urea cycle by producing ornithine and urea (22). At this point, arginase 2 which is located outside the liver becomes a competitor enzyme against NOS and limits the activity of it (22). In addition, aspartate further participates in other biochemical pathways as a substrate such as TCA cycle and protein catabolism so, the availability of it is affected by those pathways likewise.

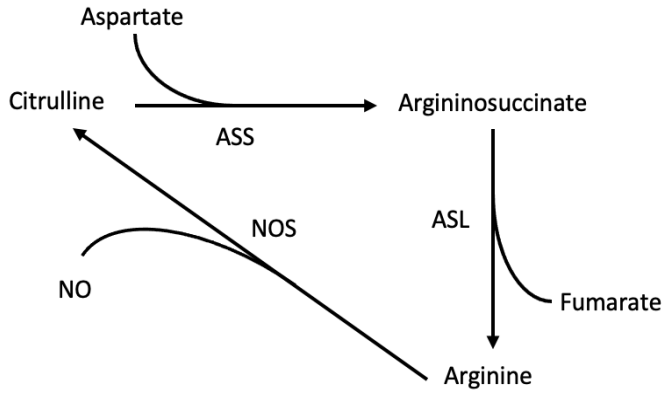


Figure 3: Arginine and NO Synthesis

The encoding gene for ASS is located at the chromosome 9q34 locus. Mutations in related gene region diminishes the enzyme production which is referred as ASS deficiency, being autosomal recessively inherited. Since this deficiency causes accumulation of its substrate, the disease is also called as citrullinemia (CTLN). There are two types of citrullinemia. The first type has a neonatal form, known as classic citrullinemia, late-onset, a postpartum and an asymptomatic form. The ASS gene is mutated in this situation. But type 2 CTLN has a totally different pathophysiology. Causing mutation in CTLN type 2 is at the SLC25A12 gene at chromosome 7q21 locus which encodes a polypeptide called citrin, the inheritance pattern of the mutations causing citrin deficiency is autosomal recessive similar to other types. Citrin is a calcium dependent aspartate-glutamate transporter located at the outer mitochondrial membrane (23). It carries solutes from cytoplasm to mitochondria as a part of the malate aspartate NADH shuttle. When there is no citrin to carry aspartate inside the

mitochondria, there is no substrate for ASS thus, citrin deficiency causes ASS deficiency and citrulline accumulation indirectly (24).

Signs and symptoms of the CTLN type 1 are similar with the previous ones. Infants become lethargic, lose their appetite, generally vomit, and can show neurological insults like seizures, loss of consciousness, spasticity caused by increased intracranial pressure, because of ammonia accumulation after being totally normal postpartum (25). The ones with the partial enzyme deficiency experience milder manifestations of the disease starting during infancy or later in life may show distorted growth and development, ataxia and other periodic symptoms coming with ammonia increase. Citrin deficiency has different clinical presentations depending on age as follows: neonatal intrahepatic cholestasis in newborns and infants, developmental delay and lipid metabolism disorders in older children and adult onset CTLN type 2 in adults (26). Adult onset CTLN mainly affects the neurologic system, related symptomatology is experienced as a result.

Excess amount of ammonia and in the blood is the primary laboratory parameter suggesting ASS deficiency. Quantitative amino acid analysis, urine orotic acid level measurements can be done at the next steps. Increased levels of citrulline in citrullinemia type 1; increased levels of citrulline, arginine, methionine, threonine, tyrosine, lysine are found in the plasma analysis (15). The confirmation of the diagnosis is done by detailed family history, enzyme activity measurements using red blood cells, liver or fibroblast specimens and detection of the mutations with the molecular testing. The differentiation between whether the mutation is affecting ASS gene and the SLC25A13 gene is extremely important since two types of citrullinemia are different from each other in terms of pathophysiology and treatment process.

The treatment consists of protein restriction, vitamin supplementations, arginine supplementations, scavenger drugs for excess nitrogen for citrullinemia type 1. Additionally, because there is a defect in the NADH shuttle in the type 2 citrullinemia, the diet of the patients includes low carbohydrates but high protein and lipids (24).

2.5. Argininosuccinate Lyase Deficiency

Cleavage of argininosuccinate to fumarate and arginine is catalyzed by ASL in the cytosol. Liberated arginine is subsequently used in the proceeding steps of the urea cycle whereas fumarate establishes a connection between urea and TCA cycles. Carbons of fumarate can be converted to malate to be further converted to glucose or oxaloacetate in the fasting states. The ASL gene is located at chromosome 7cen-q11 locus. The inheritance pattern of the ASL deficiency is by autosomal recessive transmission, partial defect seen in the heterozygote patients and it is the secondly common UCD seen after OTC deficiency.

The clinical picture of the ASL deficiency is known as argininosuccinic aciduria. Considering the neonatal onset patients, clinical picture rapidly deteriorates from digestive symptoms to seizure, coma and respiratory arrest requiring immediate recognition and intervention (27).

More severe forms occur in early periods after birth as a result of complete deficiency while milder forms reveal themselves later in life with a partial defect in the urea cycle. Symptoms and signs are similar to the other UCDs. Together with respiratory alkalosis and tachypnea; hepatomegaly, progressive liver diseases, fibrosis and cirrhosis can be seen as liver manifestations. As a result of the deficiency, arginine deprivation disturbs the NO synthesis. Since it is a potent vasodilator, systemic hypertension can also be seen in the lack of NO. Additionally, different from other cycle defects, trichorrhexis nodosa, a condition which causes node formations along the hair shaft making the breakage easier to occur because there is lack of arginine in the hair, is observed in half of the ASL deficient patients (28).

Fundamental finding in the biochemical tests is high ammonia levels in the blood. Plasma amino acid measurements typically indicates increased amount of citrulline, accompanying ammonia. Increased levels of alanine, glutamine, glycine as a result of defect in the nitrogen excretion; orotic aciduria as a result of diminished ornithine turnover can be seen in some individuals (28). Elevated argininosuccinic acid (ASA) levels in urine or blood samples are pathognomonic for ASL deficiency. Finally, the confirmation can be done by detecting the disease causing alteration in the genome with sequence mutation analysis or by enzymatic activity testing using fibroblast or red blood cell samples.

Two main goals of the therapy, curing of the acute manifestations of hyperammonemia and preventing long term outcomes, stay identical with the other UCDs. What to be careful about long term diet arrangements is that not to jeopardize the growth and development of the child while applying a low protein diet. Supporting the amino acids, arginine in particular is needed.

2.6. Arginase 1 Deficiency (Argininemia)

There are two very similar isoforms of arginase in the body: arginase 1 is located mostly in the hepatocyte cytosol, participating in the urea cycle and arginase 2 which is mitochondrial, mostly expressed outside the liver, mainly in the kidneys (29). Arginase 1 is in charge of the last step of the urea cycle. It catalyzes the hydrolytic clevage of arginine and produces the final products, urea and ornithine in the cytoplasm. ARG1 is the responsible gene, located at the chromosome 6q23 locus and the deficiency is inherited in autosomal recessive manner.

In arginase deficiency, patients do not present with hyperammonemia crisis, they do not show acute symptoms in infancy period. Instead, affected children develop intellectual disability, growth retardation, seizures, progressive spasticity in their two to four years of age (30).

Biochemical abnormalities of the deficiency include hyperargininemia with 15-fold increase in the levels in the blood, increased guanido compounds originating from arginine, orotic aciduria because of activation of NAG synthesis by excess arginine and subsequent activation of carbamoyl phosphate synthetase 1 (31). Contrary to other UCDs, hyperammonemia is not a biochemical hallmark but it can still be seen in some patients. Under the light of the biochemical and clinic profile, final diagnosis can be confirmed with enzyme analysis or genetic investigations. It was reported that the mutations recorded for this gene are missense mutations which alter the active site of the enzyme thus, generates the enzyme deficiency (31). Enzyme activity is analyzed through erythrocytes for arginase 1 deficiency.

As mentioned above, arginase deficient patients do not experience episodes of hyperammonemia often, if there is an episode, it should be treated with an approach to decrease the ammonia concentration with proper drugs to channel the excess nitrogen to other pathways and the body needs to be nourished with fats and carbohydrates while making protein restriction in order to prevent protein catabolism (32). The long term management is the same with the previously mentioned disorders.

2.7. Hyperammonemia- Hyperornithinemia- Homocitrullinuria (HHH) Syndrome

After the activity of arginase, ornithine needs to go back to mitochondria to ensure the continuity of the cycle. The entrance gate for ornithine in exchange for citrulline is a transporter called ornithine translocase, also known as mitochondrial ornithine transporter 1 (ORNT1) (33). The absence of ORTN1 is caused by mutations in the SCL25A15 gene located at chromosome 13q14 locus. The mutations are transferred as an autosomal recessive way. When there is no passage from cytosol to mitochondria for ornithine, the urea cycle cannot be concluded which causes hyperammonemia; ornithine cannot enter the next cycle and accumulates reasoning hyperornithinemia. Lastly, ornithine has a role in the synthesis of citrulline and the two amino acids: glutamate, proline and it is believed that homocitrulline emerges as a side product of those mentioned amino acids' reactions while orotic acid is formed secondary to reduced OTC activity which both of them shows increased levels in the urine of the affected patients (34).

Age of onset can show variability from patient to patient. Neonatal presentation takes its source from excessive ammonia levels; showing vomiting,

refusal to eat, muscle stiffness, paresis, somnolence, poorly controlled breathing and epileptic seizures whereas clinical picture outside the neonatal period includes mostly neurological deficits and liver dysfunction (35).

The biochemical findings are already stated at the name of the syndrome as increased levels of ammonia and ornithine in the plasma and homocitrulline in the urine. Additionally, rise in the levels of glutamine, alanine, liver transaminases in the blood, ornithine in the blood and urine can be seen. After suggestive biochemical markers, recognition of pathogenic variants pertaining to both of the SCL25A15 alleles concludes the final diagnosis.

Management of the complaints is similarly handled with the mentioned disorders. Further, citrulline supplementations are administered. Routine follow up of growth and development until adolescence, mentioned biochemical tests and periodic neurological control remain important for the evaluation of the prognosis.

Table 2: Summary of the Urea Cycle Disorders

Deficiency	**Alternative Name of the Deficiency**	**Frequency**	**Genetics**	**Biochemistry**
CARBAMOYL PHOSPHATE SYNTHETASE 1	CPS1 deficiency	1/50,000 -1/300,000	AR 2q35 CPSI	**Blood:** ↑ glutamine ↑ alanine ↓ citrulline ↓ arginine
				Urine: ↔ orotic acid
N-ACETLYGLUTAMATE SYNTHASE	NAGS deficiency	<1/1,000,000	AR 17q21 NAGS	**Blood:** ↑ glutamine ↑ alanine ↓ citrulline
				Urine: ↔ orotic acid
ORNITHINE TRANSCARBAMYLASE	OTC deficiency	1/56,500	X-linked Xp21.1 OTC	**Blood:** ↑ glutamine ↑ alanine ↓ citrulline ↓ arginine
				Urine: ↑ orotic acid

ARGININOSUCCINATE SYNTHETASE	ASS deficiency- Citrullinemia type 1	1/44,300 -1/200,000	AR 9q34 ASS	**Blood:** ↑ citrulline ↓ arginine **Urine:** ↑ orotic acid
CITRIN	Citrullinemia type 2	1/100,00 – 1/230,000 (Seen in Japanese descent (36))	AR 7q21 SLC25A12	**Blood:** ↑ citrulline ↑ arginine **Urine:** ↔ orotic acid
ARGININOSUCCINATE LYASE	ASL deficiency- Argininosuccinic Aciduria	1/70,000 – 1/218,000	AR 7cen-q11 ASL	**Blood:** ↑ citrulline ↑ argininosuccinic acid ↓ arginine **Urine:** ↑ argininosuccinic acid ↑ orotic acid
ARGINASE 1	Hyperargininemia	1/300,000 – 1/1,000,000	AR 6q23 ARG1	**Blood:** ↑ arginine **Urine:** ↑ orotic acid
ORNITHINE TRANSLOCASE (ORNT1)	HHH syndrome	<1/1,000,000	AR 13q14 SCL25A15	**Blood:** ↑ ornithine ↑ glutamine ↑ alanine **Urine:** ↑ homocitrulline ↑ orotic acid
↑, increased; ↓, decreased; ↔, unaffected; AR, autosomal recessive				

3. Prenatal Testing and Newborn Screening Programs

Prenatal testing especially in the families of previously affected individuals are important for reporting to parents and more importantly getting prepared for the upcoming treatment conditions postnatally. Either mutation detection from chorionic villus or amniotic fluid cell sampling and measurements of citrulline or argininosuccinic acid levels from amniotic fluid for ASS and ASL in particular are convenient ways of prenatal detection (37).

Centers for Disease Control and Prevention (CDC) defines the purpose of newborn screening for any disease as early recognition, diagnosis and intervention for the particular genetic, endocrine, metabolic and physical conditions that affect the mortality and morbidity of the child (38). It started in the 1960s, with the screening for the detection of an amino acid disorder, phenylketonuria (PKU), using the Guthrie test developed by Robert Guthrie. The test is completed by taking a small amount of blood from the baby's heel, collecting the blood on a special card called Guthrie card, and sending it to the laboratory for testing. The results come back in five to seven days thereafter. Applied screening programs and the interpretation algorithm of the results differ from country to country, even from state to state in some countries. Diseases included in the screening programs are expected to have relatively high incidence; an easily applied, possibly non-invasive, cheap, sensitive and specific screening test parameter.

Considering all the UCDs, neonatal presentations are known to be most severe and rapidly deteriorating especially in the absence of the treatment. The important issue to emphasize is that the severity of presentations occurring after the results of NBS tests can be alleviated with the presymptomatic treatment, moreover the patients can be transferred to proper institutions in case they are situated in a place where the urgent intervention such as hemodialysis, intravenous ammonia scavenger drugs etc. cannot be provided (39). Unfortunately, intervention to cases presenting before the availability of the results or showing sudden clinical symptoms without a screening test are firstly related to the ability of the clinician to suspect the presence of an inherited metabolic disorder and manage accordingly then, if there is a result, it contributes to the diagnosis and speeds up the process. Those mentioned diseases are prone to deteriorate and result with drastic complications or even death particularly if there is a delay in the treatment attempts.

For the UCDs, only some of them are tested in some countries around the world. In the view of the fact that the biochemical and clinical hallmark parameters for UCDs, such as hyperammonemia and liver dysfunction, overlap with many disorders, the sensitivity and specificity of the screening does not provide a solid outcome for every case (40). Importance of early detection

of UCDs is mentioned above. In addition to that, even milder cases with late onset disease, expressing only one pathological allele which provides enough amount of enzyme or transporter activity can experience lethal outcomes of hyperammonemia which proposes one more powerful reason for UCDs to be included in the NBS programs (41).

4. Summary and Future Investigations

UCDs are a group of rarely seen metabolic disorders studied under a bigger title, inborn errors of metabolism, caused by various defects throughout the ammonia detoxification mechanism. The pathologic part of the metabolism can also disturb the other pathways participating in the reactions of different parts in the body so, awareness of all the clinicians remains highly significant. Primary laboratory hallmark of the UCDs is extreme levels of hyperammonemia supported with ancillary testing. Even though ammonia toxicity firstly affects the neurological system, the clinical presentation is particularly nonspecific and requires immediate action. To confirm the initial diagnosis, genetic and enzymatic tools are being used. After the diagnosis, most of the related patients need a lifelong strict treatment regimen to regulate their bodies to the deficiency.

In a recent study, it has been conducted that not being able to show full potential intellectually and experiencing problems like attention deficiency or autism can challenge patients socially and additionally, while early onset UCD patients are more prone to undergo difficulties in getting a job or maintaining a relationship, late onset patients are more near normal (42). To sum up, early diagnosis of the UCDs remain paramount to prevent a person from living a potentially low quality life by applying updated diagnostic and treatment approaches which is hoped to be improved from day to day.

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CHAPTER XI

MAD HONEY IN RECENT HISTORY

Perihan Şimşek[1] & Abdülkadir Gündüz[2]
[1](Asst. Prof. Dr.) Trabzon University, e-mail: p_simsek19@hotmail.com
Orcid: 0000-0002-0216-3968
[2](Prof. Dr.) Karadeniz Technical University,
e-mail: gunduzkadir@hotmail.com
Orcid: 0000-0001-8591-9769

1. Introduction

It is well known that mad honey poisoning dates back to ancient times. Written documents of this type of poisoning appear in the last century as well. This issue is frequently discussed in both scientific journals and non-scientific food and plant journals. The articles vary in content like the in vitro effects of the poison at the cellular level, systemic effects, clinical status, and plant species that may or should not cause poisoning. These mad honey-containing articles that have been written so far offer significant awareness to today's medical knowledge and research, and the relevant documents published in the last century are both complementary and confirming the previously published documents. When we wish to chronologically review the articles on mad honey published from the beginning of the nineteenth century to the present, we also embark on an exciting journey about nature, humans, plants, poison, and food, such as *Rhododendron*, the black sea, and the spread of *Rhododendron* in the world.

2. Old Records of Mad Honey Poisoning Cases

In old records, the plant from which the poison in mad honey originates has been an interesting research topic throughout history. Regarding this issue, Trabzon consul Alfred Biliotti (1833-1915) claimed in his report dated 1879 that a kind of honey that cannot be eaten was produced in Trabzon and that the poison in the honey was collected from the Datura Stramonium plant, which is widely grown in the coastal region. However, in the same period, it is recorded that there were no complaints of poisoning with honey collected from beehives in Datura Stramonium growing regions. Moreover, those who observed the plant noted that no bees approached the plant. This is explained by the fact that the

flowers are pollinated not by bees, but by long-nosed insects flying at night since the nectar of the plant is at the bottom of the long pollen tube (1).

In addition to observations, scientific studies were also carried out to find the source of the poison in the mad honey produced in Trabzon. Alfred Biliotti sent honey samples from the region to England for examination. Scientific studies in England proved that the samples do not contain alkaloids and do not dilate the pupil (1). These results supported the idea that the source of the poisonous honey in Trabzon is Azalea Pontica, not the Datura Stramonium plant (2), which contains atropine, hyoscyamine, and scopolamine, which causes anticholinergic poisoning (1).

A book on beekeeping, published in 1916, warns that the quality of honey in the hives will decline when it is time for various plants to bloom. In the book, it is stated that various daisy-type plants (bitterweed or sneezeweed) bloom after the end of the clover harvest, and the honey produced from these flowers is bitter and often mixed with high-quality white clover honey, spoiling its taste. The book also states that honey collected from *Mountain laurel* (*Kalmia latifolia*) and *Rhododendron* (*Rhododendrons*), which grows widely in the Virginia Mountains and surrounding states, is poisonous. The book also mentions that honey collected from mountain laurel (*Kalmia latifolia*) and *Rhododendron*, which grows widely in the Virginia mountains and surrounding states, is poisonous. For this reason, it is recommended that beekeepers should be careful in choosing a place for their hives, that beekeepers should learn when these plants bloom, and that they should remove all the good quality honey from the hive before the bees start collecting nectar from them (3).

The United States of America (USA) is one of the countries where mad honey poisoning is experienced. According to a book written in 1920 on honey-producing plants, the source of mad honey in this country is Mountain laurel (Kalmia latifolia). Mountain laurel, a common shrub that grows at high altitudes in the Southern States of America, from New England and Ontario south to the Gulf states, is also known as conifer bush, poison ivy, or spoon tree. Poisonous kinds of honey are also produced in America besides mad honey. For example, the Manchineel tree (Manchineel tree; Hippomane Mancinella, Euphorbia; a flowering plant species from the Euphorbiaceae family), which grows widely in the beaches and marshes of South Florida, in the Bahamas, and Tropical America, is recorded to be the most poisonous of American trees. It is even known that the Caribbean used the milky juice of this plant to make their arrows poisonous. It is emphasized that this tree, which produces abundant nectar in some seasons, is also used in honey production. The book also mentions that among the Maori Indians in New Zealand, there are frequent cases of poisoning and sometimes death due to the honey produced from the puka-puka and wharangi bush (Melicope ternata). According to the book, unripe honey is

responsible for honey poisoning, and glazed and ripe honey can be consumed as healthy food (4).

Ancient documents on mad honey poisonings report that in some areas in Edinburgh sheep were poisoned after eating *Rhododendron* leaves. It was observed that the main symptoms observed in poisoned sheep were drooling, vomiting, weakness, drowsiness, and death in a few cases. German scientist Plugge reported that the toxic component effective in these cases is Andromedotoxin, also found in other Ericaceae species (5).

Andromedotoxin was first obtained from Andromeda Japonica by Eykman in 1882 and was named "Asebotoxin" in reference to the Japanese name for the plant. In the same period, Plugge discovered this compound and named it Andromedotoxin. The chemical and pharmacological systematic research of the compound was made by H. De Zayer in 1886 and a summary of his work was published by Plugge in 1887 (6).

Hardikar isolated the "Andromedotoxin" compound from *Rhododendron* leaves with a method like Zayer's method and published the results listed below in 1921.

1. Andromedotoxin is an active compound of *Rhododendron* and affects the endpoint of the N. vagus. As a result of this interaction, first arousal then paralysis develop. The most obvious effects resulting from this situation are as follows:
    a. Short-term respiratory inhibition, change in respiratory movements and slowing of respiratory rhythm.
    b. Asthmatic dyspnea due to bronchial muscle spasm partially because of stimulation of afferent fibers and partially N. vagus.
    c. Increase in bronchial secretions.
    d. A rapid heartbeat and a rise in blood pressure following the slowing heartbeat and falling blood pressure.
    e. Frequent emptying of the bowels.
2. Andromedotoxin paralyzes motor nerve endings in striated muscles. Muscles and nerves tire easily while paralysis develops, but they regain their excitability after rest. At a more intense concentration, the muscles can permanently lose their excitability. This type of fatigue is seen earlier and more clearly in the muscles and nerves that need to be in constant motion, especially in the phrenic nerve and diaphragm.
3. Death due to andromedotoxin occurs in two ways:
    a. In high doses, it develops rapidly, and because of direct involvement of the heart, heart stops at the ventricles in diastole or partial systole.
    b. At lower doses, respiratory failure develops due to paralysis of the phrenic nerve and usually the diaphragm.

4. There is a narcotic effect in the upper brain centers. The spinal cord is not affected.
5. Heart block or ventricular arrhythmia may occur. The time required for ventricular diastole increases and diastole cannot be completed (5).

