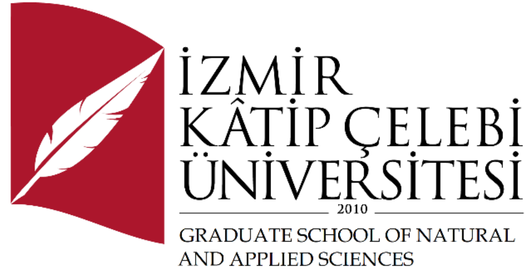

THE EFFECT OF FIBROUS DENTAL BARRIER MEMBRANE ON OSTEOGENIC DIFFERENTIATION OF HUMAN MESENCHYMAL STEM CELLS

Submitted to the Graduate School of Natural and Applied Sciences
in partial fulfillment of the requirements for the degree of

Master of Science

Biomedical Engineering

by

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July, 2022

This is to certify that we have read the thesis **The Effect of Fibrous Dental Barrier Membrane on Osteogenic Differentiation of Human Mesenchymal Stem Cells** submitted by **Bahar UTAR**, and it has been judged to be successful, in scope and in quality, at the defense exam and accepted by our jury as a MASTER'S THESIS.

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Declaration of Authorship

I, **Bahar UTAR**, declare that this thesis titled **The Effect of Fibrous Dental Barrier Membrane on Osteogenic Differentiation of Human Mesenchymal Stem Cells** and the work presented in it are my own. I confirm that:

- This work was done wholly or mainly while in candidature for the master's degree at this university.
- Where any part of this thesis has previously been submitted for a degree or any other qualification at this university or any other institution, this has been clearly stated.
- Where I have consulted the published work of others, this is always clearly attributed.
- Where I have quoted from the work of others, the source is always given. This thesis is entirely my own work, with the exception of such quotations.
- I have acknowledged all major sources of assistance.
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Date: 06.07.2022

The Effect of Fibrous Dental Barrier Membrane on Osteogenic Differentiation of Human Mesenchymal Stem Cells

Abstract

Bone healing is one of the important issues in clinical areas such as oral, maxillofacial, orthopedic, and plastic surgery. Especially in the implantation process to be performed in dental applications, the structure of the jawbone and the quality of the bone density are very important for the performance of the process. In the area of tissue engineering, there are biodegradable bone implants with biocompatible synthetic polymers that give successful results in many areas. The dental barrier membrane is a synthetic bone graft based on Poly (DL-lactide). It is used in many dental applications in the medical device field. Dental Barrier Membrane is a bioabsorbable polylactic acid (PLA) membrane designed for use in lots of applications within guided tissue regeneration (GTR) and directed bone regeneration procedures. The polymer of the membrane is the metabolite lactic acid and is reduced to CO_2 and H_2O. The base polymer has a long history of safe medical use. The Dental Barrier Membrane functions to support the initial blood clot and maintain the adequacy of collateral circulation. It provides a structure designed to appeal, capture, and retain fibroblasts, and epithelial cells, while protecting the area around the teeth for the development of bone and periodontal support tissues. In this study, it was aimed to assess the properties of the Dental Barrier Membrane to prevent cell migration and support osteogenic differentiation by using Bone Marrow Stem

Cells (BMSC) with high differentiation and proliferation properties. It is expected that the positive effect of the Dental Barrier Membrane on cell adhesion and differentiation thanks to its microfiber structure and on the prevention of cell migration thanks to the film layer surface of the Dental Barrier Membrane will be observed by experiments using BMSC.