In historical records, it is seen that the name "mad honey", which basically describes kinds of honey produced from *Rhododendron* type plant nectars and which has a toxic effect due to the grayanotoxin it contains, is also used for honey produced from different sources. According to a 1921 book on pharmacopoeias, "During the blooming season of the opium poppy, bees make a honey with narcotic properties known as "Mad Honey". Those who eat this honey wander aimlessly, speak incoherently, and look insane. It is non-drug and has apparently quite different properties from morphine" (7).

Poisonous honey is frequently mentioned in the old sources about beekeeping. In a study published in 1923, one of these sources, some plants with poisonous nectar are listed as Kalmia latifolia, tobacco (Nicotiana tabacum), yellow jasmine (Gelsemium sempervirens), sweet pepper bush (*Clethra alnifolia*), and *Rhododendrons*. The publication indicates that *Mountain laurel, Yellow jasmine,* and *Rhododendrons* are abundant in the lower reaches of the Appalachian Mountains (a mountain system in eastern North America), where more beekeeping is done than elsewhere in the United States. However, it is emphasized that there are not many cases of honey poisoning in the region, and that unfounded rumors about the subject should not be taken into account unless the plants causing honey poisoning are determined. The article also focuses on the possibility that recorded honey poisoning may not be caused by the poison in honey but by people being sensitive to honey (8).

Scientific publications mention that some kinds of honey produced in the mountainous regions of central and northern Japan in summer or early spring have "narcotic" properties and cause poisoning cases. It has been recorded that this poisonous honey produced in Japan is light in color and does not have any strange smell, but when it is eaten with a spoonful, it causes coughing and irritation of the stomach. The severity of poisoning cases experienced in this region varies according to individual characteristics and the amount consumed, and the main symptoms are retching, vomiting (rarely diarrhea), headache, palpitation, and relaxation of voluntary muscles. When honey is extremely poisonous, ataxia, weakening of the heartbeat, extremity coldness, mild spasm, dilated pupils, and increased deep tendon reflex can be seen. In cases of poisoning in Japan, recovery is generally observed in a few hours or a few days, and no fatal case has been reported (9).

Japanese scientists working on poisonous honey from Sado Island determined that the minimum lethal dose of their sample was 3.5 mg per kilo

in rabbits and 20 mg in mice when given by subcutaneous injection. In their research on mice, scientists listed the main symptoms caused by the poison as restlessness, vomiting effort, drooling, and partial paralysis and emphasized that the poison can lead to death (9).

Japanese scientists argued that the toxic substance in honey is found in nectars collected from the Tripetaleia Paniculata and Hotsutsuji (Elliota paniculata) from the family Ericaceae, which spreads to the mountains and hills in regions such as Naganoken, Iwateken, Sado Island in Niigata-ken. That is because, poisonous honey is produced in the summer season, which is the flowering period of Tripetaleia Paniculata and Hotsutsuji plants from the family Ericaceae, and these plants are widely grown in places where poisoning cases are common. In addition, the production of poisonous honey at short intervals in the region was found to be quite compatible with the short-term blooming of heather bushes (Tripetaleia-shrubs). Scientists have revealed the following results in their research on the poison that they assume passes through the nectars of the family Ericaceae to honey:

- "The poison can be isolated with alcohol in an almost pure form. There are at least two poisonous substances in honey, "Crystalline" and "Amorphous". These substances are also found in the alcoholic extract from the flowers and leaves of Tripetaleia. The poison cannot be removed by boiling it at 100 ^{o}C for an hour."
- "There is no trace of nitrogen in the poisonous component of honey, and it cannot be judged that the origin of the poison in its content comes from plants such as wolfsbane and datura, which bloom in summer and contain alkaloids as poisonous substances."

The production of poisonous honey in the region only in summer has enabled beekeepers to collect their honey just before or after the spring or autumn period. In addition, thanks to the fact that the Tripetaleia plant grows in limited areas in the mountains with only a few inhabitants or where nomadic beekeepers continue their beekeeping activities, poisoning cases have been observed in limited numbers (9).

German botanist Prof. Dr. Kurt Krause (1883-1963) visited Anatolia and made a research on Anatolian flora and described the information he gained about honey poisoning during these trips as follows: "Today, poisonous honey is widely known in the northern part of Anatolia. In May 1926, I had the opportunity to travel with Berlin entomologist Dr. H. Bleschoff, especially in the hinterland of Trapezum (Trabzon), Kerasund (Giresun), and Samsun. Symptoms of honey poisoning have been known since ancient times. Discomfort, nausea, vomiting are the main symptoms. I witnessed that a Turkish doctor who had

personally experienced honey poisoning confirmed these symptoms. Weakness usually does not last long. However, nausea persists for a while. Dizziness is a common symptom, especially in severe poisoning cases. Turks call poisonous honey "mad honey". This name reflects a general characteristic of the honey produced around Trabzon. Toxic honey can be produced in the northern coastal areas of Anatolia, the northwest, the European side of the Bosphorus bridge, and the Turkish-Bulgarian border" (10).

Remarkably, Prof. Dr. Krause's publications on poisonous honey received worldwide attention. In an article published in The Science Newsletter in 1927, the issue of poisonous honey is conveyed as follows referring to Prof. Dr. Krause: "Having just returned from his trip in Anatolia, Dr. K. Krause reported that the same poisonous honey that attracted attention in Ancient Greece is still found in that region. Dr. Krause explained that as a result of his experiments, "poisonous dessert" caused drowsiness, sometimes short-term loss of consciousness and weakness. Where the bees get the poisonous nectar from is still an unanswered question, but the doubts are mostly on the two *Rhododendron* species growing in that region and whose leaves are known to be poisonous to livestock" (11).

It is possible to come across cases of honey poisoning in many different parts of the world. G. Mosolevsky, in his article in the Bee World magazine in 1929, suggested that the most common places for honey known as poisonous were the regions around the Black Sea and the Caspian Sea. Mosolevsky continued his article as follows: "Honey poisoning in some parts of the Caucasus occurred with the start of the consumption of comb honey. It lasts for a certain period each year but occurs more in the dry season than in the rainy season. The first symptoms generally appear three or four hours after eating honey. Cases resulting in death are more common in children, which may be because children tend to consume more honey relative to their weight than adults" (12).

The first information about poison honey in historical records was provided by Xenophon, who described the unforgettable retreat of ten thousand Greeks from Persia. The famous French botanist Tournefort, excited to learn the truth of what Xenophon was talking about, did some studies while he was in Trabzon. As a result of his studies, he stated that the poisonous honey was collected from a flowering bush, which is abundant in the environment, the smell of its flowers is similar to honeysuckle, and it has toxic effects. It cannot be clearly understood from Tournefort's definition whether the plant is a poisonous plant native to the Anatolian region, such as Rose Laurel or Azalea Pontica. The mentioned plants and poisonous honey were also found in the region of Mingrelia (a region in Georgia) (13).

Honey collected in Philadelphia in the fall and winter of 1790 was found to be deadly harmful to health. It was therefore come to the attention of the American Government and was ordered to be investigated. As a result of the

investigation, the source of the poisonous honey was reported to be Kalmia Latifolia flowers (13).

Dr. Barton enumerated several other species of Kalmia, Azalea, *Rhododendron*, and *Andromeda*, which are the sources of the poisonous honey, and expressed that experiments were done showing that this honey is also poisonous to dogs. He also noted that poisonous honey causes vertigo, vision loss, intoxication, stomach and intestinal pain, convulsions, excessive sweating, foaming at the mouth, vomiting, diarrhea, and sometimes temporary paralysis and although rare, it causes death. According to Dr. In Barton's articles, two people in New York died after eating wild honey, which was thought to have been collected from the dwarf laurel flowers abundant in the American forests (13).

In the following years, a case of honey poisoning in Tahawndam village in Upper Burma is mentioned in the book titled "Plant Hunter's Paradise" written by Kingdon Ward. In this case, which took place in early May, two people were affected by the poisonous honey, and one of them suddenly fainted. The symptoms disappeared after a short time, and it was described as a situation similar to acute alcohol intoxication. The honey in question was thought to have been collected from local *Rhododendron* plants. According to the writings, it was understood from these cases that *Rhododendron* species other than R. Ponticum can also produce poisonous nectar (14).

Mad honey poisoning is also seen in the Caucasus. *Rhododendron* and Azalea are widely grown in the Caucasus, near Batumi. Honey producers in the region know that the nectar collected from these flowers is poisonous and they do not use the collected honey when these plants bloom in the spring. However, the fact that honey obtained from these plants is not always poisonous has raised doubts about its toxic effects, and it has been claimed that poisoning is caused by bees crushed in honey. However, since it is known that bee venom is easily destroyed by saliva and stomach ferments, even in large quantities, these claims have not found much support (15).

The bee's stinger has a poisonous chamber near its sting, and if it falls into the honey, it can cause injury hours or even days later. The poisonous substance in the chamber is volatile but retains its potency well in honey. It is reported that there are bee stings buried in broken hives from time to time, and people who eat this honey have sores in their mouths. If a severed stinger meets the body surface, the venom in the reservoir may drain into the wound and travel under the skin layers or the mucous membranes (15).

It is noteworthy that historical records present different information about plants that can be the source of poisonous nectars. An article by FN Howes on this subject, published in the Kew Bulletin in 1949, contains the following information: "The first records of honey poisoning probably date back to 4000

BC and are about the soldiers who were poisoned when Xenophon set up camp in Trabzon on the Black Sea coast during the Return of the Ten Thousand. Here, the effect of the poison on the soldiers is explained and it is assumed that the poison of honey is generally caused by the *Rhododendron* plant. Other poisoning cases thought to be caused by *Rhododendron* have also been recorded in this region and neighboring areas" (16).

Pontic Rhododendron is cultivated in many countries. The British Isles are also included in these countries. This plant was suspected to be the source of the harmful honey in several cases of poisoning in the UK. Herrod Hempshall, who examined honey samples from Cobham and Kent, allegedly produced from *Rhododendron*, indicated that people who tasted honey stated that honey has emetic properties. In another example in Camberley, a well-known beekeeper said that ingestion of fresh comb honey for breakfast resulted in symptoms of poisoning and that all family members who ate the same honey were affected. It is known that many *Rhododendron* species grow in the region where the case is seen. Another *Rhododendron* poisoning in England was seen in Nottinghamshire. In this case, vomiting and abdominal pain were reported in children who plundered the wasp nest (16).

In cases of honey poisoning seen in North America, it was thought that the source of the poison could be many plant species such as Mountain Laurel, Yellow Jasmine, Pieris species, Andromeda, and Leucothoe. Among these plants, in the eastern parts of the United States where Kalmia Latifolia is common, beekeepers and farmers tested the first harvested honey in dogs, and children and other family members were allowed to eat it if no harmful effects were observed as a result of the test (16).

In the articles in Beekeeping Magazine, Dr. Grammar, a surgeon in the Confederate Army in the United States in the 1875s, reported that the bees in the places where mountain laurel grows are very fond of this plant. Toxic honey is extremely narcotic, and its effects depend on the amount taken. Expressing that he had the opportunity to see how it affected people during the war, and who also experienced mad honey poisoning, Dr. Grammer explains that the cases seem to be intoxicated, the innervation in the voluntary muscles is significantly affected, and a partial recovery can be achieved within a few hours with general drugs used for narcotics, but the effects of the poison last for two or three days (16).

In addition to this information about the poisonousness of honey produced from the family Ericaceae, the article explains that other members of the family Ericaceae are known to give good honey, such as broom bush (Calluna Vulgaris) and various types of Ericaceae (Erica spp.) are the sources of many kinds of honey produced in Western Europe. Honey is seen poisonous and harmful only in the hive when it is not yet ripe or not glazed, and it is believed that honey loses

its harmful properties when it is properly glazed and matured. However, it is underlined that this situation is not always valid for *Rhododendron* honey (16).

An interesting outbreak of poisoning occurred in 1945 in Pongakawa, off the coast of the Plenty Region on the North Island of New Zealand, and was investigated by Palmer Jones et al. Vomiting, loss of consciousness, and abdominal pain were observed in people who ate poisonous honey, which is a February product. Temporary memory loss has also been detected in a few people. A cook working in a mill and eighteen people around him who ate some of this honey were affected. The severity of the cases varied according to the amount of honey consumed (16).

In another case, a man who ate just one teaspoon of honey was affected three hours later. After eating the honey, he went pig hunting and had difficulty returning home. After arriving home, he lost consciousness for a while. The man's wife, who sweetened her tea with only half a teaspoon of honey, was affected after eight to twelve hours. In another example, the day after the honey was consumed, the case "went berserk". The symptoms seen in this event are described as loss of consciousness, temporary memory loss, vomiting, and abdominal pain. A chemical substance called mellitoxin ($C_{15}H_{18}O_7$) was isolated from honey, which causes poisoning. In the experiments, it was observed that the effects of Mellitoxin on Guinea pigs were the same as the effects of honey, and it was thought to be one of the picrotoxin class poisons. There was also evidence of a second toxic substance in honey (16, 17).

3. Laboratory Research on Mad Honey in Recent History

The toxic effects of *Rhododendron* and *Laurel* were detected in the 1900s (18). Eykman isolated the toxic compound from Andromeda Japonica in 1882 and named this compound "Asebotoxin" (19). During the same period, Plugge probably isolated the same compound from several species of Andromedan and other members of the Ericaceae family. Plugge named this compound "Andromedotoxin". The first study on the pharmacological properties of Andromedotoxin was also done by Zayer and Plugge (6). Later, Archangelky (1901) (20), Hardikar (1922) (18), Makino (1927) (21), and Chu (1931) (22) researched the subject.

Plugge studied frogs and rabbits and reported respiratory depression, subsequent convulsions, and cardiac arrest (6). In addition to the findings reported by Pluggen, Archangelsky reported that curare-type drugs stopped the hearts of frogs in systole, similar to the effect of digitalis on the motor nerves and the cardiac muscle (20). Hardikar conducted a more comprehensive systematic review of the effects of andromedotoxin and detected respiratory depression, bradycardia, hypotension, cardio acceleration in mammals, and cardiotoxic

effects vomiting, and paralysis in skeletal muscles in frogs and rabbits (18). Chu confirmed Hardikar's findings (22). Makino worked on a compound obtained from the *Rhododendrons* and named rhodotoxin. The results were similar to those of Hardikar with Andromedotoxin (21).

There is a consensus in the literature that Andromeda, Rhodendron, Kalmia species contain active physiological compounds. However, there are differences in the definition of the isolated active agents. Moran et al. (1954) researched andromedotoxin, a nitrogen-free, neutral, crystalline substance obtained from Rhodendron maximum. In these studies, bradycardia, hypotension, and respiratory depression developed as a result of intravenous infusion of andromedotoxin. Moran et al. reported that atropine is effective in the improvement of bradycardia and hypotension (23).

Moran et al. found that Andromedotoxin, a nitrogen-free, crystalline substance obtained from the leaves of *Rhododendron maximum*, has complex cardiovascular effects quite similar to those of some veratrum alkaloids. The research results revealed that the most significant cardiovascular effect was Vaso depression, and the hypotensive state contained at least two components. First, reflex reduction in blood pressure and heart rate, which can be eliminated by cervical vagotomy; the second is a gradual, longer-lasting depressor effect that occurs at a dose slightly higher than the first component, associated with blockade of the carotid sinus depressant reflex in vagotomy-treated animals. In the study of Moran et al., the effect of andromedotoxin on the carotid sinus was investigated in vagotomy dogs. The main results obtained are listed as follows (24):

1. "The IV dose of Grayanotoxin (8-20 micrograms) that causes hypotension in dogs undergoing vagotomy does not cause any response or produces a pressor effect after denervation of the bilateral carotid sinus area.
2. In dogs undergoing vagotomy with unilateral carotid sinus denervation, injection of andromedotoxin in minute doses (0,05-0,1 micrograms) into the carotid sinus adventitia layer on the side with ongoing innervation causes a permanent depressing effect with partial or complete blockade of the carotid pressor reflex. Injections of equal volumes (0,1 ml) of saline administered as a control are ineffective. Rapid denervation of the carotid sinus in the hypotensive phase causes an abrupt rise in blood pressure to the hypertensive level, and subsequent administration of andromedotoxin by IV or carotid sinus injection is insufficient to reduce blood pressure.
3. In two dogs with bilateral N. vagus incision but no operation applied to the carotid artery area, andromedotoxin administered IV or to the carotid sinus adventitia layer did not cause a depressor response. Thus, in this second step of the experiment, it appears to play no role in the hypotensive action.

4. Neogermitrine and protoveratrine also exhibited the same type of response, which was very similar to that between andromedotoxin and these veratrum alkaloids.
5. It was concluded that the hypotensive effect of Andromedotoxin is purely reflexive. No vasodepressor effect was observed under central control" (24).

In a study by Maling and Moran (1956), in dogs with bilateral N. vagus and N. carotid sinus incisions, 10 to 40 microgram/kg doses of Andromedotoxin caused a significant increase in cardiac contractility, blood pressure, and heart rate, while circulatory shown to have opposite effects in dogs with intact reflexes. The cardiovascular stimulatory effect can be largely eliminated by cervical spinal cord block in dogs undergoing nerve incision, but it is not significantly reduced by adrenalectomy alone or thoracic sympathectomy alone (25).

In a study by Tallent et al., a procedure was developed for the detection of acetylandromedol in plant extracts, using borate buffer solutions and paper electrophoresis. Positive results were found in only some but not all species of *Kalmia, Leucothoe, Lyonia, Pernettya, Pieris,* and *Rhododendron*. Especially *Caroliniana*, a *Kalmia Angustifolia* species, was found to be a good source of acetyl andromeda. Evidence from this study indicates that the empirical formula for Andromedol is $C_{20}H_{34}O_6$ and Acetylandromedol, Grayanotoxin I and Rhodoxin are identical and have the formula $C_{22}H_{36}O_7$ (26).

Most of the nectars that caused honey poisoning in history were collected from the Ericaceae family. *Rhododendron*, azalea, and andromeda (rosemary) plants are particularly significant in that they carry poisonous nectar. Some plants whose nectar contains poison have also been reported to be toxic to bees, and *Rhododendron* species have been suspected in some cases. The data on a major epidemic case in which bees were poisoned by *Rhododendron* on Colonsay Island in Scotland are noteworthy. This outbreak led Carey et al. (1959) to study the properties of the poisonous substance and to discover from which *Rhododendron* it was produced. Carey et al. reported the effects of nectar obtained from some *Rhododendron* species on animals in their study as follows: "The bees became sluggish, some of them struggled to fly for a short time, and from time to time they fell on the floor of the cage. When they fell, they lay on their side or on their back, their stomachs lifted as if they were supported by their wings. Ataxia and disorientation were seen. Instead of climbing up, as they always do, bees tend downwards. When removed from the cage, they typically spun around and fluttered their wings quickly, but were unable to fly. Increasing weakness and extreme fatigue followed by death, often with tongue sticking out." (27)

Carey et al. discussed their findings in the light of the literature of that period as follows: "Research has shown that some *Rhododendron* nectars are

toxic to bees, cats, and mice. Especially R. thomsonii, some hybrids and R. arboreum, album, and R. Pratti are poisonous. Isolation and identification of Acetylandromedol (andromedotoxin) from R. Thomsonii nectar; The fact that toxic nectars and acetylandromedol show similar effects when tested pharmacologically indicates that the poisonous substance is acetylandromedol. However, it is not possible to predict whether a particular *Rhododendron* may secrete toxic nectar. For example, nectar secreted by the R. Redwing hybrid obtained from four species, three of which are poisonous, was found to be non-toxic" (27).

Grayanotoxins are known to be found only in the Ericaceae family. However, many members of the Ericaceae family that grow in the Pacific Northwest and are said to be poisonous were never investigated whether to contain these compounds. To explore whether it is possible to identify and detect Grayanotoxins in some plant species and thus confirm the compound responsible, at least in part, for the reported toxicity, Constantine et al. screened seven species of Ericaceae plants reported to be poisonous by thin-layer chromatography to detect the presence of Grayanotoxins. While Grayanotoxin-I was found in five of the scanned plants, Grayanotoxin-II and Grayanotoxin-III were not found (28).

In the study of Scott et al., a poisonous honey sample from Grouse Mountain in British Columbia (Canada) was examined and found to contain Grayanotoxin II and Grayanotoxin III. It was stated that these compounds are closely related to Grayanotoxin I (andromedotoxin) and desacetyl pieristoxin B. Analysis was done by thin-layer chromatography and mouse bioassay (29).

In the study of Narahashi and Seyama, the depolarization mechanism caused by Grayanotoxin-I on cuttlefish axon membranes was investigated with internal perfusion and voltage-clamp techniques. Some of the findings obtained in the study are listed below.

1. No depolarization was observed in the absence of sodium ions in both the external and internal environment, indicating that the depolarization of Grayanotoxin-I in the normal environment is due to a specific increase in resting sodium permeability.
2. The resting sodium permeability as measured by the voltage-clamp technique through internal/intracellular administration of 1 x 10-5 m of Grayanotoxin-I increased to approximately 1.39 × 10-6 cm/sec. There has been an increase of approximately 90 times.
3. Tetrodotoxin counteracted the resting sodium permeability-increasing effect of Grayanotoxin-I non-competitively (30).

Brown et al., suggested that, theoretically, the cause of Grayanotoxin-induced arrhythmias is the induction of automatic activity as a result of increasing the amount of sodium leaking into the cell and accelerating phase-4 depolarization, or induction of trigger activity due to an increase in sodium current associated with TI current and formation of OAPs (Optical Action Potentials). To separate these possibilities, Brown et al. examined the electrophysiological effects of another biologically active Grayanotoxin, Grayanotoxin-III, on cat-isolated Purkinje fibers under a variety of conditions known to increase or block the production of OAPs. As a result, it was revealed that the underlying mechanism of Grayanotoxin-induced arrhythmias is the production of activity that starts as vibration after tension (31).

In the study of Zushi et al., it was concluded that the effect of Grayanotoxins on the neuromuscular junction is probably due to an increase in the membrane permeability of sodium and depolarization of both presynaptic and postsynaptic membranes (32).

Seyama et al. pointed out that GTX's exhibit various pharmacological effects such as extensive transmitter release at the neuromuscular junction in frog, positive inotropic effect on the myocardium, and negative chronotropic effect in SA node cells, and indicated that the findings were a potential candidate for a pharmacological tool to study the Na channel of GTX (33).

According to Seyama et al., since the increase in Na permeability induced by GTX is due to the modification of the Na channel, it is conceivable that the peptide bonds in the Na channel protein critical for GTX binding may be cleaved by some proteases so GTX cannot depolarize the membrane. Therefore, Seyama et al. designed an experiment to obtain information about the molecular structure of the Na channel by associating the enzyme-substrate specificity with the loss of the depolarization response to GTX. They obtained data revealing that the effect region of the GTX is not limited to the channel transition and that there is a part of the Na channel that has both voltage sensor and ion filter functions. Seyama et al. showed that Grayanotoxin derivatives obtained from the leaves of the Ericaceae family particularly increase the permeability of the sodium-sensitive inducible membranes (33).

4. Mad Honey Related Animal Poisoning in Recent History

Rhododendron species plants have been recorded to cause animal poisoning in the form of a series of rural epidemics as well as honey poisoning. In toxicological studies on animals, the rumen content was generally examined. Postmortem examinations of sheep and goats poisoned by *Rhododendron* showed that despite the emetic effect of the toxic compound, sufficient andromedotoxin-containing content remained in the rumen for examination. If rumen contents or vomit

were not present, liver, or intestinal contents were found suitable for chemical examination, although they usually contain low levels of andromedotoxin (34).

If a plant carries Grayanotoxins, the whole plant, namely leaves, twigs and pollen grains, contains Grayanotoxins. Animal poisoning is also caused by this situation. It is worth noting that not all *Rhododendron* species produce toxic nectar, but it is difficult to predict which one is causing the problem and which one can be safely consumed (35).

The article written by Kenneth F. Lampe in 1988 summarizes the information on mad honey poisoning. It is stated in the article that the compounds responsible for mad honey poisoning are Grayanotoxins. These compounds are disclosed as nitrogen-free terpenes, polyhydroxy cyclic hydrocarbons. Grayanotoxins vary according to the plant species, and Grayanotoxin-I, which was known as andromedotoxin, acetylandromedol, and rhodotoxin in earlier years, is the most common GTX type (36).

The article provides the following information about the mechanism of action of Grayanotoxins: "Grayanotoxins bind to the receptor II site in a region of the sodium channel that participates in voltage-dependent activation and inactivation of the sodium channel in the cell membranes. These compounds prevent inactivation; Thus, excitable cells (nerve and muscle cells) remain in a state of depolarization, and during this time calcium entry into the cells can be facilitated. All observed responses of the skeletal and myocardial muscles, nerves, and central nervous system are associated with membrane effects." (36).

The article also addresses the findings and treatment of mad honey poisoning, and provides the following information: "In humans, symptoms of poisoning occur after a latency period lasting from a few minutes to two hours or more, depending on the dose, and this period includes drooling, vomiting, and extremity paresthesias. Significant hypotension and sinus bradycardia develop. In severe poisoning, loss of coordination and progressive muscle weakness occur. Extra systoles and ventricular tachycardia can occur with both atrioventricular and intraventricular conduction disturbances. Convulsions related to honey poisoning have also been reported." (36).

"Poisoning is rarely fatal and usually lasts no more than 24 hours. No intervention may be required. Severe hypotension usually responds to fluid administration and correction of bradycardia. Vasopressor therapy is rarely necessary. Sinus bradycardia and conduction defect usually respond to atropine therapy; However, it may be necessary to place a temporary pacemaker" (36).

Animal poisonings caused by grayanotoxin have also recently been encountered in Canada. The "Heath Family Ericaceae" shrubs growing in Canada produce neurotoxic diterpenoids with a variety of conditions, such as andromedotoxin and grayanotoxin. These toxins are found throughout the plant, including the nectar, and are toxic to bees. Signs of poisoning in animals

are vomiting, general depression, hypotension, irregular heartbeat, colic, convulsions, inability to coordinate voluntary muscles. The leaves of most of these species are very thick or bitter, and their flavor is known to be quite low. Records show that cattle rarely graze these plants unless other forages are scarce, so although most animal species such as cattle, horses, llamas, and goats are susceptible to andromedotoxin and grayanotoxin poisoning, cases of poisoning are rare. Sheep are the animals most affected. Animals should be removed from areas with these plants. Although no specific antidote is known, subcutaneous injection of morphine has been reported to be used successfully in goats (37).

Table 1. Demographic, Clinical Findings of
Mad Honey Poisoning Cases in Last Three Decade

Ref. no	**Country where honey is produced**	**Number of case**	**Sex /age**	**Findings/symptom-ps**
38	Nepal	4		Bradycardia, Chest pain, Dizziness, Fainting
39	Turkey	2	(27)	Hypotension Sinus bradycardia, AV block, Syncope, Nausea, Vomiting
			(34)	Hypotension Nodal rhythm, Syncope, Dizziness
40	N. America	9	Male (3) Female (6) Age:10 months-9 years	Vomiting, Diarrhea, Palpitations
41	Eastern Black Sea Region/ Turkey	16	2 Female 14 Male (Mean age 41)	Hypotension, Sinus bradycardia, Nodal hythm, Wolff-Parkinson White syndrome, Complete AV Block Syncope, Dizziness, Nausea Vomiting

Ref. no	Country	Number of case	Sex /age	Findings/symptom-ps
42	Turkey	29		Chest pain, Complete AV block, Sinus bradycardia, Nodal rhythm, Blurred vision, Dizziness Headache, Weakness, Syncope, Nausea, Vomiting
43	Black Sea Regi-on/Turkey	23	21 Male 2 Female (7 month-61 year)	Sinus bradycardia, Complete AV block, Wolff Parkinson White syndrome, Impaired consciousness Dizziness, Nausea, Vomiting
44	Turkey	1	Male (49)	Sinus bradycardia, Dizziness
45	Turkey	1	Male (60)	Hypotension Brady-cardia, AV block
46	Nepal Brazil	2	Male (51)	Bradicardia, Hypo-tension, Syncope
			Male (61)	Bradicardia, Hypo-tension, Syncope, Weakness
47	Turkey	1	Male (8)	Sinus bradycardia Impaired consciousness, Dizziness Seizure, Weakness, Nausea, Vomiting
48	Turkey	1	Male (42)	Sinus bradycardia, Syncope, Dizziness, Nausea, Vomiting

Ref. no	Country	Number of case	Sex /age	Findings/symptoms
49	Black Sea Region/ Turkey	1	Male (58)	Complete AV block, Nodal rhythm, Dizziness, Presyncope, Nausea
50	Black Sea Region/Turkey	1	Male (28)	Sinus bradycardia, Numbness
51	Eastern Black Sea Region/ Turkey	1	Male (56)	Bradycardia, Hypotension, Complete AV block, Presyncope
52	Réunion Island	1	Female (28)	Complete AV block, ST elevation, Asthenia, Nausea, Vomiting
53	Korea	3		Bradycardia Hypotension, Nodal rhythm, Syncope
54	Eastern Black Sea Region/ Turkey	8	2 Male 6 Female (Mean age 58.8)	Bradycardia Hypotension, Complete AV block, Nodal rhythm, Nausea, Vomiting
55	Western Black Sea Region/ Turkey (Kastamonu)	66	53 Male 13Female (Mean age:51.9)	Sinus bradycardia, Dizziness, Syncope, Weakness, Nausea, Vomiting
56	Korea (*poisoning from drinking Rhododendron liquor*)	2	Male (50)	Bradycardia, Hypotension, Dizziness, Nausea, Vomiting
			Male (20)	Bradycardia, Hypotension, Dizziness
57	Turkey	1	Male (56)	Complete AV block, Presyncope

Ref. no	Country	Number of case	Sex /age	Findings/symptom-ps
58	Eastern Black Sea Region/ Turkey	2	Male (48) Male (74)	Weakness, Dizziness, Faintness, bradycardia, Hypotension, Sweating
59	Black Sea Region/Turkey	1	Male (46)	Dizziness, Presyncope, Nausea
60	Korea *(The patient had ingested about 10 azalea blossoms)*	1	Male (76)	Bradycardia, Hypotension, Drowsiness, Dizziness
61	Black Sea Region	1	Male (54)	Sinus bradycardia, Unconsciousness
62	Turkey	1	Male (64)	Hypotension, Sinüs bradycardia, AV block Chest pain, Vertigo, Nausea, Vomiting
63	Black Sea Region/ Turkey	1	Male (66)	Sinus bradycardia, Hypotension, ST elevation Chest pain, Dizziness
64	Korea	1	Male (67)	Chest pain, Junctional rhythm, Weakness, Dizziness
65	Western Black Sea Region/ Turkey	1	Male (29)	Sinus bradycardia, Dizziness, Nausea, Vomiting
66	Hong Kong	1	Male (57 days)	Bradycardia, Unresponsiveness, Twitch, Hypotension, Nausea, Vomiting

Ref. no	Country	Number of case	Sex /age	Findings/symptom-ps
67	Turkey	1	Male (54)	Nodal rhythm, Hypotension, Syncope, Dizziness
68	Turkey	1	Male (41)	ST segment depression, Chest pain, Acute non–ST-segment elevation myocardial, infarction
69	Turkey	1	Male (56)	Hypotension, Chest pain, Complete, AV block, Somnolence, Nausea, Vomiting
70	Reunion island	7	6 Male 1 Female (Mean age: 40.4)	Hypotension, Bradycardia, Chest pain, Confusion Dizziness, Nausea, Vomiting, Diarrhea
71	Eastern Black Sea Region/ Turkey	5	Female (17)	Sinus Bradycardia, type II second degree AV block Dizziness, Presyncope
			Female (65)	Sinus Bradycardia, type I second degree AV block, Dizziness, presyncope
			Male (34)	Sinus Bradycardia, type I second degree AV block Dizziness, presyncope
			Male (37)	Sinus Bradycardia, type I second degree AV block Dizziness, presyncope
			Male (21)	AV Block, Dizziness, presyncope

Ref. no	Country	Number of case	Sex /age	Findings/symptoms
72	West Black Sea Region/ Turkey (Zonguldak)	1	Male (48)	Complete AV block, Bradycardia, Hypotension Dizziness, Syncope, Weakness
73	Black Sea Region/ Turkey	1	Male (43)	Chest pain, Nausea, Vomiting
74	Turkey	21	18 Male 3Female (Mean age: 55)	Chest pain, junctional nodal rhythm, Left bundle branch block, Atrial fibrillation Dizziness Syncope Blurred vision, Weakness, Nausea, Vomiting
75	Nepal	1	Male (56)	Sinus bradycardia, Chest pain, Weakness, Dizziness Nausea
76	Black Sea Region/Turkey	47	40 Male 7 Female (Mean age 56.3)	Sinus bradycardia, Nodal rhythm, Complete AV block
77	Nepal	7	Male	Palpitations, Hypotension, Unconsciousness Nausea, Vomiting

Ref. no	Country	Number of case	Sex /age	Findings/symptom-ps
78	Nepal	7	Male (25)	Weakness, Diplopia, Blackouts, Pupils were bilaterally dilated and sluggishly reacting to light Nausea, Vomiting, Burning sensation in the throat
			Male (24)	Blackouts, Dizziness, Headache, Unconsciousness Pupils were bilaterally, dilated and reacting sluggishly to light Burning sensation in the throat Nausea, Vomiting
			Male (24)	Blackouts, Diplopia, Blurring of vision, Unconsciousness
			Male (22)	Dizziness, Blurred vision
			Male (22)	Nausea, Dizziness, Blurred vision
			Male (23)	Dizziness, Blurred vision, Diplopia, Pupils were bilaterally dilated and sluggishly reacting to light Vomiting
			Male (24)	Dizziness, Blurred vision, Diplopia, Vomiting

Ref. no	Country	Number of case	Sex /age	Findings/symptom-ps
79	Korea	1	Male (58)	Sinus bradycardia, Hypotension, Change in consciousness Nausea, Vomiting
80	Turkey	1	(56)	Hypotension Sinus bradycardia, Syncope, Nausea Vomiting
81	Eastern Black Sea Region/ Turkey (Rize)	33	30 Male 3 Female (age 42-68 years)	Sinus bradycardia, Dizziness, Confusion, Blurred vision, Syncope, Nausea, Vomiting
82	Black Sea Regi-on/Turkey	1	Male (70)	Second degree heart block, Brief loss vision Syncope, Nausea Vomiting
83	Turkey	2	Female (73)	Hypotension, Sinus bradycardia, Dizziness Nausea, Epigastric discomfort
			Male (74)	Hypotension, Brad-ycardia, Dizziness, Headache
84	West Black Sea Region/ Turkey (Zonguldak)	72		Sinus bradycardia, complete AV block
85	Eastern Black Sea Region/ Turkey (Rize)	1	Male (60)	Mobitz type 1 block Dizziness

Ref. no	Country	Number of case	Sex /age	Findings/symptom-ps
86	Western Black Sea region/ Turkey (Kastamonu)	42	33 Male 9 Female (Mean age 48.5)	Sinus bradycardia, Complete AV block, Nodal rhythm, Dizziness, Syncope, Seuzire, Nausea Vomiting
87	Turkey	1	Male (50)	Palpitations, Junctional rhythm Dizziness, Presyncope, Blurred vision
88	Turkey	1	Male (46)	Atrial fibrillation, Wolf Parkinson White syndrome, Palpitations, Tachycardia, Dizziness
89	Turkey	21	13 Male 8 Female (Median age 55)	Bradycardia, Nodal rhythm, Atrial fibrillation Dizziness, Weakness, Nausea, Vomiting
90	Turkey	2	Male (79)	Complete AV block, Dizziness, Nausea, Vomiting
			Male (55)	Nodal rhythm, Dizziness, Syncope, Nausea, Vomiting
91	Turkey	1	Male (70)	Paroksismal Atrial fibrillation intermittent left branch block, Confusion, Weakness, Nausea, Vomiting
92	Turkey	1	Female (87)	Sinus bradycardia, Left branch block, Long QT interval Syncope

Ref. no	Country	Number of case	Sex /age	Findings/symptoms
93	Turkey	2	Male (50)	ST elevation, Complete AV block, Chest pain
			Female (42)	ST elevation Nodal rhythm, Chest pain
94	Black Sea Region/Turkey	1	Female (63)	Chest pain, Sinus bradycardia, Dizziness, Nausea
95	Eastern Black Sea Region/ Turkey (Trabzon)	1	Female (52)	Sinus bradycardia, Hypotension, Atrial fibrilation, AV complete block
96	Eastern Black Sea Region/ Turkey	30	24 Male 6 Female (Mean age 57)	Sinus bradycardia, Nodal rhythm, Complete AV block, Atrial fibrillation, Sinus arrest, Dizziness Syncope, Nausea Vomiting
97	Black Sea Region/ Turkey	1	Male (79)	Hypotension, Bradycardia, Nodal Rhythm, Dizziness, Syncope, Vomiting
98	Eastern Black Sea Region/ Turkey	1	Male (36)	Bradycardia, Atrial fibrillation, Dizziness, Syncope
99	Turkey	1	Male (46)	Hypotension, Bradycardia, Dizziness, Blurred vision, Fainting
100	Turkey	1	Female (55)	Complete AV block, Bradycardia, Hypotension, Dizziness, Syncope, Nausea

Ref. no	Country	Number of case	Sex /age	Findings/symptom-ps
101	Western Black Sea Region/ (Turkey) Zonguldak	37	31 Male (Mean age:56.17) 6 Female (Mean age 69.66)	Hypotension, Bradycardia, Complete AV block Chest pain, Dizziness, Syncope, Nausea, Vomiting
102)	Black Sea Region/Turkey	1	Male (53)	Atrial fibrillation, Weakness, Nausea Vomiting
103	Eastern Black Sea region/Turkey	1	Female (45)	Complete AV block, Dizziness, Asthenia, Nausea, Epigastric pain
104	East Black Sea region/Turkey	1	Male (54)	Chest pain, Atrial fibrillation, Dizziness, Nausea Vomiting
105	Western Black Sea Region/ Turkey (Zonguldak)	1	Male (76)	Chest pain, 1. degree AV block, ST elevation Dizziness, Nausea, Vomiting
106	Turkey	1	Female (51)	Bradycardia, HypotensionNodal rhythm, Dizziness, Nausea, Vomiting
107	Turkey	5	Mean age: 40.8	Vertigo, Presyncope, Nausea, Vomiting
108	Turkey	1	Male (74)	Sinus bradycardia Hypotension, ST elevation Chest pain, Dizziness
109	Himalaya	1	Male (48)	Sinus bradycardia ST elevation, Dizziness, Blurred vision

Ref. no	Country	Number of case	Sex /age	Findings/symptoms
110	Turkey	38	30 Male 8 Female (averageage:51)	Chest pain, Dizziness, Unconsciousness, Nausea, Vomiting
111	Western Black Sea Region/ Turkey (Zonguldak)	1	Male (21)	Bradycardia, Hypotension, Dizziness, Nausea
112	Eastern Black Sea Region/ Turkey (Bayburt)	1	Male (24)	Sinus bradycardia, Dizziness, Nausea, Vomiting
113	Black Sea Region/Turkey	1	Male (35)	Sinus bradycardia, Dizziness, Numbness
114	Turkey	1	Female (39)	Bradycardia, Acute inferoposterolateral myocardial infarction,. Presyncope
115	Turkey	1	Male (15)	Dizziness
116	Eastern Black Sea Region	46	36 Male (Mean age: 50.2) 10Female (Mean age 59.4)	Complete AV block, Syncope, Unconsciousness
117	Black Sea Region/Turkey	1	Male (58)	Sinus bradycardia, Negative T Wave, Dizziness, Blurred vision, Nausea, Vomiting

Ref. no	Country	Number of case	Sex /age	Findings/symptoms
118	Eastern Black Sea Region/ Turkey	4	Male (38)	Dizziness, Blurred vision, Confusion, Nausea Vomiting, Abdominal pain
			Female (35)	Dizziness, Nausea, Vomiting
			Female (10)	Dizziness, Nausea
			Female (5)	Nausea
119	Turkey	2	Female (48)	Sinus bradycardia, Presyncope, Nausea, Vomiting
			Male (55)	Sinus bradycardia, Presyncope, Headache, Nausea
120	Turkey	1	Male (64)	Sinus bradycardia, Hypotension, Dizziness, Syncope Nausea, Vomiting
121	West Black Sea Region/ Turkey (Akçakoca)	1	Male (70)	Hypotension, Complete AV block, Dizziness, Presyncope, Weakness
122	Eastern Black Sea Region/ Turkey	1	Male (67)	Hypotension, Bradycardia, Unconsciousness, Weakness, Nausea, Vomiting
123	Turkey	1	Male (65)	Biphasic T wave, Dizziness, Somnolence

Ref. no	Country	Number of case	Sex /age	Findings/symptoms
124	Turkey	1	Female (66)	Sinus bradycardia, Dizziness, Double vision, Perioral Numbness, Paresis, Disorientation, Nausea, Vomiting
125	Western Black Sea region/Turkey	3	Male (65)	Sinus bradycardia, Dizziness
			Male (42)	Sinus bradycardia, Syncope
			Male (40)	Sinus bradycardia, Fainting, presyncope, Weakness
126	Black Sea Region/Turkey	1	Male (55)	Sinus bradycardia, Unconsciousness, Weakness, Nausea, Vomiting
127	Turkey	16	10 Male 6 Female (Mean age: 58.5)	Sinus bradycardia, nodal rhythm, first degree AV block, Atrial fibrillation Impaired consciousness, Dizziness, Syncope, Vision loss, Nausea Vomiting
128	Nepal/Himalaya	15	10 Male 5 Female (Mean age:52.2)	Sinus bradycardia, Junctional bradycardia complete AV block, Atrial fibrillation
129	Turkey	1	Male (52)	Hypotension, Sinus bradycardia, Syncope, Vomiting
130	Turkey	1	Female (89)	Sinus bradycardia, Weakness, Nausea

Ref. no	Country	Number of case	Sex /age	Findings/symptoms
131	East Black Sea region/Turkey	4	Male (67)	Hypotension, Bradycardia, Dizziness, Nausea
			Male (51)	Hypotension, Bradycardia, Dizziness, Nausea
			Male (70)	Hypotension, Bradycardia, Dizziness, Nausea
			Female (40)	Hypotension, Bradycardia, Dizziness, Nausea
132	Eastern Black Sea region/ Turkey (Rize)	82	66 Male 16 Female (Mean age: 53)	Sinus bradycardia, 1st degree AV block, Nodal rhythm, Preexcitation, Atrial fibrillation, Chest pain Dizziness, Vertigo, Blurred vision, Change in, consciousness, Nausea, Vomiting
133	Turkey	1	Female (55)	Chest pain, Sinus bradycardia, Myocardial infarction Weakness, Dizziness, Nausea
134	Black Sea Region/Turkey	1	Female (18) (Pregnant)	Hypotension, Bradycardia AV Block
135	Turkey (Erzurum)	1	Male (19)	Dizziness, Confusion, Nausea, Vomiting
136	Japoya	1	Male (61)	Sinus bradycardia, Dizziness, Nausea, Abdominal discomfort

Ref. no	Country	Number of case	Sex /age	Findings/symptoms
137	Eastern Black Sea Region/ Trabzon	3	Male (66)	Sinus bradycardia, Dizziness, Nausea, Vomiting
			Male (57)	Sinus bradycardia, Loss of consciousness, Headache
			Female (79)	Sinus bradycardia, Dizziness, Nausea, Vomiting
138	Nepal	2	Male (43)	Bradycardia, Syncope, Hypotension, Mobitz type I heart block
			Male (44)	Bradycardia, Hypotension, Junctional bradycardia
139	Eastern Black Sea Region/ Turkey	1	Male (58)	Sinus bradycardia, Hypotension, Chest pain, ST elevation, Nausea
140	Turkey	1	Male (16)	Sinus bradycardia, Confusion, Nausea, Abdominal pain
141	Korea (*poisoning from drinking Rhododendron liquor*)	6	Male (54)	Impaired mental status
			Male (47)	Visual disturbance
			Male (48)	Visual disturbance
			Male (48)	Chest pain, Dizziness
			Female (47)	Dizziness
			Female (34)	Visual disturbance, Nausea, Abdominal pain

Ref. no	Country	Number of case	Sex /age	Findings/symptoms
142	Sungdel and Taplejung regions/ Nepal	2	Female (53)	Sinus bradycardia, Hypotension, Dizziness Nausea, Vomiting
			Female (67)	Sinus bradycardia, Dizziness, Vomiting
143	The case from Germany; honey from Turkey	1	Female (age:46)	Hypotension, Bradycardia, ST elevation, Dizziness Syncope, Nausea
144	Western Black Sea Region/ Turkey (Düzce)	36	Male (27) Female (9) Mean age: 58.1	Anterior MI, Sinus bradycardia, Ventricular, Tachycardia, Dizziness, Fatigue, Nausea, Vomiting Throat Burns, Abdominal pain, Exhaustion
145	Western Black Sea Region/ Turkey (Zonguldak)	38	Male (23) Female (15) Mean age: 55.4	Hypotension, Sinus Bradycardia, Nodal Rhythm Complete AV Block, Slow Ventricular Response AF Chest pain
146	Eastern Black Sea Region/ Turkey	1	Male (60)	AV block, wolff parkinson white syndrome, Dizziness, Asthenia
147	Eastern Black Sea Region/ Turkey (Trabzon)	25	18 Male 7 Female (mean age:56)	Dizziness, Syncope, Nausea

Ref. no	Country	Number of case	Sex /age	Findings/symptoms
148	US/ Pennsylvania (*poisoning from eating Pieris japonica)*	1	Female (2)	Lethargy, Nausea, Vomiting
149	Nepal	1	Female (age:52)	Hypotension, Bradycardia, first-degree atrioventricular block, burning sensation in the chest, Blurred vision, numbness
150	Western Black Sea Region/ Turkey (Düzce)	3	Male (1) Female (2) Mean age: 9.5	Hypotension, Sinus Bradycardia, Dizziness, Blurred vision, Vomiting, Abdominal pain
		1	Male Age:16	Hypotension, Sinus Bradycardia, Dizziness, Syncope, Blurred vision
151	The case from France; honey from Nepal	1	Female (age:32)	Hypotension, Bradycardia, ST elevation, Dizziness perioral paraesthesia Nausea, Vomiting

As seen in Table 1, there has been a rapid increase in the reporting of mad honey poisoning cases in the last three decades. The clinical and demographic characteristics of the cases reported during this period are given in Table 1. The most common symptoms in mad honey poisoning cases published in this period were hypotension and bradycardia, cardiac conduction disorders, dizziness, blurred vision, sweating, nausea, and vomiting. In addition, the rate of men in mad honey poisoning cases is higher than women. Most of the cases were detected in Turkey, in the Black Sea Region. However, cases of poisoning have also been reported in Nepal, Korea, the United States, Japan, Hong Kong, and the islands of Réunion. The spread of cases across the world seems to be related to the fact that *Rhododendron* species, which are known to be the most important source of mad honey poisoning, have a very wide distribution area throughout

the world. However, the reasons for the remarkable high number of cases in Turkey is an issue that needs to be investigated. Consciously or unconsciously consumed honey as an alternative treatment method in Turkey may be a reason for the high incidence rates in Turkey.