Keywords: Dental Barrier Membrane, Bone Marrow Mesenchymal Stem Cell (BMSC), Osteogenic differentiation, Bone Regeneration

Fibröz Yapılı Dental Bariyer Membranın İnsan Mezenkimal Kök Hücrelerinin Osteojenik Farklılaşması Üzerine Etkisi

Öz

Kemik iyileşmesi oral, maksillofasiyal, ortopedik ve plastik cerrahi gibi klinik alanlarda önemli konulardan biridir. Özellikle diş uygulamalarında yapılacak implantasyon işleminde çene kemiğinin yapısı ve kemik yoğunluğunun kalitesi işlemin performansı için çok önemlidir. Doku mühendisliği alanında birçok alanda başarılı sonuçlar veren biyouyumlu sentetik polimerlere sahip biyobozunur kemik implantları bulunmaktadır. Dental bariyer membranı, Poli (DL-laktid) bazlı sentetik bir kemik greftidir. Tıbbi cihaz alanında birçok dental uygulamada kullanılmaktadır. Dental Bariyer Membran, yönlendirilmiş doku rejenerasyonu ve yönlendirilmiş kemik rejenerasyon prosedürleri dahilinde birçok uygulamada kullanılmak üzere tasarlanmış, biyolojik olarak emilebilir bir Polilaktik Asit (PLA) membrandır. Membranın polimeri metabolit laktik asittir ve CO_2 ve H_2O'ya indirgenir. Baz polimer, uzun bir güvenli tıbbi kullanım geçmişine sahiptir. Dental Bariyer Membran, başlangıçtaki kan pıhtısını destekleme ve kollateral dolaşımın yeterliliğini koruma işlevi görür. Kemik ve periodontal destek dokularının gelişimi için dişlerin etrafındaki alanı korurken fibroblastları ve epitel hücrelerini çekmek, yakalamak ve tutmak için tasarlanmış bir yapı sağlar. Bu çalışmada, farklılaşma ve çoğalma özellikleri yüksek Kemik İliği Kök Hücreleri (BMSC) kullanılarak Dental Bariyer Membran'ın hücre göçünü önleme ve

osteojenik farklılaşmayı destekleme özelliklerinin değerlendirilmesi amaçlanmıştır. Dental Bariyer Membranının mikro fiber yapısı sayesinde hücre tutunması ve farklılaşmasına ve film tabakası yüzeyi sayesinde hücre göçünü önlemeye olumlu etkisinin BMSC kullanılarak yapılan deneylerle gözlemlenmesi beklenmektedir.

Anahtar Kelimeler: Dental Bariyer Membran, Kemik İliği Mezenkimal Kök Hücresi, Osteojenik farklılaşma, Kemik Rejenerasyonu

To my lovely family…

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List of Abbreviations

TE	Tissue Engineering
BTE	Bone Tissue Engineering
MSC	Mesenchymal Stem Cell
BMSC	Bone Marrow Mesenchymal Stem Cell
2D	2 Dimensional
3D	3 Dimensional
GTR	Guided Tissue Regeneration
GBR	Guided Bone Regeneration
PTFE	Polytetrafluoroethylene
Ti	Titanium
PLA	polylactic acid
PGA	Polyglycolic acid
PCL	Polycaprolactone
PLGA	Poly (lactic-co-glycolic acid)
SEM	Scanning Electron Microscope
FBS	Fetal Bovine Serum
UV	Ultraviolet
PBS	Phosphate Buffered Saline
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
ALP	Alkaline Phosphatase
DNA	Deoxyribo Nucleic Acid
EDTA	Ethylenediaminetetraacetate

ECM	Extra Cellular Matrix
ORCID	Open Researcher and Contributor ID

List of Symbols

mL	Milliliter
μL	Microliter
μm	Micrometer
Nm	Nanometer
mm	Millimeter
℃	Celsius Degree
H_2O	Dihydrogen Monoxide
CO_2	Carbon Dioxide
mM	Millimole
CaP	Calcium Phosphate
kV	KiloVolt
N	Newton
MPa	Mega Pascal

Chapter 1

Introduction

1.1 Bone Tissue Engineering

Tissue engineering (TE) is an interdisciplinary science that works by coordinating many sub-branches of science with the engineering principle to ensure tissue regeneration. TE studies aim to regain, protect, and support functionality of tissues that have lost their function for various reasons. It aims to provide tissue restriction by applying both engineering principles and medical sciences [1]. Today, new treatments continue to be developed and maintained as a research area for many disease conditions using TE studies. TE can basically be divided into two groups. The first is soft TE, which deals with tissues such as skin, blood vessels, nerves, skeletal muscle, and the second is hard tissue engineering, which works with bone [2].