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CHAPTER XII

THE JOURNEY OF NUCLEOTIDES: DNA SEQUENCING

Abdussamed Yasin Demir
(Asst. Prof. PhD), Erzincan Binali Yıldırım University, Faculty of Medicine
Department of Medical Genetics, Erzincan, Turkey
e-mail: ayasindemirr@gmail.com
Orcid: 0000-0002-0420-5017

1. Introduction

DNA sequence analysis; it refers to the detection of nucleotide sequences, which are the primary (basic) structures of DNA. In this respect, DNA sequence analysis is the most important factor that determines the path to be followed at the beginning and the continuation of the treatment process. In order to understand the mechanisms related to the occurrence and treatment of hereditary diseases, the relevant gene regions should be determined (1).

Today, it is known that many diseases are caused by certain mutations that are common in society. Changes in the bases of a living thing's DNA for various reasons are called mutations. These changes can be in the form of a single base being replaced by another base (point mutation), or it can be in the form of deletion or addition of one or more bases. Some mutations do not directly cause a disease, but only increase the susceptibility to the disease. In the studies carried out in line with this information, it is aimed to find the genes that are effective in diseases and the mutations on these genes. For this reason, the determination of genes and mutations related to diseases is a guide for both diagnosis of the disease and understanding of the mechanism of the disease and developing treatment methods (2,3). The development of faster and cheaper DNA sequencing methods has led to the realization of projects to reveal the sequencing of the entire genome of many living species. With DNA sequence analysis, important information about the structure and organization of the genes of many organisms has been obtained and whole genome maps of many living species have been defined. The most important of these is the Human Genome Project (HGP) which started in 1990 and was completed in 2003. Genome projects enable the determination of the genes carried by the living thing by studies on the obtained sequences. HGP; provided an understanding of many

genes, various mutations and their relationship with many diseases (4,5). As the limit of what can be done with genetic information expands, technologies that enable access to this information become more important. Although the DNA sequence of the genomes of many living things, including humans, has been determined with existing technologies it is clear that much faster and cheaper technologies will be needed in the future.

Various DNA sequencing methods have been used since the 1970s. It is clear that different methods will emerge with the rapidly developing technologies. In this chapter; first of all, the structure of DNA and the polymerase chain reaction, which is a breakthrough in molecular genetic studies, are briefly mentioned. Then, traditional and new generation DNA sequencing methods, which are frequently used in routine applications, are reviewed.

2. DNA Structure

The DNA molecule, like many organic molecules in our body, is in a polymer structure. The structure formed by adding the same or similar building block molecules to each other is called a polymer. The building blocks that make up DNA are also called nucleotides. Although the nucleotides that make up DNA have a basically similar structure, they consist of four types of nucleotides (A/adenine, T/thymine, G/guanine and C/cytosine) with minor chemical differences (6).

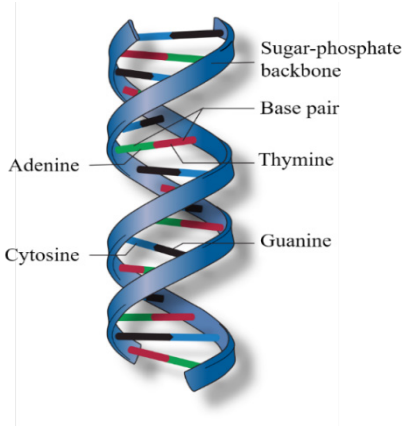

Figure 1: The DNA double helix as proposed by Watson and Crick (7)

Decoding DNA means accurately determining the type and order of bases (A, T, C, G) that make up a DNA molecule. The DNA molecule is formed by the coiling of two chains (double helix) formed by the sequence of bases. These two chains stay together thanks to the interaction between the bases, so that the bases on the two chains are positioned opposite each other. This reciprocal positioning of the bases in the DNA double helix is A to T, C to G. That is, the two DNA

strands that make up the double helix have sequences that complement each other (6) (Figure 1).

3. Polymerase Chain Reaction (PCR)

Before the polymerase chain reaction (PCR) was found, copies of the DNA fragments to be sequenced were made by the bacterial cell. First, the DNA fragment to be sequenced was attached to another ring-shaped piece of DNA called a vector and transferred to the bacterial cell. As the bacterial cell reproduces, it makes a copy of this vector, as well as a copy of its own DNA. Therefore, with each division, the number of vector copies increases in parallel with the increasing number of bacteria. The vector carrying the DNA to be sequenced is then isolated from the bacteria and the sequence of the DNA is determined. This laborious and long-term process can be performed in a few hours with the use of PCR (8).

PCR is a technique for enzymatically replicating a specific region between two segments of known sequence in DNA, outside the cell. This method is simply; it is based on the principle of multiplying nucleic acids in a tube under suitable conditions. PCR, also called a kind of in vitro cloning; it consists of a certain number of iterations of the cycle consisting of denaturation at 92-95 °C, annealing at 45-65 °C, and elongation at 72 °C (9) (Figure 2).

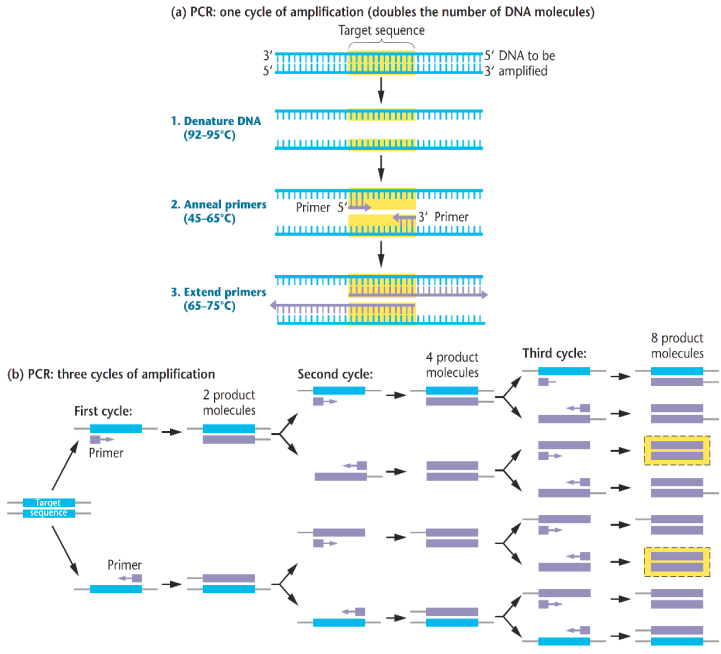

Figure 2: Steps in the polymerase chain reaction (PCR) (10)

Some basic components such as template DNA, polymerases, primers, nucleotides and buffer are needed for the PCR process to take place. The template DNA carries the DNA region to be replicated and acts as the master template for this region. DNA polymerase enzymes required for experimental studies are obtained from various microorganisms. Polymerases catalyze the synthesis of polynucleotide chains from four types of nucleotides, using the base information in the original template strand to form a DNA strand complementary to the template strand. Primers, which are precursor sequences, are pre-synthesized to be specific to the DNA sequence to be amplified. Primers are synthetic oligonucleotides that define the region to be replicated on the template DNA and bind to the complementary sequence in the template molecule to initiate synthesis. Nucleotides and buffer are commercially available. Nucleotides provide the energy and nucleotide source for the polymerization reaction. The buffer provides the appropriate conditions for the reaction to take place (10, 11).

In the PCR process, firstly, the temperature is increased to separate the DNA double strands. Meanwhile, there are free primer sequences in the medium. When the temperature is lowered, many DNA sequences are matched to the primer sequence, as there are plenty of primer sequences in the environment. When the temperature is raised again, the enzyme DNA polymerase synthesizes new DNA strands by adding nucleotides to the ends of the primers, using the primers as a starting point. As a result, the new DNA molecule, which is the same as the original double-stranded DNA molecule, is copied. These processes, which double the amount of target DNA in the reaction medium, are called cycling. By repeating these steps, a large number of copies of DNA are obtained in a very short time (9) (Figure 2).

4. Sequencing Methods

In the 1970s, the nucleotide sequences of DNA began to be determined quickly and effectively. In line with emerging requirements and developing technologies, many sequencing methods have been developed and used until today (12). These methods can be grouped under two headings; traditional sequencing methods and next generation sequencing methods.

4.1. Traditional Sequence Methods

Two different methods, also called first generation nucleotide sequencing methods, were developed almost simultaneously. The first of these is the chain termination method developed by Sanger and Coulson in England and the other is the chemical degradation method developed by Maxam and Gilbert in the USA. Although different from each other, both methods are equally valuable. However, although it emerged a short time after the Sanger method, the chemical degradation method will not be discussed since it is almost never used anymore (11).

4.1.1. Chain Termination / Sanger Sequencing

The first step in the chain termination method is to obtain single-stranded DNA. This is done by cloning the DNA to be sequenced, usually into an M13 vector. After this step, an oligonucleotide is used as the primer, which serves as the starting point for DNA polymerase I's complementary strand synthesis. The primer matches either the adjacent sequences of the M13 vector from which the sequence to be sequenced was cloned, or the complementary sequences at the 3' end of the DNA sequence. For strand synthesis enzyme, four radioactive ^{32}P-labeled deoxyribonucleotides and modified dideoxy nucleotides are added to the reaction mixture. These modified nucleotides participate in the structure of the synthesized complementary chain. However, from the point where this nucleotide is added, the synthesis ends. This is because the 3'-OH group is absent on the sugar portion of the dideoxyribonucleotide. As is known, in DNA synthesis, DNA polymerase adds new nucleotides to the 3'-OH group of the synthesized complementary strand. If the nucleotide added to the reaction medium is ddATP, the synthesis reaction is terminated at the positions on the template where thymines are present. However, the synthesis is not always interrupted at the first thymine position, because there is normal dATP in addition to ddATP. Therefore, new strand synthesis is terminated at the positions of thymines, and DNA fragments with the length of each thymine nucleotide are synthesized on the template. Similarly, DNA fragments of length that determine the location of guanine, cytosine and adenine nucleotides in the template strand are synthesized using ddCTP, ddGTP and ddTTP in different reactions (11, 13, 14) (Figure 3).

The next step is to separate the DNA fragments of different lengths, each of which corresponds to a nucleotide position in the template DNA, resulting from four different reactions. The separation process is carried out by polyacrylamide gel electrophoresis. After electrophoresis, the position of the bands separated by autoradiography is determined and DNA sequence reading is performed. Since DNA fragments are separated according to their length in electrophoresis, the fastest traveling band corresponds to the shortest DNA fragment. When these bands are read in order from smallest to largest, the order of the nucleotides in the template DNA is determined (11, 13, 14) (Figure 3).

The Sanger method has now been completely automated. The traditional Sanger method requires that radioactively labeled DNA fragments be read in autoradiography. However, after the development of fluorescent markers, the use of radioactive materials was abandoned. In the automated system, each of the four types of ddNTP is labeled with different fluorescent dyes. Therefore, there is no need for four different reactions as in conventional sequencing. It is possible to put four different ddNTPs in a single reaction. The products

formed as a result of the reaction are separated by capillary gel electrophoresis containing polyacrylamide. DNA fragments are excited by a laser as they pass through the capillary. The laser excites the fluorescent dye in each piece of DNA so that different wavelengths of light are emitted. These emitted rays are captured by a detector and this information is amplified and transferred to the computer. The computer converts the light motif into a DNA sequence called an electrophorogram. The resulting data appears as a series of peaks, each corresponding to a nucleotide in the sequence (10, 16) (Figure 4).

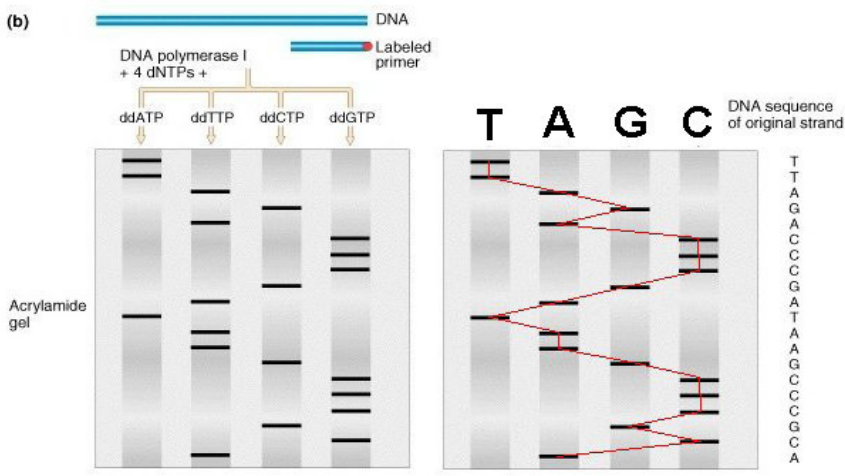

Figure 3: Chain termination method (classical approach) (15)

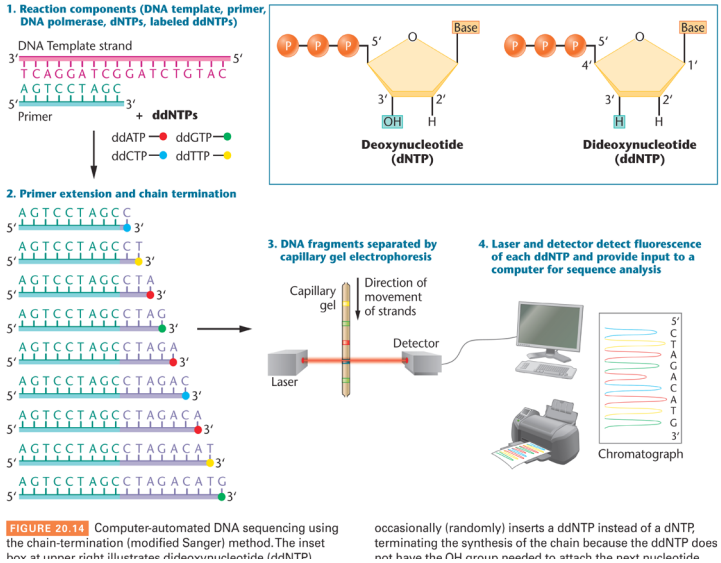

FIGURE 20.14 Computer-automated DNA sequencing using the chain-termination (modified Sanger) method. The inset box at upper right illustrates dideoxynucleotide (ddNTP) occasionally (randomly) inserts a ddNTP instead of a dNTP, terminating the synthesis of the chain because the ddNTP does not have the OH group needed to attach the next nucleotide

Figure 4: Chain termination method (automated approach) (10)

Automatic DNA sequencing devices contain up to 96 multiple capillaries. Each capillary can identify several thousand base sequences. In this way, it is possible to sequence more than 2 million base pairs per day.

4.2. Next Generation Sequencing Methods

Advances in genomics have led to the development of next-generation sequencing approaches, as DNA sequences consisting of millions of bases need to be determined in a shorter and faster way. These approaches use state-of-the-art fluorescence imaging systems that can simultaneously synthesize DNA from tens of thousands of identical DNA strands and then identify newly synthesized sequences and generate an average sequence data from the large number of sequenced molecules. In this context; different technologies are used such as pyrosequencing, sequencing by synthesis and nanopore sequencing. Here we will focus on the technology of sequencing by synthesis which has the most applications in practice (10, 17).

4.2.1. Sequencing by Synthesis / Illumina Sequencing

The Illumina sequencing method is often used in sequencing entire genomes of organisms and in genome-wide association studies (GWAS) research. GWAS studies aim to detect disease-associated single-nucleotide changes (SNPs). It is also used in exome sequencing studies (18, 19).

The Illumina sequencing method consists of three main steps: amplification, sequencing and analysis. The process begins with the purification of DNA. The purified DNA is fragmented and adapters serving as reference points are attached to these fragments. Bridge replication is performed after the fragments are attached to the adapters. Bridging amplification is a PCR process where DNA fragments are attached to a solid phase. Primers and modified nucleotides are then added to this solid phase. Because these nucleotides have a reversible 3' fluorescence blocker, DNA polymerase can add only one nucleotide at a time to DNA fragments. After each synthesis step, a camera takes a picture of the solid phase. The computer determines which base is attached via the wavelength of the fluorescent label and records it for each point on the solid phase. Uncombined fragments are removed after each synthesis step. A chemical deblocking step is then performed to remove the 3' fluorescent terminal block group. This process continues until the entire DNA molecule has been sequenced. Let's try to take a closer look at the steps in the procedure (20, 21).

Genomic library preparation: After the DNA is purified, a genomic library is created. The tagging method is used to create a genomic library. In the labeling

method, DNA is fragmented and adapters are added randomly by the transposase enzyme (22) (Figure 5).

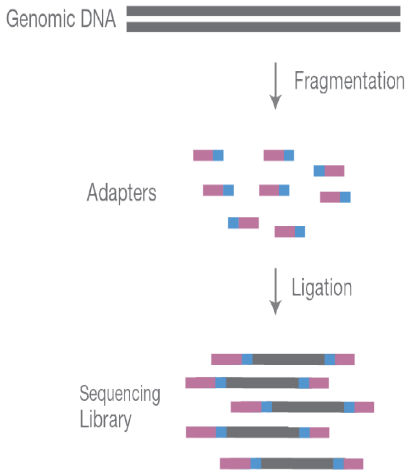

Figure 5: Genomic DNA library preparation (21)

Adapters: Adapters are oligonucleotide sequences that are attached to the ends of DNA or RNAs and contain specific sequences. The first task of the adapters is to connect the formed DNA fragments to the solid phase where the replication process will take place. The second task of the adapters is to establish the identity of the fragments with a six-nucleotide tag. After the fragments are attached to the adapters, the solid phase is washed to avoid errors in the experiment; thus removing fragments not bound to the adapter (21) (Figure 6).

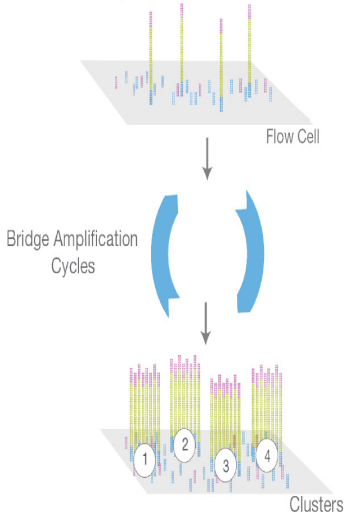

Figure 6: Adding adapters to DNA fragments (21)

Bridge amplification: This step starts after the solid phase bonding process has taken place. The purpose of this method is to obtain hundreds of identical DNA fragments. Fragments amplified after this process may belong to forward or reverse chains. For this reason, two types of adapters are used, left and right. Fragment clusters are created with this PCR method. The linked fragments are mapped by a conventional PCR procedure and a double-chain structure is formed. The replicated samples are also initially bridged and bound to the solid medium. After the coupling process is complete, the chains are denatured and made into a single chain, resulting in separated forward and reverse chains. This whole process is called bridge replication (21) (Figure 7).

Cluster amplification: Bridging is the process of multiplying the number of forward and reverse chain fragments generated during PCR. The groups formed by different forward and backward fragments are called clusters, and in this step, the fragments in each cluster are replicated and replicated to become double-stranded. The purpose of this step is quality control. Forward and reverse chains must be complementary to each other and all forward and reverse reading frames must match. If a read sequence is not sufficiently similar to other read sequences in the same cluster, an error may have occurred. Some laboratories use a minimum similarity threshold of 97% (21) (Figure 8).

At the end of clonal amplification, all backward chain fragments are removed by washing, leaving only forward chain fragments. The primer added to the medium binds to the primer binding site in the adapter region of the forward chain. DNA polymerase then adds a fluorescently labeled dNTP to the chain. The synthesis is blocked due to the presence of the fluorophore at the 3 end and the polymerase can only add one nucleotide per cycle. Each type of nucleotide in the medium is assigned a different color, so the computer can distinguish nucleotides from emission differences. After each signal is sent to the computer, that is, after the nucleotide type is determined, the fluorophore is removed and another nucleotide is added to the newly synthesized strand. This process is repeated until the synthesis of the new chain is terminated (21) (Fig. 9).

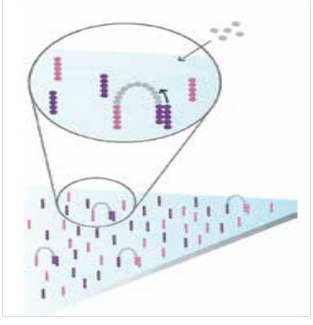

Figure 7: Bridge amplification (21)

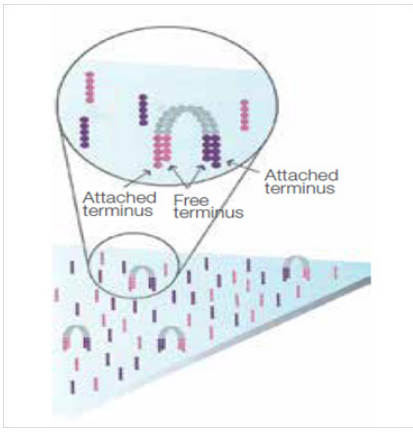

Figure 8: Cluster amplification (21)

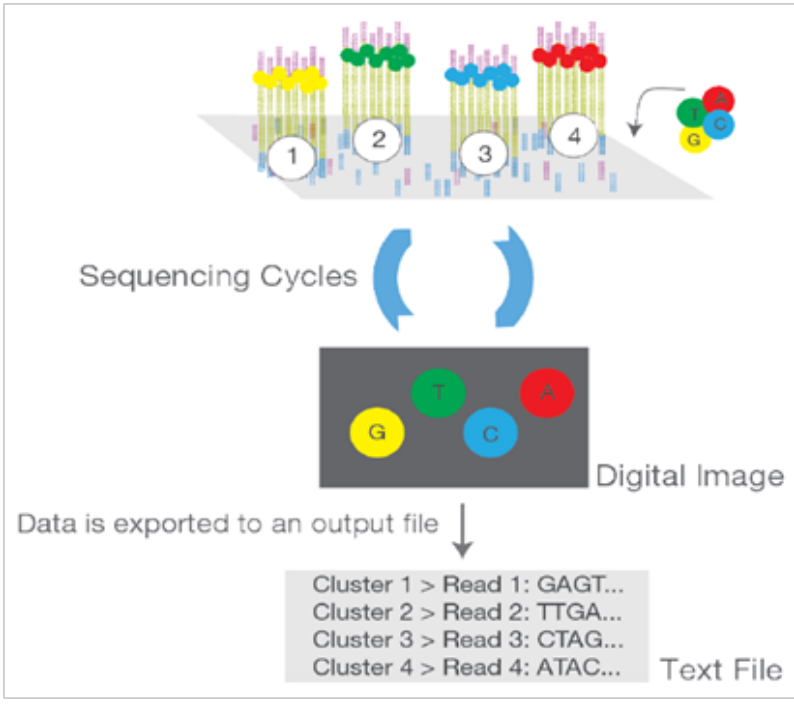

Figure 9: Sequencing cycle (21)

Data analysis: This step is the process of superimposing overlapping regions of sequences with identical regions, called contigs and reading the sequences. Thousands of contig sequences are overlapped and sequenced in this way, resulting in a DNA sequence with high sequence accuracy. With this method, if there is a reference fragment SNPs, variants can be detected and GWAS studies can be performed (23) (Figure 10).

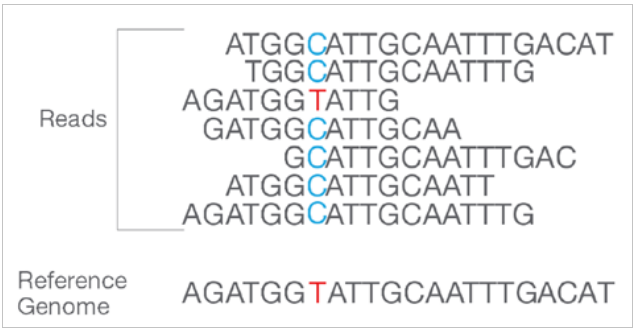

Figure 10: Data analysis (21)

The main advantage of Illumina over the Sanger method, from which it originated, is that it is relatively faster because many fragments are used at the same time. The sequencing process takes longer in the Sanger method as only one type of fragment is used in each iteration, but the process is faster in Illumina. In addition, it is possible to check how accurate the sequencing is, as Illumina uses fragment clusters. In addition, the Illumina sequencing method can yield 1 to 5 million data reads per sample, a number that cannot be reached with Sanger. However, Sanger sequencing stands out as an important option in cases where the results obtained with NGS need to be confirmed, revealing the value and importance of this method.

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CHAPYER XIII

INVESTIGATION OF THE THERAPEUTIC EFFECT OF ACUPUNCTURE, ON THE IN VIVO CYTOPATHOLOGICAL/ CYTOTOXIC EFFECT CAUSED BY DECUBITUS ULCER

Mustafa Çiçek
(Asst. Prof. Dr.), Department of Medical Biology, Faculty of Medicine,
Kahramanmaras Sutcu Imam University,
e-mail: mustafacicek_GOP@hotmail.com,
Orcid: 0000-0001-8925-0230

1. Introduction

Wound healing is a complex repair process aiming to restore the structural integrity of the damaged tissue that involves the interaction of cells, mediators, growth factors and cytokines. The basis of wound healing is constituted of cell growth and regeneration. Renewal of the dead cells in an organism and repair of local damage is critical in survival[1]. The orderly amplification of the epithelium of the skin and internal organs depends on the continuity of the basal membrane. Maintenance of the integrity of the basal membrane affects the nature and polarization of the cell type, as well as, cell migration, growth and morphogenesis during repair[1]. If the extracellular matrix is damaged due to wounding, the tissues may only heal with scar formation. The healing process begins when platelets contact with collagen following laceration[2]. Sufficient blood flow is a crucial parameter affecting healing. If the wound cannot receive sufficient blood flow, it is not nourished properly, thus becomes a candidate for infection and necrosis[3].

Acupuncture is a highly effective, scientific treatment method used for many diseases[4]. In order to understand the effect mechanism of acupuncture in the Eastern approach to philosophy and medicine, the balance system of the

body should be comprehended. There is a balance of contrasts in the human body. It is called the balance of Yin and Yang in the Chinese philosophy. According to the Chinese philosophy, it is the functional disorders in the system that generates the malfunctions in the body[5]. The self-healing power of the body is immense. There are precise points that trigger this power in the body, which are called the acupuncture points[4]. By the stimulation of the acupuncture points, the energy circulation in the body is returned to normal and thus the disease state is eradicated[6].

The most commonly known and used influences are its analgesic effect, sedative effect, homeostatic effect, which helps to establish balance within sympathetic, parasympathetic and endocrine systems, enhancing effect of the immune system by raising antibodies and gamaglobulins that increase the body's resistance, psychological effect that elevate dopamine and seratonine levels, motor repair effect accelerating the motor recovery following paralysis, and regeneration effect that accelerates cell renewal by increasing remote and regional blood flow[7]. In this study, it was aimed to investigate the effect of acupuncture on wound healing via increasing the blood flow and stimulating the regeneration mechanisms.

2. Material and Method

2.1. Experimental Procedure

This study was performed in the laboratories of the Department of Experimental Medicine Research, Gaziosmanpaşa University with the approval of the Animal Experiments Ethics Committee of the Faculty of Medicine, Gaziosmanpaşa University (no HADYEK-19 dated 08/05/2015). Twenty-two female rats of the Wistar Albino type weighing 250-300 grams were used in this study. All experimental animals were housed in room conditions to provide standard environmental conditions. They were fed with standard rat feed and tap water. No prophylactic antibiotherapy was performed. Rats were anaesthetized under ketamine (60 mg/kg) and xylazine (10 mg/kg). Depth of the anaesthesia was assessed by testing their withdrawal reflexes. After shaving the areas of surgery, the wound model identified by Park and colleagues was applied[8]. On the backs of all the rats, open ulcers were created via punch biopsies of 3-4mm depth and 10mm diameters at the point of origin of the last costa where the processus spinosus of the thoracic and lumbar 1-2 vertebrae are located and also coincide with the posterior of the vertebra. A total of 22 experimental animals were used in four groups in this study. Experiment duration for Group 1 and 2 was estimated as 8 days, and 25 days for Group 3 and 4. Group 1 was the early period control group and Group 2 the early period acupuncture group, which were treated with

acupuncture on days 1, 3, 5 and 7 following the ulcer generation. Group 3 was the late period control group and Group 4 the late period acupuncture group, which were treated with acupuncture on days 1, 3, 5 and 7 following the ulcer generation (Table 1).

Table 1: Study Groups

Experimental and Control Groups	Number of animals in the group	Total of animals/ groups
Early Period Control Group	**6**	**6**
Early Period Acupuncture Group	**6**	**6**
Late Period Control Group	**5**	**5**
Late Period Acupuncture Group	**5**	**5**

Acupuncture was performed on the Ashi points in the ulcerated areas with 8 0.2mm thick 25 mm long Hua Long brand acupuncture needles (Figure 1).

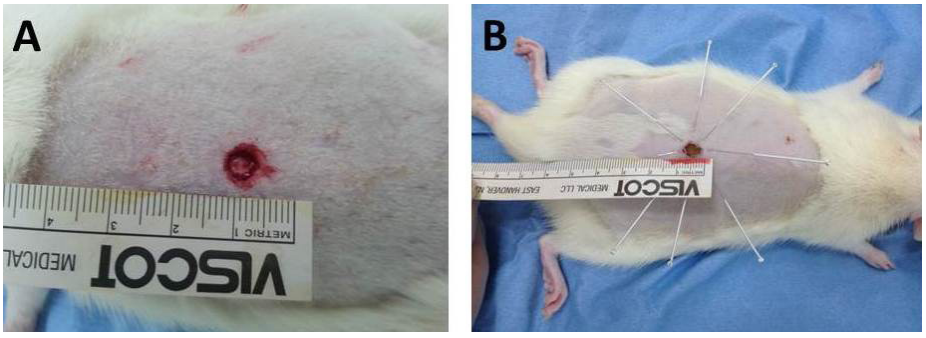

Figure 1: Experimental method
A) Seconder wound formation B) Acupuncture practice

2.2. Taking Samples

Tissue samples were obtained from all the experimental animals (in the early group comprising the whole wound and in the late group of 10mm wide and 20mm long samples corresponding to half of the wound), embedded to parrafin blocks and 5mm sections were taken for histological evaluation (Leica RM2125 RTS Rotary microtom). In addition, for the tensile damage analysis, 10mm wide and 20mm long tissue samples were gathered from the experimental animals.

2.3. Histological Examination

Hematoxylin-eosine (HE), Masson Trikrom (MT) and for immune staining Type I collagen (ABCAM, Anti-Collagen I antibody, Rabbit polyclonal to Collagen I, Cambridge, UK) and Matrix Metalloproteinase-2 **(**MMP-2) (ABCAM, Anti-MMP2 antibody, Rabbit polyclonal to MMP2, Cambridge, UK) stainings were performed on the sections and they were appraised under light microscope. Findings were evaluated with the H scoring method. For the H scoring method, cells in 5 animal slides taken from each group were clustered to 0 +, 1+, 2+ and 3+ according to their staining intensities with subjective evaluation, then counted and the findings were converted to percentages conferring to total cell count. After that, acquired values were multiplied with the coefficients of the intensity levels and thus H scores were calculated[9].

2.4. Tension Test Analysis

Tension test is the experiment specimen, which had been prepared according to standards on a single axis, being subjected to stretching with a constant pace and temperature until break off. During the experiment, the elongation of the standard specimen was noted down simultaneously with the application of a constantly increasing tensile force. Data is given as kg/mm² digitally from the USSC Sutures tensile strength device.

2.5. Statistical Analysis

Statistical evaluations were performed using the IBM SPSS 21.0 package program. In the data appraisement, aside supplementary statistical methods (mean, standard deviation), for intergroup comparisons Kruskal Wallis test and Mann-Whitney U test, for qualitative group data comparisons chi square test, and quantitative group data comparisons Student's t tests were used. Values of $p<0.05$ were considered statistically significant.

3. Results

3.1. Macroscopic Findings

In the acupuncture-applied group, less scab formation was observed and in the later period, due to the epithelialization from the edges of the wound lips to the subgrade, brighter and lively looking wound healing areas were encountered. Moreover, compared to the control group, in the acupuncture-applied group, edges of the wound observed to be brighter red and contained more vessels due to the increased levels of neovascularization of the wounds (Figure 2).

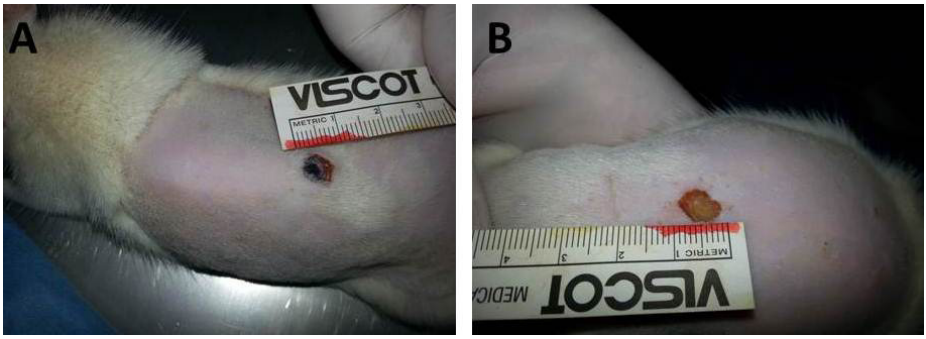

Figure 2: Macroscopic findings A) Control Group B) Acupuncture Group

3.2. Histological Findings

Histological evaluations were performed according to Table 2.

Table 2: Wound healing score evaluation criteria

Score	Reepithelialization	Granulation tissue	Collagen accumulation	Inflammatory cells	Angiogenesis	Ulcer
0	None	None or immature	None	None	None	Wide and deep ulcer, abscess
1	Partial	Low	Low	Low	<5 vessels	Wide ulcer
2	Complete but immature or thin	Moderate maturation	Moderate	Moderate	6-10 vessels	None or very small
3	Complete and mature	Mature	Abundant	Abundant	> 10 vessels	None

3.2.1. Haematoxylin- Eosin Staining

In acute early period, wound areas were covered with granulation tissue in both control group and acupuncture-applied group. In the acupuncture-applied group, the inflammatory infiltration was less in and around the subgrade of the wound, conversely, in the control group, accompanying increased inflammatory infiltration, denser migration of the leukocytes with polymorphic nuclei to the wound. In addition, fewer, scattered macrophages were detected (where in the wound area) in the acupuncture-applied group. In the early period acupuncture group, increased level of vessel formation was noted compared to the control group (Figure 3).

In the rats of the late period groups, animals in both groups showed complete reepithelialization and the wound areas were cicatrized with

mature epithelial cells. In the animals of the control group, more collagen fibers and fibroblast cells were found than in the acupuncture-applied group (Figure 3).

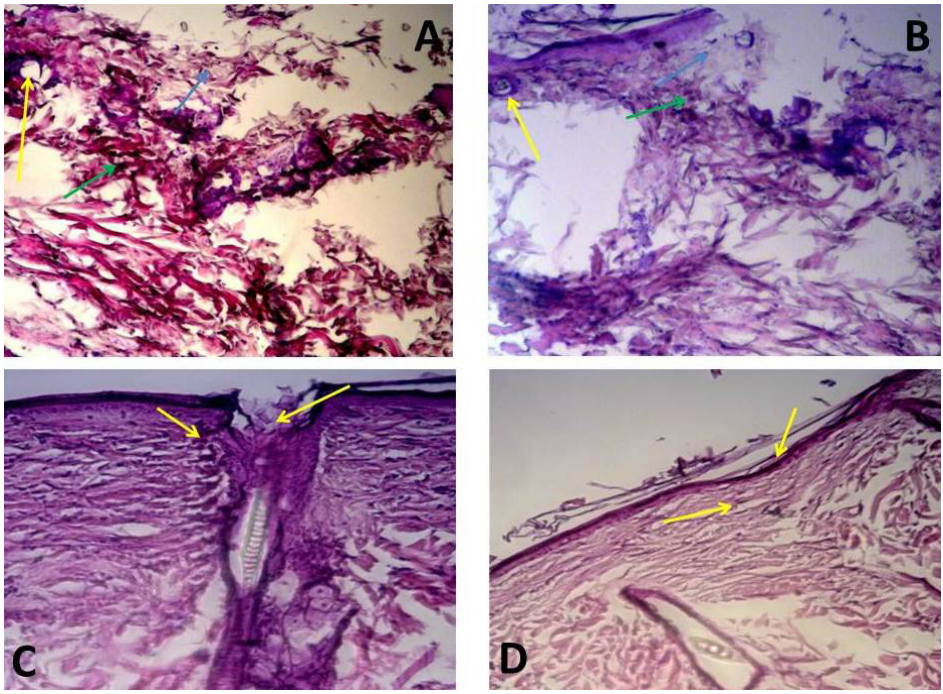

Figure 3: Wound healing H&E staining Early and Late period, 10X A) Early Period Control Group B) Early Period Acupuncture Group C) Late Period Control Group D) Late Period Acupuncture Group (Yellow arrow; blood vessels, Green arrow; PNL migration, Blue arrow; scab and scar formation)

3.2.2. Masson's Trichrome Staining

In the rats of the late period group, total collagen intensities in the wound sections were shown with Masson's Trichrome staining. In the late control group, more collagen fibers were observed in the subepithelial region than in the acupuncture-applied group. In the acupuncture group, collagen fibers appeared more areolar (reticular) similar with the normal connective tissue and organized, yet the fibers in the control group extended parallel to epidermis and formed stratifications. Collagen bundles were thicker in the acupuncture group than in the control group (Figure 4). Masson's Trichrome staining was not performed for the early period groups to give information on the total collagen intensity.

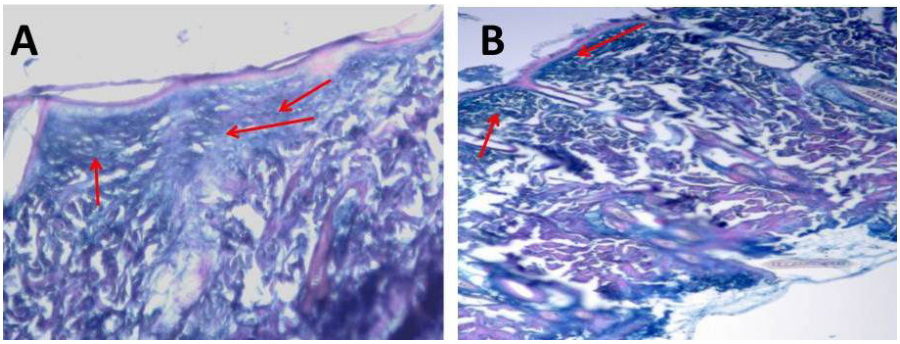

Figure 4: Late period Masson's Trichrome staining, 10X
A) Late Period Control Group B)Late Period Acupuncture Group

Semi-quantitative evaluation results related to total collagen intensity are given in Table 3. A significant increase (+++) in the total collagen intensity in the subepithelial region was observed in the control group, while a decrease (+) in the acupuncture group (Figure 4). In the control group, the total collagen intensity showed a statistically significant increase compared to the acupuncture group (p=0.003).

Table 3: Masson's Trichrome staining and Type I Collagen immunreactivity

		Group				Total		
		Control		Acupuncture				
	intensity	n	%	n	%	n	%	P
Masson's Trichrome Staining Late Period	+	0	0	7	100	7	100	0,001*
	+++	7	100	0	0	7	100	
Type I Collagen Immunreactivity Late Period	++	0	0	7	100	7	100	0,001*
	+++	7	100	0	0	7	100	

3.2.3. Immunohistochemical Findings

3.2.3.1. Matrix Metalloproteinase-2 (MMP-2) Staining

The MMP-2 levels of the wound sections were shown immunohistochemically in the late period healing group rats. No immune reactivity was observed in negative controls. MMP-2 showed immunepositivity in the keratinocytes of the epidermis, many cell types of the subepidermal layer (macrophages and fibroblasts etc.), hair follicles and vessel endothelial cells. H-score results are given in Figure 6. In the acupuncture group, MMP-2 immunereactivity was significantly decreased compared to the control group (p=0.02) (Figure 5).

3.2.3.2. Type I Collagen Staining

In the rats of the late period groups, type I collagen protein levels were shown immunehistochemically on the wound sections. No immune reactivity was observed in negative immunohistochemical stainings. Results were evaluated semi-quantitatively, which are given in Table 3. While type I collagen immune reactivity was significantly increased (+++) in the control group, in the acupuncture group a moderate (++) staining was observed (Figure 5). Type I collagen amounts were significantly increased in the control group than the acupuncture group (p=0.008).

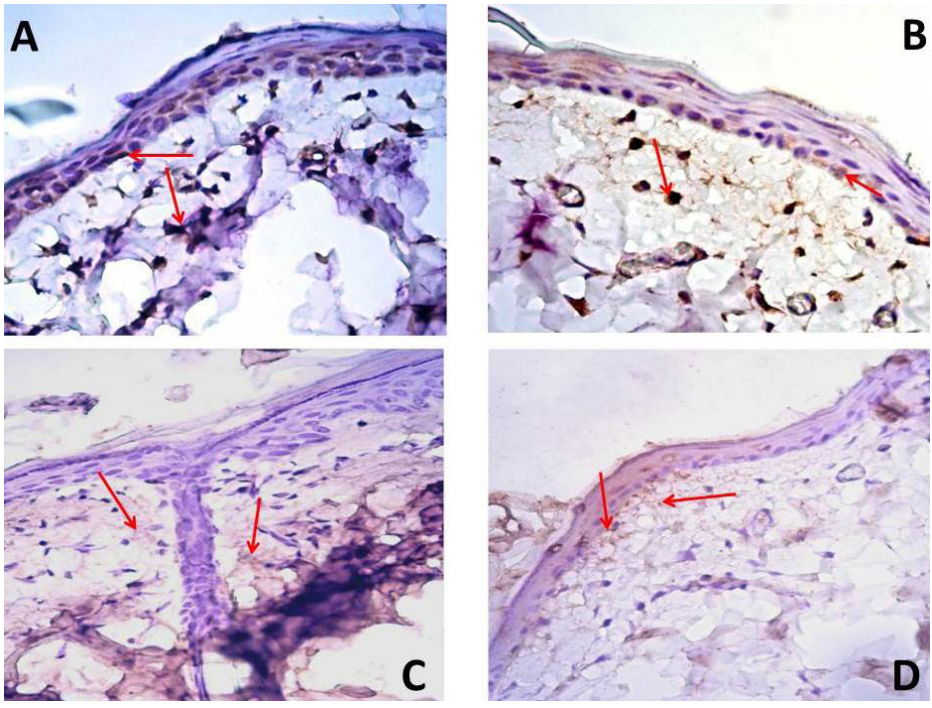

Figure 5: Late Period MMP-2 and Type I Collagen immunreactivity, 40X A) MMP-2 Control Group B) MMP-2 Acupuncture Group C) Type I Collagen Control Group D) Type I Collagen Acupuncture Group

3.2.4. Tension Test Findings

The tension test results of each group performed on the 25th day are given in Table 8. In the acupuncture group, forces needed to separate the edges of the wound lips were greater than in the control group (Figure 6). The difference between two groups was statistically significant (p<0.001).