Bone is the tissue that is the subject of various studies in the field of TE for the elimination of medical conditions such as bone deformation and insufficiency. Bone Tissue Engineering (BTE) is an engineering technology that develops day by day. Bone tissue problems or bone defects may occur due to infection, tumor, osteolysis, osteomyelitis, periodontitis, or traumatic fractures [3]. There are studies aiming to address and solve many clinical issues such as spinal fusion, joint replacement, tumor treatment, pathological bone loss and fracture repair. Bone grafts and substitutes are often needed to resolve bone tissue problems [4]. Another important issue in bone tissue studies is bone regeneration. In the working area of BTE, it is known that the formed scaffolds support regeneration. Therefore, bone tissue regeneration is becoming a demand in BTE applications. Engineered bone grafts are used in many bone tissue studies. There are various methods and

applications in graft applications. There are 3 main characterizations in the bone grafting process. These are osteogenesis, osteoinduction and osteoconduction. Osteogenesis is carried out by osteoblasts, which are derived from the new bone formation matter itself. Osteoinduction is the capability to stimulate the formation of osteoblasts by the growth of bone from the surrounding tissue of a graft host site. Osteoconduction is the promotion of bone growth on the surface of a graft material [5].

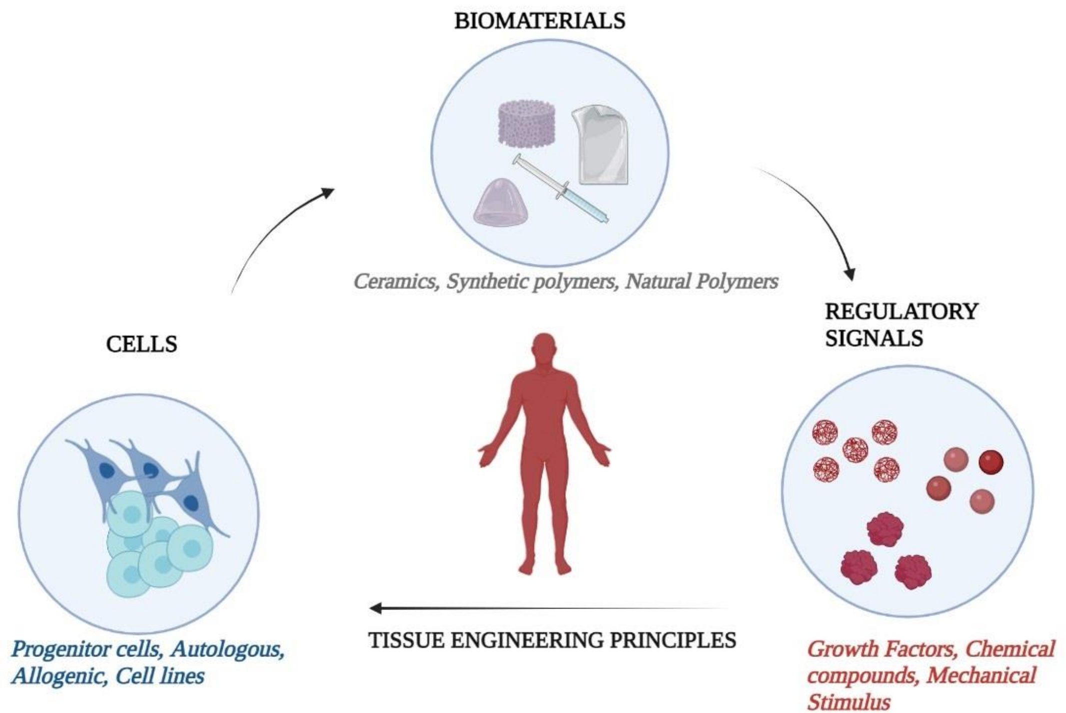

Figure 1: Basic representation of TE principle as illustration.

Bone grafts are quite diverse. Some of them are autograft, allograft, xenograft and alloplasts. The technique of bone transplantation from one's own bone tissue to another injured location of the same individual is known as autogenous bone grafts, or autografts. For the reason that autografts are derived from the host, they are immune to infection. Cortical or spongy autogenous grafts, or a combination of the two, can be used. The use of autografts is limited by factors such as the number of grafts available and the harvesting technique. Other sorts of grafts could be formed as a result of these restricting limitations. Allografts, also known as allogeneic, homologous, or homograft, are made up of tissue from a different member of the same species. Material from another species is used in xenografts, also seen as heterografts or xenogeneic transplants. Another form of graft is alloplastic grafts, frequently known as synthetic grafts. Synthetic grafts, also known as alloplastic

grafts, are artificial or manufactured materials that can be classified based on their origin and chemical makeup [6-8]. These materials come in a wide range of combinations. Such applications have made biomaterials more known. A wide variety of biomaterials currently used in BTE are available on the medical device market. In materials used for bone repair, it is important that the material is safe and biocompatible, has properties such as bioactivity and biodegradation. Bone applications are among the treatment methods frequently used in dental surgery as well as being used in interventions in various parts of the human body [9].

Table 1: Bone grafts classifications according to sources.

Human Bone Graft Tissues	**Non-Human Source Materials**	**Synthetic Materials (Alloplasts)**
Autografts	Xenografts	Bioactive Glasses
Allografts		Calcium Phosphate
		Polyglycolic Acid / Poly (L-lactic acid)/ Poly (caprolactone)
		Calcium Sulphate

1.2 Bone Marrow Mesenchymal Stem Cell (BMSC)

Nowadays, bone tissue studies are very important for many fields such as biomedical technologies, regenerative medicine, and health sciences. Stem cells, which are very popular in studies of bone tissue, have an ability to maintain the population and to regenerate themselves to create more stem cells. They are also the major cells that make up all multicellular organisms' tissues and organs, and they can heal damaged tissue by following the damaged areas. Stem cells, thanks to their active telomerase enzyme activity, they can be divided for a long time. Unlike other cells, it has the ability to produce at least one similar cell (self-renewal) that carries the characteristics of the original cell. When they receive appropriate signals, they can differentiate into one or more cell lines (multi-lineage differentiation) and functionally reconstruct a

tissue [10,11]. Based on their division-differentiation properties and origins, stem cells can be categorized in two ways. With these general qualities, one of the most extensively employed forms of stem cells is Mesenchymal Stem Cell (MSC). MSCs are multipotent which has a property to differentiate to various cell types. MSCs are stem cells that establish the foundation for stroma cells in the connective tissues, which can be differentiated in every environment and can easily pass to the damaged tissue from the tissue where it is located [12].

Thanks to its differentiation, MSC can produce many connective tissues, especially bone. The main reason for this differentiation and adaptation is the different conditions provided by the environments. Because of the connective tissue origin of MSCs, it can contribute to the development and function of the tissue cells. Thus, it has a characteristic to differentiate to connective tissue cells, bone, muscle, cartilage, tendon, and ligament cells and to other tissue cells. The Bone Marrow Stem Cell (BMSC) itself may be differentiated from ectodermal origin neurons other than mesodermal-derived adipocytes, chondrocytes, osteoblasts, and myoblasts, thanks to its renewal and differentiation properties. It has a very important role in bone repair thanks to its ability to be directed to different cells [13-16]. Thanks to BMSC high proliferation rate, differentiation, and regeneration properties, BMSC a cell line that is frequently preferred in BTE studies, especially in laboratory examinations of bone tissue biomaterials. Especially for the development of bone reconstruction techniques, MSCs were first placed in 3D biomaterials, and it was observed that they undergo osteogenesis after implantation and directly contribute to the repair of many bone defects. In another approach, it has been observed that systemic or intraosseous infusion of MSCs to patients with various bone diseases such as osteogenesis imperfecta, and osteoporosis leads to attenuation of such disease phenotypes through osteogenic differentiation of MSCs [17-23].