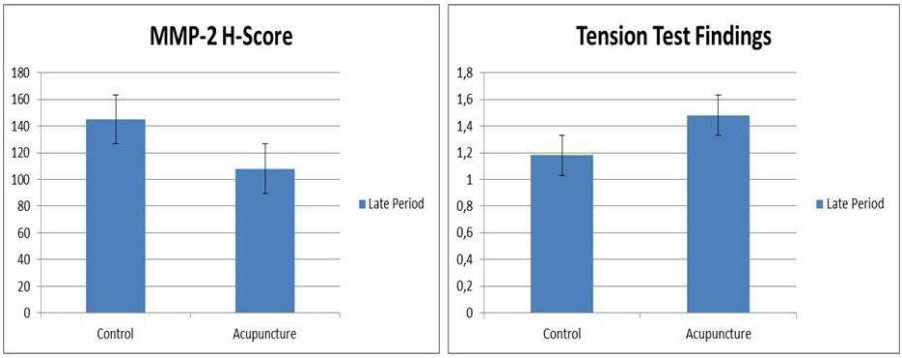

Figure 6: MMP-2 H-Score and Tension Test Findings

4. Discussion

Wound healing is a complex process comprised of three steps. Together with the development of these steps, many important cellular parameters are also altered[10]. In this study, it was aimed to display the early and late period results of wound healing. For that, wound samples were collected in the 8th and 25th days in order to show the histopathological changes in a cellular level and functionally by testing the detachment force required with a tensile test.

The sufficient nourishment of the wounded tissues and providing blood supply are the most important factors affecting the wound healing process[11]. There are some studies focused on angiogenesis aiming to increase the generation of new blood vessels and reducing the adverse effects caused by local ischemia[12]. In many studies conducted on wound healing, growth factors were used in order to accelerate angiogenesis, fibroplasia and proliferation of the granulation tissue[13]. These factors have been shown to have positive impacts on epithelialization and granulation in ischemia and pressure sores[14]. It has been demonstrated that wound healing process is accelerated by the stimulation of angiogenesis and endothelial cell migration[15]. In this study, histopathologically haematoxylin-eozin stained slides in the acute group showed increased angiogenesis in the acupuncture applied wound group.

Another main reason for wound healing disruption is fibroblasts being affected. Fibroblasts being effected, and decreased proliferation and migration of fibroblasts, lead to insufficient and functionally ineffective collagen production[16]. Thus, collagen gene expression and secretion of growth factors decreases, and collagen maturation falls behind[17]. In this study, in the subepithelial regions of the acupuncture-applied subjects, elevated levels of collagen fibers and fibroblasts were observed. In the early period, in the light of the histopathological evaluations, new blood vessel formation was found higher in the acupuncture group than the control group. The possible underlying reason may be due to its notable effect

on neovascularization. In a study using recombinant human granulocyte colony-stimulating factor and recombinant human macrophage stimulating factor to stimulate cells during wound healing, it has been shown to accelerate wound healing by increasing collagen synthesis[18]. In our research, an increase in collagen was shown in the early period acupuncture group with HE staining.

Studies on the application of platelet rich plasma products to wounded regions showed that growth factors accelerate the healing process, infections can be taken under control by adding bactericidal proteins to other proteins that expedite healing, it shortens hospitalization durations, and it decreases pain and swelling[19]. However, platelet rich platelets do not produce a very solid fibrin clot due to their high platelet concentrations yet low fibrinogen concentrations[20]. Therefore, it is thought to accelerate the process by means of growth factors instead of preventing wound healing[21]. In one study, it was reported that platelet-rich plasma caused extracellular matrix accumulation due to TGF-β and PDGF, and increased connective tissue cell proliferation[22], whereas Anitua et al., (2004) observed that thrombocyte rich plasma that they applied to titanium implants reduced inflammation[23]. In this study, it was observed that in the early period acupuncture group, the inflammation layer in the wound area was less intense than in the control group, and that fewer macrophages were scattered. This decrease in inflammation may be due to the activating effect of acupuncture on platelets. The PGDF released from the platelets may possibly shift the wound healing process towards faster in the wound area by increasing the inflammatory process, proliferation of the connective tissue cells and collagen production.

Acupuncture has been shown to significantly shorten the systemic and local inflammatory processes[24]. In this study, it was also observed that inflammation in the acupuncture group was considerably less than in the control group. Granulocytes in the human body may cause acute neutrophilic inflammation. Furthermore, formation of the granulation tissue and contraction are important steps in the open wound healing process. During wound healing, myofibroblasts gradually develop from fibroblasts in the granulation tissue, and alpha smooth muscle actin (SMA), which is a sign of smooth muscle differentiation, becomes evident temporarily[25]. Fibronectin, one of the components of the extracellular matrix, is a cell adhesion protein that serves in cell migration and collagen accumulation, and accrues continuously during wound healing[26]. Moreover, bFGF reduces alpha SMA expression and induces fibronectin expression[27]. In their study, Lee et al., observed a decrease in the expression of injured alpha-SMA and an increase in bFGF and fibronectin expression following acupuncture treatment. Previous studies have reported that acupuncture increases bFGF expression in the dorsal root ganglion following partial dorsal root ganglionectomy[28]. They noted that acupuncture promoted wound healing and by inducing bFGF expression, induced healing without a scar. In our study,

collagen fiber formation in the subepidermal and dermis layers in the late period acupuncture-applied rats was found to be less intense than in the control group. In addition, the collagen fibers in the acupuncture group were similar to the normal connective tissue with their areolar appearance and advanced organization, while in the control group collagen fibers were extended parallel to the epidermis layer and tightly stratified.

The primary goal in wound care is rapid closure of the wound with functional and acceptable scarring. According to the results of the tension test in our study, it was determined that the breaking force required to separate the wound edges in the acupuncture group was significantly higher than the control group. This may be due the fact that acupuncture lead to less intense yet more functional scarring.

Park and his colleagues examined the effect of acupuncture therapy on healing of an ulcerated wound area, cell proliferation and granulation tissue formation[8]. Previous studies showed that acupuncture has a therapeutic effect on pain in Ashi-point and nose and bone fractions[29,30]. Park et al. (2012) in their study of wound healing when treated with acupuncture, they observed the wounds' healing on day 1, 3, 5 and 7, and reported enhanced healing in the acupuncture-applied group compared to the control group. In the same study, they stated that acupuncture develops angiogenesis and granulation formation positively in cells by forming immune chain in phenotype markers such as proliferating cell nuclear antigen (PCNA), CD-31 and α-SMA[8].

Stronger repairing is supported by the improvement of the amount of collagen type I, the most important collagen in skin[31]. In this study, the intensity of the immunoreactivity of the type I collagen amount in the acupuncture-applied late-perid animals was found less than in the control group. It seems that acupuncture does not increase the amount of total type I collagen excessively, yet it produces less collagen which is more organized and more resistant to tension.

In a study conducted by Zhang et al., acupuncture was reported to accelerate wound healing, reduce pain, and inhibit the formation of keloid scarring in pressure wounds[32]. A significant amount of keloid scarring has been observed on a 27-year-old woman with a colles fracture, even after six months after the closure of the wound. With the acupuncture treatment applied, scar formation and local tenderness were significantly eliminated[33]. In our study, it was found that the excess collagen accumulation, which is the reason of scar formation, was less than in the late period acupuncture group that of the control group and the organization of the collagen fibers in the dermis was almost like a normal connective tissue.

In the study performed by Yang et al., (2013) on cerebral ischemia and reperfusion, by using two important acupuncture points showed decreased infiltration in the infalmmatory enzymes and MMP-2 expression, which is a proinflammatory enzyme. MMPs, like other proteases are fold-growing molecular

inflammatory products. Blood brain barrier tight junction proteins developed from the endothelial barrier and basal lamina proteins are vulnerable against an attack from the MMPs. In this study of Yang et al., it has been clearly shown that acupuncture converts the MMP-2 upregulation significantly to the inverse[34]. In our study, the immunoreactivity of MMP-2 in epidermal keratinocytes, fibroblasts and macrophages in the subepidermal layer, hair follicles and vascular endothelial cells were significantly decreased in the acupuncture group compared to the control group. In the study of Murti et al. it was observed that according to the results of the tensile test, the tensile endurance showed the maximum activity for wound healing with a statistical significance[35]. In this study, the detachment force applied to separate the wound edges after tensile test was greater in the acupuncture group than in the control group. Collagen fibers are the most common fibers in the connective tissue. These fibers are not elastic and are more resistant to steel owing to their molecular arrangement. Therefore, it provides unique durability to the tissues with collagen. Collagen fibers consist of tightly packed fibrils in mammals, which collagen fibers are formed by their parallel arrangement. The morphologically thick and wavy structure of the collagen fibers determines their functional effectiveness. The normal collagen structure in the normal intersisal area is supported by the reticular fibers, which are thinner fibers, and gains a stronger structure due to the widespread network of reticular fibers.

Strong tensile test results rendered by acupuncture are probably due to the fact that newly formed collagen fibers are distributed in a similar manner as in normal dermis tissue, they are thicker than in the control group and are more supported by the nets formed by the reticulated fibers.

5. Conclusıon

Finally, for the reason of acupuncture improving blood flow during wound healing and positively contributing to the wound healing process by its regeneration effect, by preventing keloid scar formation elevating collagen generation especially in the dermis layer to the standards of normal dermis connective tissue, by providing healthier wound healing and accelerating wound healing we suggest that it can be benefited from as an additional treatment after surgical operations.

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CHAPTER XIV

EXPERIMENTAL INVESTIGATION OF AGING-INDUCED TYPE I COLLAGEN AND MMP-2 INTERACTIONS IN MANDIBULAR BONE TISSUE WITH CELLULAR AND PHYSIOLOGICAL CHANGES AND IN VIVO CYTOTOXICITY

Mustafa Çiçek[1] & Mehmet Kemal Tümer[2] & Velid Unsal[3]

[1](Asst. Prof. Dr.), Department of Medical Biology, Faculty of Medicine, Kahramanmaras Sutcu Imam University, Turkey

e-mail: mustafacicek_GOP@hotmail.com,

Orcid: 0000-0001-8925-0230

[2](Assoc. Prof. Dr.), Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Alanya Alaaddin Keykubat University, Turkey

e-mail: dr_kemaltumer@yahoo.com

[3](Asst. Prof. Dr.), Department of Nutrition and Dietetics, Faculty of Health Sciences, Mardin Artuklu University, Mardin, Turkey

e-mail: velidunsal@hotmail.com

1. Introduction

Among the most important changes that aging has on the organism, the changes that occur in the oral cavity and changes that occur in the tissues that surround the cavity are among the most significant [1]. With aging, calcified tissue in all bones, often in women, decrease, which results in porosity and increases the brittleness of the bones. Blood flow in both maxilla and mandible is also decreased in elderly [2]. Mandibular subluxations or dislocations occur as a result of trauma more often in elderly individuals and fractures develop more often due to thinned chondyle structure. Toothless and elderly individuals who suffer from Costen syndrome show symptoms

such as headaches, dizziness, ear aches, ear ringing and irritated tongue due to displacement of mandibular condyle apex in the posterior-superior direction, which is caused by the over-closure of the mouth[3]. Bone tissue consists of non-mineralized organic structures such as bone extracellular matrix (ECM) and Type I collagen, as well as components such as calcium and phosphate in the form of inorganic hydroxyapatite crystals. [4]. The main metabolic effect of calsitonin is to keep llevels of calcium and phosphate in the plasma in normals levels and the parathyroid hormone (PTH) increases the amount of ionized calcium that is decreased in the blood plasma, thus keeping it at physiological limits [5]. Inflammatory responses increase levels of proteases, including matrix metalloproteinases (MMP) and reduce the levels of protein matrix components, such as collagen and gelatine. In addition to inflammatory biomarkers such as TNF-α and IL-1B, aging also affect the synthesis of MMPs [6].

Recent studies have attempted to establish a relationship between the levels of PTH, which plays an important role in the transformation of bones and the levels of bone-specific Alkaline phosphatase (bALP), Ca, calcitonin and type I collagen, which are the the biochemical indicators of bone production[7]. In this study, we aimed to show the biochemical changes, the presence of expression of Type I collagen and MMP-2 and the related histopathology and immunohistochemical changes, which are all effects that aging has on the mandibular bone, an element of the temporomandibular joint.

2. Material and Method

2.1. Procurement of Animals

This study was carried out in Gaziosmanpaşa University experimental medicine research unit after obtaining the local ethics committee permission (HADYEK-28). In the study 14 Balb/C species white mice (50-80 g) were used. Animals were divided into two equal groups of seven. Group 1 included young animals which were 2 months old (n=7) and Group 2 included old animals which were 18 months old (n=7). Prior to application of the experiment, the mice were kept at room temperature (22 ± 1 ° C) and 40-50% humidity. The light pattern was adjusted to 12 hours of day and 12 hours of night. Eating and drinking habits of the animals were left untouched. The rats were kept under observation for a week and their daily physical examinations were carried out[8].

2.2. Taking Samples

Mice without any health problems were put down using the excretion technique while they were under an anesthesia of ketamine / xylazine (50/10

mg / kg). Plasma of blood samples of 14 mice from young and old groups were separated and frozen at -80 ° C. The ramus mandible was removed alongside mandibular condyle of the mice of both groups, and the right and left portions were divided into two parts by using the tuber mental line as the alignment. To allow biochemical analysis from the right mandibular bone tissue, it was flash frozen using a liquid nitrogen tank and it was kept frozen at -80 ° C for the preparation of the tissue honogenate. Left mandibular condyle was placed inside of a 10% EDTA solution for immunohistochemical and histopathological evaluations.

2.3. Biochemical Analysis

The taken bone tissue homogenate samples and the calcium levels were checked using a Abnova Calcium Assay (KA0812) brand commercial kit; bone-specific ALP levels were checked using MyBiosource Mouse Bone-specific Alkphase B(ALP-B) ELISA Kit (MBS264117); PTH levels were checked using RayBio® Human/Mouse/Rat PTH Enzyme Immunoassay Kit; calcitonin levels were checked using Antibodies-Online Mouse Calcitonin ELISA Kit (ABIN1113887) and TNF-a levels were checked with ELISA method using the Antibodies-Online Tumor Necrosis Factor ELISA Kit (ABIN456701).

2.4. Histological Examination of the Mandibular Bone Tissue

Tissues were kept in the EDTA solution until the histological sections could be obtained. After routine histological follow-up, the tissues were embedded in paraffin. 4-5 Hm thick sections were taken from paraffin-embedded tissues (Leica RM 2155; Leica Inc., Nussloch, Almanya) and they were stained with hematoxylin-eosin (H & E) method. The stained sections were examined under Euromex Oxion light microscope[9].

2.5. Immunohistochemistry Analyses

The 4-5 mm sections taken from the paraffin blocks were placed on slides with polylizine. Deparafinize edilen dokular dereceli alkol serilerinden geçirilerek dehidrate edildikten sonra, distile suya alınan dokular antigen retrieval için sitrat tampon solüsyonunda pH:6'da mikrodalga fırında (600W) 5 dakika kaynatıldı. To prevent endogenous peroxidase activity they were treated with H_2O_2. To prevent surface staining, primary antibody was incubated for 60 minutes using (Rabbit polyclonal to Aquaporin 1 Abcam, **AB15080**, Camridge, UK; Rabbit polyclonal to Aquaporin 3 Abcam, **AB15117**, UK, Rabbit polyclonal to MMP2 Abcam, **AB37150**, Camridge, UK; Rabbit polyclonal to Collagen I Abcam, **AB34710**, UK) after the application of Ultra V Block (Ultra V

Block, TA-125-UB, Thermo Fisher Scientific Inc., USA) solution. After the application of the primary antibody, (biotinylated anti-mouse IgG, Diagnostic Biosystems, KP 50A, Pleasanton, USA) was administred to the secondary antibody for 30 minutes, streptavidin horseradish peroxidase was administred for another 30 minutes and ve 3-Amino–9-ethyl carbazole chromogen was applied and contrast painting was carried out using Mayers hematoxylin. In the tissues prepared for negative control, phosphate buffered saline (PBS) was used instead of primary antibody, and the other steps were performed in the same manner. The tissues that were put through PBS and distilled water were sealed using an appropriate sealing solution (Tablo 3). The prepared preparates were examined and photographed using the research microscope (Zeiss Axio Lab A1).

Evaluation of immunohistochemical markers was performed using H-SCORE analysis [9]. Type I Collagen and MMP-2 immunoreactivity intensity were semi-quantitatively evaluated using the tracked intensity categories: 0 (no staining), 1+ (weak but detectable staining), 2+ (moderate or apparent staining), and 3+ (intense staining). For each tissue, an H-score value was obtained by first calculating the total percentages of cells according to the intensity category of their staining (MMP-2). After that, this stain weighted value was multiplied by ΣPi (i l +) using the H-SCORE value in which "i" represents the intensity values and "Pi" represents the related percentage of cells. Each slide was evaluated under a light microscope (X40 magnification). The percentage of cells in each density within these areas was determined by two researchers at different times, who were not aware of the type and source of tissues. The mean score of both observers was used.

2.6. Statistical analysis

Normality control was performed by Shapiro-Wilk test. The data were found to be consistent with normal distribution. Statistical significance was accepted as $P<0.05$. Two independent sample T tests were used to compare the groups.

3. results

3.1. Biochemical Findings

Ca^{+2}, ALP, PTH, Calsitonin ve Tnf-α values were determined by the ELISA measurements carried out on mandibular bone tissue homogenates and they are as provided with the Table 1. Ca^{+2}, ALP ve calcitonin levels found in bone tissue and bone metabolism was determined to be higher in young mice in comparison to the elderly mice. Tnf-α and PTH levels, which are intracellular inflammatory cytokines, were significantly increased in elderly mice compared to young mice ($p <0.05$) (Fig. 1).

Table 1: Results of biochemical analysis of mandible bone tissues of young and old mice

Results of Biochemical analysis of Mandible			
Group			
Parameter	**Young Mice**	**Old Mice**	**p**
	$\bar{x} \pm SD$	$\bar{x} \pm SD$	
ALP	175,87±12,32 (IU/L)	76,13±3,85 (IU/L)	**0,001**
Ca^{+2}	13,86±0,61 (mEq/L)	7,53±0,39 (mEq/L)	**0,005**
TNF-α	333,02±3,72856 (ng/mL)	472,24±12,41 (ng/mL)	**0,001**
Calcitonin	854,23 (pg/mL)	687,31 (pg/mL)	**0,005**
Parathormon	12,47 (pg/mL)	36,56 (pg/mL)	**0,001**

Ca^{+2}, ALP ve calcitonin levels found in bone tissue and bone metabolism was determined to be higher in young mice in comparison to the elderly mice. Tnf-α and PTH levels, which are intracellular inflammatory cytokines, were significantly increased in elderly mice compared to young mice ($p < 0.05$) (Fig. 1).

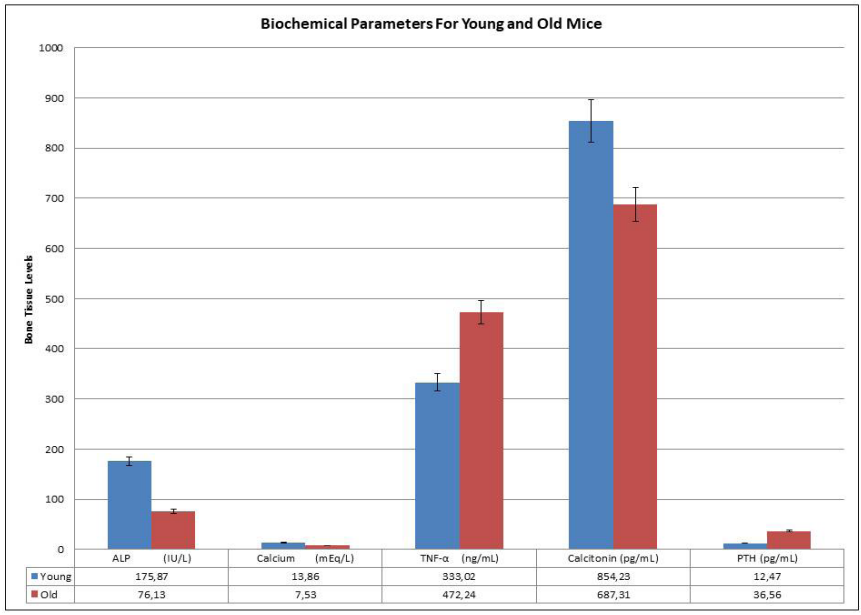

Fig. 1: Graphical illustration of biochemistry results of mandible bone tissue in young and old mouse

3.2. Histopathological Findings

In all the sections examined, it has been observed that the mandibular bone is composed of proliferation, chondrogenic and hypertrophic segments. The increased thickness of proliferative, chondrogenic and hypertrophic layers in young mice has been remarkably observed. Irregularly distributed mature chondrocytes with large, spherical shapes were observed in the hypertrophic region of the older mice, whereas in younger mice, these cells were found to be more numerous and in a specific order. It has been observed that the bone marrow of the mandibular bone is more numerous in young mice and the boundaries of spongious formtrabeculae are more pronounced in older mice. In young mice, blood vessels were observed with ease and in older mice there was a decrease in the count blood vessels and these vessels had structural deformations and expansions in arterioles and veins. A significant amount of cartilage deterioration was observed in aged mice. Bone tissue and connective tissue around the bone were observed in all mice, young and old. Substantia compacta layer of younger mice was found to be tighter and morphometrically larger compared to that of the older mice. In young mice, fibrous connective tissue formed a broader band than that of the aged mice. In young mice, numerous hypertrophic chondrocytes were found in the transition area from bone tissue to articular cartilage. In young mice, trabecular bone structure has been observed in almost all areas except the articular cartilage area of the epiphysis (Fig. 2).