1.3 Biomaterials

With the importance of BTE studies in clinical applications, the need for biomaterials is increasing day by day. It is known that biomaterials are used in trauma, fracture, loss in quantity and quality bone structure, surgical support of bone tissue due to tumor reasons, orthopedics, spinal, dental and trauma surgery. It is known that the focus is

on studies performed with cells in a 3-Dimensional (3D) microenvironment compared to 2-Dimensional (2D) cell culture, which forms the basis of many cell studies [24,25]. Particularly in the biomaterials field, it has been seen that providing cells with a 3D space for proliferation, interaction and differentiation contributes to the direction of cell behavior. By providing a 3D environment to cells in biomaterials, an environment that encourages cell-cell interaction, cell-material interaction, cell proliferation, and differentiation is developed [26]. Biomaterials of interest in BTE are used in many clinical studies. The 3D structure of the biomaterial and the microenvironment it provides to the cell are important for the cell to adhere, multiply, fulfill its function and exhibit the differentiation function. It has been proven that 3D systems with synthetic or natural biocompatible scaffolds support osteogenic, hematopoietic, and neural differentiation [27]. Besides the biocompatibility properties of BTE biomaterials, their mechanical strength and specific mechanical properties support the osteogenic differentiation of cells [28]. Various biomaterials and strategies are used in implant applications in dental surgery and periodontal applications. It has been stated that the quality of the biomaterial used for effective periodontal tissue regeneration can guide the formation of new tissue by providing stem cell differentiation, and proliferation [29]. Biomaterials used in BTE are quite diverse. In order for a biomaterial to be of high quality and effective, it must have biocompatibility, biodegradability, porous structure, high porosity, surface activity, good biological and mechanical performance, ease of processing, and ease of disinfection and storage. There are two types of polymers that are frequently used in tissue-engineered biomaterials. One is natural polymers and the second is synthetic polymers [30-36].

Table 2: Examples of Natural and Synthetic Biodegradable Polymers.

Natural Biodegradable Polymers	Synthetic Biodegradable Polymers
Collagen	Polyglycolic Acid
Chitosan	Polyorthoesters
Gelatin	Polyanhydride
Hyaluronan	Polyamides
Pectins	Poly (L-lactic acid)
Casein	Poly (caprolactone)
Alginate	Poly (lactic-coglycolic acid)

In biomaterials, the concepts of osteoinduction and osteoconduction, which BTE researchers especially focus on, also gave direction to biomaterial production studies. Researchers have demonstrated the osteoconductive effect of synthetic absorbable polymer materials [37].

Natural polymers are utilized in bone and cartilage tissue studies thanks to their high biocompatibility. Its abilities are known as appropriate for cell adhesion and proliferation. Materials formed from natural polymers are highly organized in structure and contain ligands capable of binding to cell receptors [38]. Natural polymers, which are used in clinical applications because of their biocompatibility, have limitations in terms of expansion and processability. Furthermore, because the natural polymers degradation level is dependent on the enzyme, that differs from person to person, the natural polymers degradation rate differs from patient to patient [39]. In contrast to this situation, synthetic degradable polymers offer more advantages over natural degradable polymers. Because the structure and properties of the substances used in biomaterials are well known, they can be synthesized without fear of uniformity, sustainability, reliability, and immunogenicity [40].