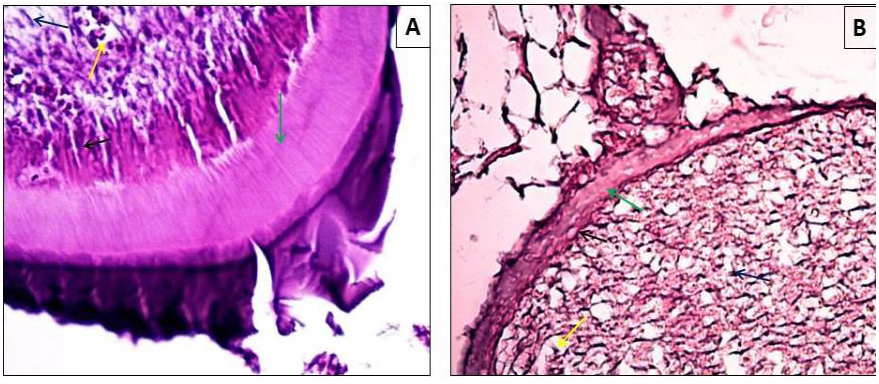

Fig. 2: Hematoxylin- eosin staining, 40X. Mandible Bone Tissue A) Young mice B) Old mice (Yellow arrow; blood vessels, black arrow; osteoblasts, green arrow; compact bone layer of mandible, blue arrow; osteocytes)

3.3. Immunohistochemical Findings of Type I Collagen and MMP-2

Type I Collagen and MMP-2 proteins were stained immunohistochemically in the mandibular bone tissue tests of both the old and young mice. The results were evaluated semi-quantitatively and they are shown on the Table 2.

Table 2: Type I collagen and MMP-2 immunreactivity of the young and old mice

Intensity of Immunoreactivity Staining					
	Group				
Parameter	Young Mice		Old Mice		
	intensity	n	intensity	n	p
Mandibulae Type I Collagen	+++	7	+	7	0,001*
Mandibulae MMP-2	+++	7	+	7	0,001*

The staining immunoreactivities that are specific for MMP-2 antibodies in osteoblast cells and for antibodies that are specific for Type I Collagen fibers in substansia compacta and substantia spongiosa regions of the mandibular bone tissues of young mice have been found to have increased significantly (+++) and it was also observed that Type I Collagen and MMP-2 immunoreactivities of old mice were at low levels (+) (p=0,001) (Fig. 3).

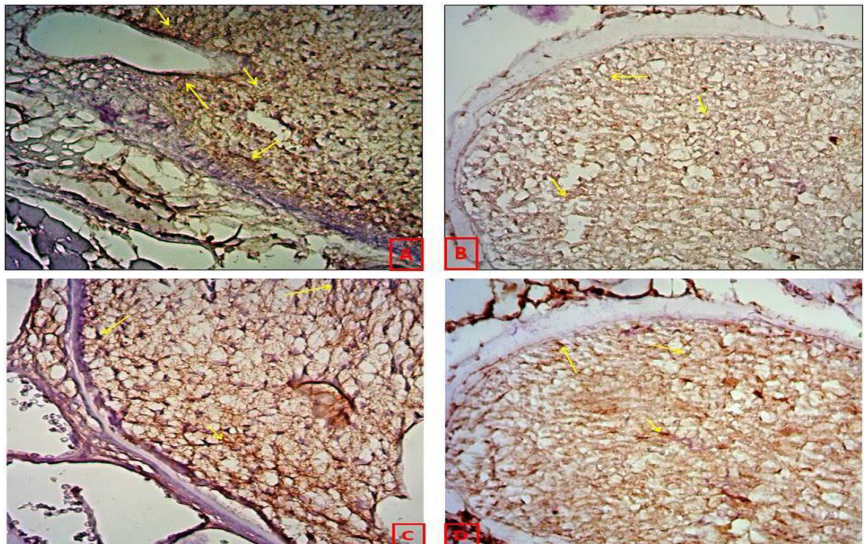

Fig. 3: Immunohistochemical staining of Type I Collagen and MMP-2, 40X A) Type I Collagen Young mice B) Type I Collagen Old mice C) MMP-2 Young mice D) MMP-2 Old mice

H-Score results that were obtained through the MMP-2 immunopositive stainings of osteoblast cells in the bone tissue of the mandibular chondyle has revealed that cell based MMP-2 Immunoreactivity of younger mice is significantly higher than that of the older mice (p = 0.001) (Table 3).

Table 3: MMP-2 H-score comparisons of the young and old mice

Comparison of H-score values						
		Group				
Parameter	Young Mice		Old Mice			
	$\bar{x} \pm SD$	n	$\bar{x} \pm SD$	n	t	p
Mandibulae MMP-2	169,27 ± 13,48	7	123,37 ± 14,32	7	5,478	0,001*

According to all our analysis results, the biochemical, cellular and physiological changes in bone tissue of young and old mice were schematized (Fig. 4, Fig. 5).

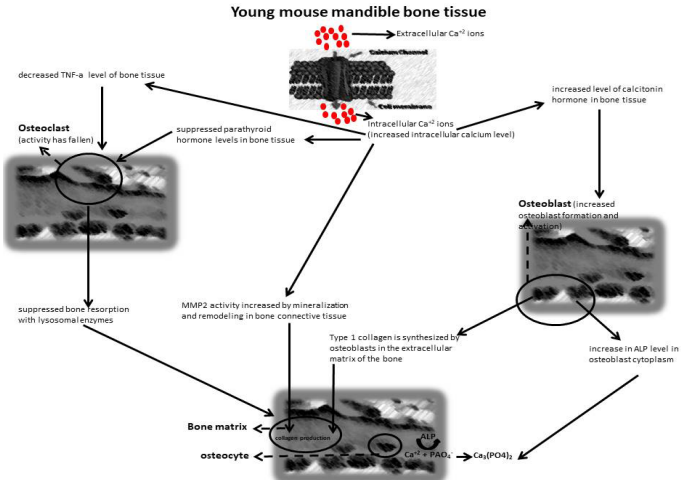

Fig. 4: Illustration of the natural development process of the mandibular bone tissue in young mice

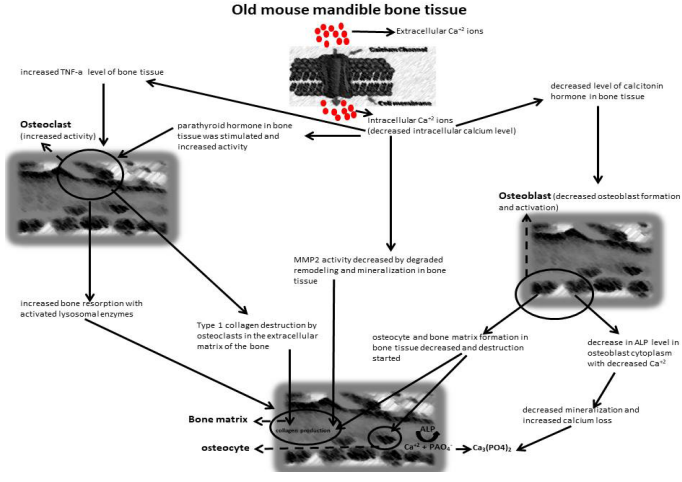

Fig. 5: Illustration of the natural development process of the mandibular bone tissue in old mice

4. Discussion

Mandibular bone increases the mobility of the chewing system. Since morphological deterioration of its structure causes chewing problems and teeth problems in both humans and animals, this matter possesses a clinical significance. Some in vitro studies have shown that the maximum force during chewing is exerted on the lateral and anterior surface of the condyle of the mandibular bone[10]. In this study, biochemical, histopathological and immunohistochemical changes in bone tissue were evaluated in order to better understand the damage caused by aging on the mandibular bone tissue. The literature review has revealed that our study is the most extensive research on the effects that aging has on the mandibular bone tissue.

Many factors play a role in growth and development of the mandible such as diet, testosterone, estrogen, gender, age and genetics[11]. In one study, changes in the cartilages of the mandibular bone and changes in the subchondral bone were simultaneously analyzed and it was determined that in young mice, age and gender based changes in the size of subchondral bone and mandibular bone were significant[12]. According to the histopathological results of our study, proliferative, chondrogenic and hypertrophic layers of the young mice were found to be thicker than the older mice. In aged mice, chondrocyte cells in the hypertrophic layer were found to be more irregular and smaller in number than in young mice. Compared with aged mice, the mandibular bone marrow of young mice was greater in number and the boundaries of trabeculae in the substantia spongiosa layer showed a less pronounced but more orderly lay-out. A significant number of pronounced blood vessels were observed in young mice. In aged mice, there was a serious degradation in the connective tissue around periosteum and around bone and cartilage tissue. The substantia compacta layer of younger mice was thicker, tighter and larger.

99% of the found in the body is within the bones and bone calcium and serum calcium are in constant mineral homeostasis, changing locations with each other constantly. Adequate amounts of calcium taken during childhood and youth period reduces bone loss rate in late menopausal period[13]. ALP functions through the release of the phosphate group and causes the mineralization for bone formation in the bone matrix [14]. Increased serum ALP levels are associated with increased osteoblastic activity. Estrogen deficiency leads to an increase in bone resorption, a decrease in PTH levels and a decrease in calcium absorption[15]. We believe that bone-specific ALP and Ca levels measured in bone tissue homogenate of our study are significantly lower in elderly mice than in young mice and this leads to morphological disorders in the mandibular bone by degeneration in bone tissue. Considering that all mice in our experimental groups are female, we describe the thickness of the proliferation and hypertrophic

layers of the mandibular bone and the intense staining of the intercellular matrix are caused by the decreased activity of ALP and Ca levels due to aging. Tnf-a promotes bone absorption by stimulating the proliferation and differentiation of osteoclasts or by activating mature osteoclasts[16]. In a study on fracture healing, whether the increase in expression of Tnf-a in osteoporosis was related to upregulation of osteoclasts was examined. As a result, it was found that upregulation of osteoplasty was correlated with increase of Tnf-a[17]. A similar situation was also noted in our study. Tnf-a levels of elderly mice were increased significantly compared to young mice. We believe that the expression of TNF-a is up-regulated and the number of osteoclasts increases in number due to aging. In the early stages of osteoblash differentiation and before the ostocalcin mRNA is expressed, gene expressions for type I collagen, osteonectin, osteopontin and alkaline phosphatase were specified[18]. In our study data, a decrease in bone-specific ALP levels was observed in aged mice and the presence of type I collagen was found to be immunopositive, whereas in younger mice, a more pronounced immunoreactivity was observed.

Aging causes collagen fibers to weaken and collapse by weakining the chemical and physical connection between them, thereby changing their microstructure[19]. According to some studies, in calcified arteries that show similarities to osteoblast cells, alkaline phosphatase is being expressed in calcified vascular cells in these arteries and type I collagens, osteopentin, osteocalcin, osteonectin and Gla proteins, which regulates the bone metabolism, are being secreted[20]. In contrast to osteoclasts, osteoblasts play a major role in the formation of new bones. In the periodontium of the elderly, osteoclasts are more common than osteoblasts, and they decrease the expression of biomarkers such as ALP and type I collagen, which are indicators for bone formation[21]. In our study, a significant decrease in aging related and bone specific ALP and Ca^{+2} levels were observed. In addition to this, as a result of our immunohistochemical stainings, it was determined that type I collagen immune reactivity was higher in young mice than in older mice. If we interpret our study data from this point of view, in the weaker points of the mandibulae, such as the mandibular chondyle, aging related tissue defects or cellular destruction can be observed in temporomandibular joint components or in the masticatory system. Some osteoblasts are generally thought to secrete bone matrix into their environment as following osteocyte transformation, they are surrounded by bone matrix Other osteoblasts continue to participate in bone block formation on the surface of the bone matrix and they transform into bone lining cells or undergo apoptosis[18]. In our study, we can explain the reason behind why type I collagen is expressed in lower amounts in older mice with the fact that cells that form the collagen protein entering apoptosis due to the physiology of aging. As a morphometric evidence of this, we can present the histopathologicaldifference in thickness

of the substantia compacta layer between young and old mice. MMP's are a group of enzymes that work in harmony with many extracellular matrix proteins that are responsible with cellular lysis during the phases of organogenesis, growth and tissue tranformation. The release and activation of MMPs from a mature tissue is limited, but there is a significant increase in various tissue pathologies such as inflammatory diseases, tumor development and metasate that cause unwanted tissue destruction[22]. In a study on gingivitis, *Boesenbergia pandurata* extract (BPE) was used as the treatment material and it has been reported to significantly reduce the synthesis of MMP-2, the number of which was increased by inflammation in elderly rats[23]. In another study, it has been reported that BPE suppresses MMP-2 expression in gingival fibroblast cells[24]. Since downregulation of osteoblast activity induces systemic bone loss, it causes rapid bone loss and has been shown to trigger an increase in MMP-2 levels[25]. Significant skeletal abnormalities characterized by impaired bone mineralization have been observed in mice as a result of disruption of MMP-2 gene[26]. It is known that enzyme activity is increased by ALP, which dephosphorylates specific areas of MMP-2[27]. The fact that our study has revealed decreases in bone specific ALP levels, increases in Tnf-a cytokine levels and PTH levels due to aging and lower MMP-2 immunoreactivity and H-Score analyses in comparison to young specimens is supportive of these studies. The decrease in MMP-2 immunoreactivity in older mice that do not have any pathological conditions other than the natural progress of aging can be explained by the decrease in osteoblast activity, cellular degradation and natural apopthosis. In addition, it can be said that structural and functional disturbances in mineralization within the bone matrix of the bone tissue, anomalies regarding structural array of collagen fibers and bone resorption all occur more often with age.

High plasma concentration or continuous infusion of PTH leads to bone destruction[28]. Osteoclastic activation includes a process that progresses very slowly. Since osteoclasts do not have PTH receptors in their cell membranes, they are stimulated especially by the IL-6 cytokine and aid in osteonecrosis[29]. Ca has an effect on the bone metabolism and it regulates the PTH serum Ca Levels, bone resorption and bone formation[29]. A study compared the long term usage of nandrol for the treatment of osteopenia and bone mineralization to a control group which did go through any treatments and it was determined that the nandrol treatment increased bone mineralization values according to the plasma PTH values[30]. In another study, it observed that daily administration of 1600 mg Ca to elderly individuals for the duration of four years have slowed down the increasing levels of serum PTH and bone resorption and decreased the rate of loss in bone tissue[31]. In a study on women, a significant increase in bone destruction, which is thought to be due to increased PTH levels, has been reported[32]. The results of our study has been similar to the studies given above. In

comparison to younger mice, significantly increased levels of PTH levels were detected in elderly mice. Since the analysis we performed is a measurement of bone tissue homogenate, it has been observed that there are large gaps filled with osteoclasts with multiple nuclei in the tissue due to age related decrease in tissue Ca content and increase in PTH levels. In this case, we think that a process that accelerates bone tissue destruction.

Since calcitonin causes some structural changes in osteoclasts by binding to the bone tissue and to the receptors in the plasma membranes of osteoclast cells, the resorption activity of these cells is lost and their number on the bone surface is reduced and suppression of bone resorption and Ca mobilization is suppressed[33]. In one study, it was stated this effect of calcitonin enables the rapid storage of Ca in the bones of young animals[34]. In another study, it was found that plasma calcitonin levels in humans were higher in adult males than in females in the same age range, and this increase was attributed to the reduction of estrogen levels due to menopause or ovariectomy[35]. In a recent study, it was noted that calcitonin inhibits the production of proinflammatory cytokines by causing a reduction in cyclogenase activity and reduces the stimulation of nociceptors in the synovial fluid and periosteum by decreasing Ca flow in the neural membrane[5]. Considering that all of the subjects we used in our study were females, a significant age related decrease in bone tissue calcitonin levels was observed in elderly mice compared to young people. We can say that calcitonin is an inhibitor of bone resorption that responds to osteoclastic function activators. According to our study data, a decrease in Ca levels and an increase in PTH and TNF-a levels were observed and this was attributed to increased osteoclast activity caused by the age related decrease in calcitonin levels.

5. Conclusion

As a result, aging causes a decrease in the amount of bone formed in the bone reconstruction cycle due to the decrease in the osteoblast support and the increases osteoclastic activity. Aging decreases the absorption of calcium from the bone tissue and decreased levels of calcium cause increase levels of PTH resorption that occur due to the need for balancing the calcium levels. And as a natural result of aging, cellular aging occurs due to dysfunctions that occur within the osteoblasts. As a result of this dysfunction the layout of the collagen fibers changes and layout deformities in fibers that should to be arranged in a manner that will increase the bone tissue endurance were observed.

6. References

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CHAPTER XV

NEONATES COVID-19 INFECTIONS: FROM SYMPTOMATICALLY TO THE TREATMENT IN PANDEMIC ERA

Cengizhan Ceylan[1*] & Şeyma Tetik Rama[2] & Hanifi Soylu[3]
[1](Res. Asst.) Selcuk University Faculty of Pharmacy,
Department of Clinical Pharmacy, Konya, Turkey
Orcid: 0000-0003-4164-9212
[1](Res. Asst.) Selcuk University Faculty of Pharmacy,
Department of Pharmacology, Konya, Turkey
Orcid: 0000-0001-5700-2419
[1](Prof. Dr.) Selcuk University Faculty of Medicine,
Department of Neonatology, Konya, Turkey
Orcid: 0000-0003-0367-859X
**Corresponding author: E-mail: c.ceylan20@gmail.com*

1. Introduction

Severe Acute Respiratory Syndrom Coronavirus-2 (SARS-CoV-2), which causes a global pandemic and originates in Wuhan, China, is a single-stranded RNA virus in the Coronaviridae family (1). While the Coronavirus disease-2019 (COVID-19) outbreak continues to affect all countries globally, according to the report of the World Health Organization (WHO) on April 20, 2021, the weekly number of confirmed cases reached 5.2 million and cumulative deaths reached approximately 3 million. The region with the highest rate was Southeast Asia (2). Human-to-human transmission of COVID-19 mainly occurs through respiratory secretions or as a result of direct contact to the infected people (3). Following an incubation period of approximately 2-7 days, most of the symptomatic patients have a fever, cough, loss of smell and taste. However, symptoms might show differences according to the virus type. In some patients, it complicates by acute respiratory distress syndrome (ARDS) (4). Neonatal COVID-19 infection is a serious concern

because neonatal mortality and morbidity, preterm birth and stillbirths were commonly seen in prior outbreaks of Severe Acute Respiratory Syndrome (SARS) and Middle Eastern Respiratory Syndrome (MERS) coronaviruses (5). The risk of viral infection in neonates is higher because their immune system is weak (6). Pregnant women may be more vulnerable to viral infection due to a variety of physiological and pathological changes. Several meta-analysis studies on viral transmission from a pregnant mother to fetus or from mother to neonate have been undertaken (7). However, our knowledge on COVID-19 in neonates is insufficient. More information is needed on maternal-to-neonate transmission, diagnosis, clinical aspects and treatment of COVID-19 infection.

In this review, our goal was to provide an overview of the diagnosis, prevention, clinical features and treatment of COVID-19 infection in neonates.

2. Diagnosis

Currently, the diagnosis of COVID-19 is made with the real-time polymerase chain reaction (RT-PCR) assay. In some studies, the sensitivity of the RT-PCR test was 63% in the nasal swab, 93% in bronchoalveolar lavage and 29% in feces (8). The details about the transmission of COVID-19 to the fetus in pregnant women and the diagnosis of COVID-19 infection in neonates is frequently discussed in perinatal medicine and every day many cases are encountered (9). COVID-19 infection can be transmitted from mother to neonate during normal or cesarean delivery through placental route or as a result of exposure to the mother's body fluids. In addition, postnatal transmission of the virus from a COVID-19 positive mother or from a healthcare worker to the neonate may be possible due to aerosol or direct contact (10).

In a retrospective analysis of six pregnant women infected with COVID-19, no virus was isolated from infant throat swabs, and COVID-19 was found to be negative in amniotic fluid, cord blood, and breast milk samples following cesarean delivery. According to another study, two out of every six pregnant mothers who are infected with COVID-19 pass the virus vertically to their baby (11,12). In another study, the baby born from a mother infected with COVID-19 did not contact the mother after birth and the results of swabs taken from the placenta were negative. However, the nasopharyngeal swab test taken from the neonate at postnatal 36 was positive (13). Finally, COVID-19 RNA was isolated from placental and fetal membrane samples by RT-PCR during delivery showing that vertical transmission is a possibility (14). However, most of the studies were reported by small sample size and we believe more studies with large sample groups are needed to reach an opinion about better clarification of vertical transmission.

The risk of COVID-19 in neonates is also related with time of the infection during pregnancy. Usually infants born to infected mother by COVID-19 are not infected or they are asymptomatic (15). In the observational study involving 44 neonates confirmed to be infected with COVID-19; 25% of neonates were asymptomatic, while the others had only mild respiratory or gastrointestinal symptoms (16). Another study found that all 18 neonates infected with COVID-19 had only mild fever (17). However if neonates show any symptom in the first days of life or if they have multiple organ involvement, it could lead to mortality and morbidity (18).

After the delivery of pregnant women infected with COVID-19, the diagnostic approach is summarized on the table (15):

COVID-19 diagnosis in neonates	Result
COVID-19 is detected by RT-PCR from blood samples either from the infant or from umbilical cord or amniotic fluid within the first 12 hours after birth.	Confirmed case
No virus is detected by RT-PCR in the nasopharyngeal swab of the neonate at birth, but anti-SARS-CoV-2 IgM antibodies are detected in the umbilical cord blood or neonate blood within the first 12 hours after birth.	Possible case
COVID-19 is not detected by RT-PCR in the nasopharyngeal swab or umbilical cord blood at birth. COVID-19 is not detected by PCR in neonate blood or amniotic fluid within the first 12 hours after birth. Anti-SARS-CoV-2 IgM is not detected in neonatal blood or umbilical cord blood.	No infection

Studies showed that the RT-PCR analysis performed 3-7 days after the infection may be negative in neonates (19). Considering the incubation time of the virus, it will be useful to perform repeated tests in the neonates to prevent errors due to false negative early test results (20). Therefore, neonates born to mothers with confirmed or suspected COVID-19 infection should be tested within the first 24 hours of life and then retested at 48 hours to minimize the possibility of missing infection (21). It becomes necessary for neonates to be closely followed by the physicians at the hospital for a while.

Accurate diagnosis is vital, especially in neonates who are more susceptible to the virus. For this purpose, antibody evaluation could also be useful in the diagnosis of neonatal COVID-19 in suspected cases. The presence of IgM antibodies resulted from fetal immune response can be found in the fetus and cord blood and could be important in the correct diagnosis of COVID-19 in the neonates (22). In addition, IgM antibodies cannot cross the placenta due to their

polymeric nature. IgM antibodies found in the neonates probably reflect fetal production. Since IgM diagnosis with Enzyme-Linked Immunosorbent Assay (ELISA) has lower sensitivity than molecular diagnostic tests, it may be more likely to encounter false-positive or false-negative results (23).

3. Prevention

Some precautions maybe needed to prevent transmission of the virus mother who has proven COVID-19 infection to infant during delivery. For this purpose physicians can discuss with the parents about immediate cord clamping during delivery, and moving it to a different room and feeding the neonate (24). Neonates born to mothers who are confirmed or suspected to be COVID-19 positive do not endanger their families or healthcare workers. According to recent research, the risk of vertical transmission from pregnant women infected with COVID-19 to the neonate is extremely low. When vaginal secretion samples from infected pregnant women are examined, the risk of COVID-19 transmission to the neonate during vaginal delivery is found to be very low because no viral RNA is found. Similarly, the vertical transmission rate is low following a cesarean section (11, 25-30).

In reports based on the consensus of experts, it is recommended that delayed cord clamping (DCC) should not be performed in mothers with positive or suspected COVID-19 to reduce the risk of transmission to the neonates during delivery (31).

According to the Canadian Pediatric Society, mothers with suspected or positive COVID-19 should not be separated from their newborns. Mothers should breastfeed their newborns while adhering to the necessary hygiene guidelines (32). While breastfeeding the newborn, the mother should be taken to a separate room. When in close contact with a neonate, all precautions should be taken to avoid contamination, and surgical masks, goggles, and gloves should be worn (33). In addition in the guideline published by the American Academy of Pediatrics (AAP) on the management of neonates born to COVID-19 positive mothers; if the mother wishes to stay in the same room with the neonate it is recommended that they be at least 6 feet (>2 m) apart (34).

Breast milk is a unique food source for neonates and has numerous benefits, especially passive antibody transmission (35). The number of data showing that viral transmission occurs through breast milk is limited (24). The viral RNA found in breast milk can be eliminated by pasteurization. In case series involving pregnant women infected with COVID-19, it has been reported that there is no virus in breast milk and it is safe for neonates (36). In addition a report has been published showing the presence in the breast milk of Ig G and Ig A antibodies that are likely to induce immunity against COVID-19 in the

neonates (37). Colostrum, which comes from the mother within 4-5 days after birth and is called the first milk, contains many immunoglobulins and immune cells that protect the neonates from viral infections (38).

However in a study on the detection of viruses in the milk samples of 46 COVID-19 positive mothers, while no virus was detected in the milk samples of 43 mothers, the virus was detected in the milk samples of 3 mothers. Only 1 of these 3 mothers' neonates was COVID-19 positive and the transmission route of the virus to this neonate could not be determined (39). Therefore, more research is needed to determine whether the COVID-19 is transmitted to the neonates through breast milk.

4. Clinical Features

Symptoms are classified as mild, moderate and severe in newborns with symptomatic COVID-19 infection. Some of the observed symptoms are high fever, difficulty in breathing, headache, difficulty in swallowing, nausea, vomiting, sore throat, pneumonia, runny nose, dry cough and rhinorrhea (40–43).

As for laboratory findings; lymphopenia, lymphocytosis, leukopenia, leukocytosis can be observed. CRP, AST, ALT, LDH, ESR, IL-6, CK-MB, D-dimer and procalcitonin values appear to be increased (44–47).

On the other hand, unilateral and bilateral findings are observed in computed tomography results (48–50).

5. Treatment

Respiratory support, oxygen, hydration and electrolyte therapy, and empirical antibiotics if bacterial coinfection is suspected are all used in the treatment of COVID-19 infection in newborns. The US Food and Drug Administration has approved Remdesivir, an RNA-dependent inhibitor of coronavirus RNA polymerase, for emergency use. Although data on remdesivir in newborns with COVID-19 are lacking, remdesivir has been used safely and effectively in the Ebola outbreak in infants under 5 days old. In addition, oral absorption is poor, therefore, it is assumed that there is probably limited absorption in neonates from the breast milk of mothers using the agent (51–53). In the presence of acute respiratory distress syndrome, nitric oxide, surfactant and high-frequency oscillatory ventilation therapy can be applied (54-57).

6. Clinical Trials

Completed clinical trials against COVID-19 in newborns are listed in the table (58).

Study Type	Number of Participants	Study Identifier	Location
A Single-Center, Retrospective, Descriptive Study	40	NCT04337320	Istanbul, Turkey
Observational (Case-Control)	202	NCT04883801	Denizli, Turkey
Observational (Case-Control)	114	NCT04362956	Buenos Aires, Argentina
Interventional (Crossover Assignment)	48	NCT04666233	Bolzano, Italy Padova, Italy
Observational (Cohort)	108	NCT04515108	Ankara, Turkey
Observational (Case-Control)	720	NCT04551690	Santiago, Chile
Observational (Cohort)	61	NCT04691934	Bursa, Turkey
Observational (Cohort)	501	NCT04368208	Poitiers, France
Interventional (Crossover Assignment)	20	NCT04359251	Poitiers, France
Interventional (Crossover Assignment)	52	NCT04417270	New York, USA
Observational (Case Only)	100	NCT04343404	Strasbourg, France
Interventional (Paralel Assignment)	227	NCT04351243	Phoenix, USA
Observational (Cohort)	470	NCT04411459	Alessandria, Italy
Interventional (Sequential Assignment)	15	NCT04400032	Ottawa, Canada
Interventional (Paralel Assignment)	405	NCT04358939	Amiens, France

Study Type	Number of Participants	Study Identifier	Location
Observational (Case Only)	313	NCT04370249	Nantes, France
Observational (Cohort)	32	NCT04863534	Milano, Italy
Interventional (Paralel Assignment)	52	NCT04581811	Alabama, USA
Observational (Other)	40	NCT04534569	Dubai, United Arab Emirates
Observational (Cohort)	132	NCT04383678	Nancy, France
Interventional (Paralel Assignment)	32	NCT04475588	Mumbai, India
Interventional (Paralel Assignment)	48	NCT04745442	Cordoba, Spain
Interventional (Paralel Assignment)	24	NCT04355728	Miami, Florida, United States
Observational (Cohort)	30	NCT04376905	Montpellier, France
Observational (Cohort)	35	NCT04435080	Istanbul, Turkey
Observational (Cohort)	22	NCT04818164	Istanbul, Turkey
Interventional (Paralel Assignment)	222	NCT04325906	Chicago, Illinois, United States
Observational (Case Only)	1589	NCT04378582	Sao Paulo, SP, Brazil
Observational (Cohort)	300	NCT04725084	Toulon, France
Interventional (Paralel Assignment)	196	NCT04311697	California, USA
Interventional (Paralel Assignment)	200	NCT04312009	Florida, USA
Interventional (Paralel Assignment)	580	NCT04311177	Minnesota, USA
Observational (Cohort)	215	NCT04449081	Meleka, Malaysia

Study Type	Number of Participants	Study Identifier	Location
Interventional (Paralel Assignment)	13	NCT04395144	Quebec, Canada
Interventional (Paralel Assignment)	1312	NCT04308668	Minnesota, USA
Interventional (Crossover Assignment)	60	NCT04442958	Istanbul, Turkey

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CHAPTER XVI

DO FAMILIES TALK WITH THEIR CHILDREN ABOUT SEXUALITY?: AN OVERVIEW OF THE LITERATURE

Zila Özlem Kırbaş
(Lecturer Dr.), Ordu Üniversitesi
e-mail: kirbas1972@hotmail.com
Orcid: 0000-0003-4030-5442

1. Introduction

Sexuality is an emotion that begins before birth and continues throughout life. A person's sexuality is closely related to that person's beliefs, feelings, personality, attitudes, behaviors, values, spiritual self, physical appearance, socialization, likes and dislikes (1).

Sexual education begins with the child asking questions about his own body in the family. Parents should follow the child's sexual development and prepare the child for the physical, emotional, spiritual, sexual and social changes they will encounter before puberty begins (2).

Sexual education should be seen by parents as a part of the child's general education. In addition, families should know that sexual education starts at birth and continues throughout life and is necessary in each developmental period of the child. Educators and parents should know that sexuality does not start suddenly with the child's adolescence, it is present from the moment the child is formed, and it will significantly affect the development of his personality (3).

In many adolescents, physical, psychological, sexual growth and developmental changes occur with puberty and menarche (4). Getting information about these changes and coping with sexuality is one of the important concepts of adolescent development. This task is usually left to unreliable sources as parents have difficulty in talking about sexuality in this period (1).

Children and young people need to have information about sexuality in order to develop desired behaviors. This is important to protect them from many

risky situations. Information and education on these issues should begin in early childhood and continue at every stage of life. The training should include topics such as body image, gender roles, sexual development, reproductive health, and interpersonal relations. With these trainings, it is aimed that individuals acquire knowledge, skills and acquire positive behavioral changes (5).

In the adolescence period, interest in sexual issues increases and the first sexual feelings and experiences are experienced. It is necessary for adolescents to provide reliable, accurate and adequate information about sexuality when appropriate, so that they can make the right decisions about their sexual life (6, 7).

Sexual education of children and young people should start in the family and the education process should continue with sexual education programs in schools. In fact, although the majority of families think that it is necessary for children to have knowledge about sexuality, sexuality is still considered taboo in many countries (8).

Preparing their children for life, transferring knowledge and values, which are among the main responsibilities of parents, have an important place in children's lives. Families are also responsible for informing their children about sexual matters. It is stated in the studies that the communication between parents and their adolescent children on sexual matters is mostly indirect, in the form of limited and uncomfortable communication (7, 9, 10).

Communication success within the family can be considered as an important factor that also reflects on sexual communication (11, 12). In studies, it is stated that adolescents have limited communication with their parents, and it is reported that sexual communication sharing is mostly related to physical development and sexually transmitted diseases (10, 13).

Parents' conversations with their children about sexual matters affect the sexual behavior of adolescents. Studies have reported that young people who receive sexual education in the family show less risky behaviors and start sexual intercourse later (14, 15, 16, 17, 18). In studies comparing the views of families and young people, it is reported that although mothers and fathers think that they have adequate communication with their children, the evaluations of young people about their parents are negative (13, 19).

Most of the families think that they are not enough to teach their children about sexual matters. In addition, since they are not sure what, when, how to talk and how to start and continue the conversation, issues related to sexuality cannot be discussed in the family. Other reasons for lack of adequate domestic sex education include parents' fears of losing authority, embarrassment, and lack of clarity about their own sexual attitudes (8, 20, 21).

Studies have reported that adolescents do not have enough knowledge about sexual matters (6, 20, 21, 22, 23, 24, 25, 26)

Some studies indicate that many families can talk to their children about sexual matters. It is also stated in these studies that adolescents mostly prefer their mothers to talk (7, 19, 27, 28, 29). However, it is stated that 34.2% of mothers do not feel comfortable talking with their children about sexual matters (27). The inability to talk about sexuality easily among family members gives negative messages to children/young people and prevents the child/young person from talking about sexuality with their families (8).

Akın et al. (2010) stated in their study that the majority of mothers think that their child should be informed about sexual matters and that the responsibility of informing belongs to the family. In addition, they report that the majority of mothers think that they have enough information to inform their children about sexual matters (30).

According to the results of the Sexual Education Treatment and Research Association (2006) study results, it is reported that 75% of the participants want sexual education to be given in schools, and 25% of them state that it is not necessary to provide sexual education. It is also stated in the study that the first information about sexual issues was obtained from the environment and friends (31). In the study of Gölbaşı and Eroğlu (2003) with 308 students, 56.2% of the students received the first information about sexuality from their friends, 64% of them stated that it was the duty of the schools, and 78.5% of them stated that giving sexual education in schools was beneficial. They report what they think will happen (24).

The parent that adolescents prefer for communication about sexuality and other issues is mostly mothers (7, 28, 32, 33, 34). Adolescents state that they find their fathers more inadequate in sexual communication than their mothers (9). It is possible to talk about the existence of the most limited communication between father and daughter (7, 32, 34, 35). It is reported that sexual intercourse is the least talked about subject (7, 33). In another study, it was reported that physical development, reproductive and reproductive health risks related to sexuality were discussed, and emotional and psychological dimensions were less emphasized (36).

2. Research on Sexual Education in the World

According to studies conducted around the world; 83% of young women in Sub-Saharan Africa, 48% of those in Asia, North Africa and the Middle East, and 56% of those in Latin America had sexual intercourse within or outside of marriage before their 20th birthday, Brazil, In Hungary and Kenya, more than a quarter of the youth aged 15-19 had sexual intercourse before the age of 15. In the USA, the proportion of males who had sexual intercourse before the age of 13 was 29.6%, and 12.8% of females. It is stated that 40.4% of girls in England,

35.7% of boys, and in Greenland, 78.8% of girls and 70.8% of boys have the age of first sexual experience (37).

According to a study conducted in Russia in 1997, 61% of adolescents stated that they learned information about sexual issues from newspapers, magazines and books, and 51% from movies and TV programs. In addition, in the results of the study, it was found that 70% of families do not talk about sexual issues with their children (38).

In a study by Nash (2002) that investigated the age of sexual experience of young people in New Zealand, it was found that the age of first sexual experience was decreasing. The rate of those who had their first sexual experience at age 13 or younger was 6.8%, the rate of those who did it at the age of 14-15 is 22.6%, the rate of those who did it at the age of 16-17, 19.4% at the age of 18-19, and 10% at the age of 21 ,8 was found. This result indicates that at least one third of New Zealand youth had their first sexual experience before the age of 16 (39).

In the study conducted by Paul et al. (2002), the rate of students who had their first sexual experience at the age of 12 and before was found to be 30.2%. As a result of this study, it was concluded that education on sexual issues is necessary to prevent sexually transmitted diseases and unwanted pregnancies (40).

In another study conducted by Main et al. (1994) in which they evaluated the effectiveness of the AIDS prevention program with adolescents, it was found that the knowledge of most of the students about HIV increased, but the program was partially effective in reducing substance abuse and sexual behavior (41).

In a study conducted by Bill (2003), it was found that 90% of adolescents received information on HIV and AIDS, and 81% on pregnancy and childbirth at school, but their knowledge on birth control methods and fertility was insufficient. In addition, it is reported that less than 1/3 of 13-year-old adolescents know about withdrawal from birth control methods, condom and birth control pills, and 2/3 of them state condom as the most effective method for protection from sexually transmitted diseases (42).

As a result of the research conducted in the USA in 2004 with 1759 parents aged 18 and over, 93% of the parents stated that sexual education greatly helps in approaching sexual problems; 3/4 of them stated that their children, who were educated on sexual matters at school, exhibited more appropriate behaviors. In addition, the subjects that parents find appropriate for sexual education to be given to adolescents; making the right decision, sexually transmitted diseases, tests for sexually transmitted diseases, waiting for the appropriate age for sexual intercourse, avoiding sexual intercourse until marriage, talking about sexuality in families, baby formation and birth control. (43).

As a result of the study by Santelli et al. (2006), in which they evaluated the sexual education given to adolescents in the USA, the rate of use of

contraception methods by adolescents increased from 38% to 58%, the rate of withdrawal from 19% to 11%, and the rate of not using any method. It has been reported to decrease from 18% to 12% (44). In their study, Dye and Upchurch (2006) stated that condom use increased in adolescents after sexual education, and 2/3 of adolescents used condoms especially in the first sexual intercourse (45).

In the study conducted by Hilton (2007), they examined the wishes of 16- and 17-year-old male students regarding the content and application method of the education program on sexual issues applied in the classroom. Male students listed their desires on content as emotions, sexuality, sexually transmitted diseases, sexual techniques, and the effects of porn. They stated that they wanted the trainings to be given with active participation learning methods and in small groups. In addition, the students stated that it is necessary for a safe and healthy sexual life to start the education on sexual issues at an earlier age and to give it intensively (46).

Kirby and Laris (2009) examined some of the programs prepared for primary and high school students in the United States. Seven of these programs are sexual abstinence, and forty-eight are programs that provide comprehensive sexual education including abstinence and contraception methods. Research results showed that of 55 programs, 15% focused only on reducing adolescent pregnancies, 45% were on STDs and HIV prevention, and about 40% covered both topics (47).

3. Researches on Sexual Education in Turkey

Studies evaluating the effect of sexual health education given to students on students' knowledge levels have reported that education is effective in informing about sexuality (48, 49, 50).

Gölbaşı (2002) conducted an experimental study to evaluate the effect of the reproductive health education program for adolescent girls. In the results of the study, there was a significant difference in favor of the experimental group students in all classes. It was found that the cases of "students finding their knowledge of all subjects insufficient" decreased significantly after the training (6).

Although the number of experimental studies in Turkey is low, various issues have been researched within the scope of sexuality and sexual education in adolescents. These; about the age of puberty, adolescent marriages and their results, sexual behaviors and attitudes of adolescents, from which source they obtained their sexual information, sexual knowledge levels, teachers, parents' sexual knowledge levels, by whom and how sex education can be given, the content of sexual education, about sexual education opinions and attitudes.

In the study of Araptarlı (1988), while measuring the knowledge levels of female adolescents about menstruation, family planning and pregnancy, menstrual patterns, menarche ages, and problems related to menstrual period were also determined. In the results of the study; Adolescents stated that they could not easily talk to their families, especially about sexual matters, and that their information about menstruation was not detailed and accurate. In addition, it was determined that the level of knowledge of adolescents about menstruation, family planning and pregnancy is very low (51).

In the study by Tapan (1995) in which the distribution of the first resources and resources used by young people on reproductive health in order of importance was examined, the first three resources for boys were found to be friends, media and mothers, respectively, and the first three resources for girls were mothers, friends and media, respectively. However, although the first sources of information of the young people participating in the research were their mothers and friends, it was seen that they wanted to get their first information from health personnel or experts related to the subject. In addition, 74.4% of the young people included in the study stated that they received their first knowledge of reproductive health between the ages of 11-14 and that they considered the second stage of primary education appropriate for reproductive health education. In the same study, it was found that female students knew more about condom, intrauterine device, the definition of AIDS, its characteristics and transmission routes (52).

In the study conducted by Kocatürk (1999) with teachers and school administrators, 79.6% of teachers encountered children's sexual questions, 63.7% had difficulty in answering these questions, 74.5% wanted to talk about sexual issues with children and it was found that 52.7 of them had a lack of knowledge on these issues. 83.5% of the teachers stated that they want to receive in-service training on sexual education. In addition, biology teachers were found to be the most knowledgeable group on this subject, and the students' approach to sexual education was found to be 100% positive (53).

In a study conducted by Tuğrul and Artan (2000) with a total of 665 mothers with children between the ages of 2-18, 40.6% of female mothers and 44.4% of male mothers stated that sexuality started in high school years. 39.4% of girls' mothers and 39.7% of boys' mothers stated that sexual education was in primary and secondary school; 29.5% of female mothers and 26.2% of male mothers stated that they should start in high school years (54).

In the study conducted by Kaptan (2001), physical and mental changes in adolescents, biological structures and functions of male and female genitalia, menstrual period and its characteristics, masturbation, sexual intercourse, sexual preferences, reproduction, birth control methods, sexually transmitted diseases and protection from them. It has been found that they have insufficient

knowledge about the roads (55). In another study conducted by Siyez and Siyez (2009), it was determined that students had moderate knowledge about sexually transmitted diseases (56). In the study conducted by Mağden (2003) to determine the level of knowledge of students about AIDS, 99% of the students answered correctly that AIDS is transmitted by blood and blood products, 99.9% of them said that it can be transmitted by sexual intercourse, and 49.6% of them said that it is possible to get into the sea. It was determined that 92.8% of them thought that it could be transmitted from mother to baby, 49.6% could be transmitted by shaking hands, and 64.0% thought that it could be transmitted by common use of glasses, forks, spoons, towels, etc. (57).

Bulut and Ortaylı (2004) interviewed 68 male workers between the ages of 23-49 to gather information on sexuality. At the end of the interview, it was determined that the main source of information about sexuality for men, although they grew up in different parts of Turkey, is their circle of friends. The interviewed men stated that it is necessary to give sexual information to their children no matter what environment they grow up in, they consider themselves inadequate in learning sexual information in the family, they are worried that talking about sexual information with children will undermine their authority, and that it is appropriate to give sexual education at school. In addition, it was stated that only two of the interviewed men received sexual education from their families (58).

In the study conducted by Biri et al. (2007), which included 128 girls between the ages of 10-19, it was seen that 85% of the participants did not receive any training on sexual issues, and 66% could not answer the question about the reproductive system (59).

In the results of the study conducted by Kaya et al. (2007) with the participation of 340 first-year students from the faculty of education; the students stated that they believed that education about sexuality was necessary, and they wanted to receive training on the first sexual experience, hymen, sexually transmitted diseases, contraception methods, pregnancy symptoms. In addition, it is stated that 89.1% of the students stated that they would like to receive this training from a specialist doctor or nurse (60).

Göçgeldi et al. (2007) conducted their study to determine the views and attitudes of mothers and fathers with children aged 3-6 about their children's sexual education. In the results of the study, the parents who participated in the study stated that their children should be educated on sexual issues, that this education should be given primarily by the child's family, and that they gave sexual education to their children. In addition, the parents who participated in the study stated that they did not have enough information about the sexual development of their children and that they would start sexual education when their children asked them questions (61).

4. Conclusion

As it is understood from the literature, the sexual health information of children and adolescents is insufficient, and they want to reach the right information on this subject. Adolescents want to get this information from reliable sources such as their families, school and health institutions. The majority of adolescents want sexual health education to be given in schools and even to make such a course compulsory. For this reason, it is inevitable to talk about a sexual education that can provide the information they need in a timely and complete manner. It may be possible to protect children and adolescents from the negative consequences of risky and unconscious behaviors in sexual matters, with a sexual health education that is initiated and continued at an appropriate age.

In Turkey, adequate and comprehensive sexual health education programs are not implemented in schools yet. For this reason, it is the responsibility of families to inform their children about sexual matters. In this regard, especially mothers have a great responsibility. However, in order for families to take an active role in sexual health education of their children, their communication skills should be developed. In addition, the information of the families should be at a sufficient level. Considering that mothers and fathers in Turkey also do not receive formal sexual health education and gain their knowledge on sexual health mostly from informal sources and experiences, it is not known how much they can help their children. The communication between the family and the adolescent on issues related to sexuality and sexual health shows how far the family can reach the adolescent.

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