1.4 Guided Tissue/Bone Regeneration (GTR/GBR) Therapy

TE applications draw attention to various and effective methods in restructuring damaged tissues in both engineering and medicine fields. One of the effective applications of TE is guided tissue regeneration (GTR). GTR is the practice of isolating the damaged area from other tissues and providing regeneration, and since it is a simpler application compared to other applications such as in vitro tissue reconstruction, both application and material research have been highly concentrated by researchers [41]. One of the areas where TE applications are highly effective is the studies aiming at repairing the damage of bone tissues caused by trauma, infection, and tumor formation. Bone is considered both a tissue and an organ. Bone insufficiency and damage presents very serious clinical challenges [42]. For this reason, stimulating, supporting, and repairing bone formation is of great importance in the field of TE. In bone tissue repair, a very common method in clinical medicine is guided bone regeneration (GBR) applications. It allows the application to be performed by isolating the damaged bone tissue to be applied from other tissues in order to ensure the regeneration of damaged bone tissue. It is used in many bone defect applications in clinical areas to support bone cell proliferation and to make an effective application [29,43]. GBR applications, like many other TE applications, require a biomaterial to support, stimulate, and direct cell formation. Especially in dental applications, GBR protocols for dental regeneration are frequently applied clinically today. Since the existing bone quality and quantity are important in the success of dental implant applications, these studies are of great importance. For the application of GBR, cell invasion is prevented by isolating the damaged bone area from other tissues, specifically from the gingival epithelium and connective tissue. In dental applications, products called dental barrier membranes are used for periodontal tissue regeneration, especially bone augmentation associated with implant treatments. Various barrier membranes have been used and reported for this application to date. The Dental Barrier Membrane products used are also biomaterials. There are many types of Dental Barrier Membrane products in the medical device market today [43,44].

1.5 Types of Dental Barrier Membrane

In both GTR and GBR studies, it serves to stabilize the blood clot, heal the damaged area, isolate the bone healing location from the soft connective tissues, and offer sufficient gap for bone healing [41]. In dental applications, dentists need barrier membranes for GBR/GTR applications depending on the extent of damage and to eliminate the major effects of discomfort such as potential bacterial infection. The barrier membrane application used to isolate and cover the bone is an application based on the principle of directed bone regeneration. It is an effective application used to prevent epithelial cell migration, which can prevent osteopromotion and osteogenesis, and to prevent fibroblasts from preventing bone formation with a dental barrier membrane product. In dental surgery applications, bone substitutes are applied for filling the bone defect area and then applying implants. Aside from the bone substitutes, there are a few more things to consider, such as dental barrier membrane products are used to support the regeneration of lost and damaged tissue and to inhibit the fibrous connective tissue from reaching the damaged area. In addition, the usage of a dental barrier membrane product is a very effective application for providing osteogenesis.

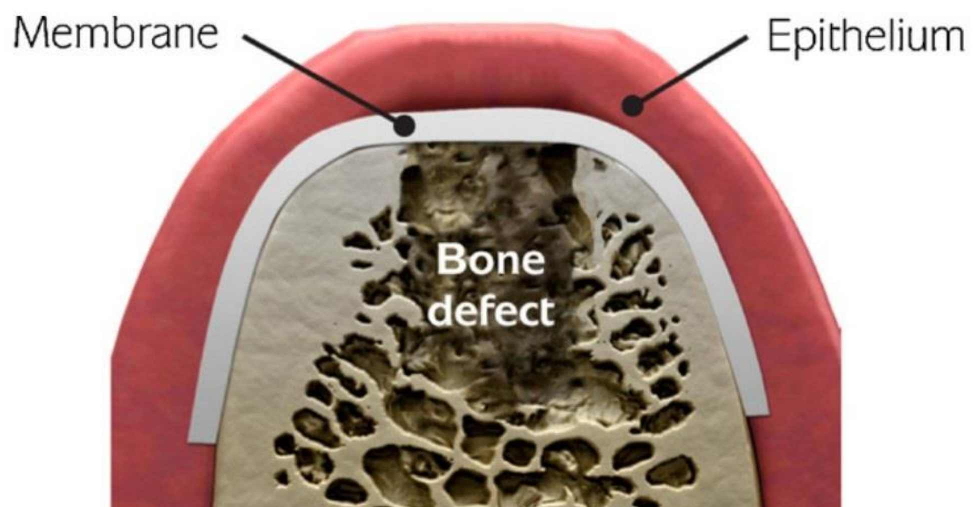

Figure 2: Illustrated representation of the Barrier Membrane placed in a GBR scenario in dental application [52].

Barrier Membranes encourage bone formation in the area where the implant will be applied, ensure that new bone tissue fills the cavity and regains its functionality, by preventing the migration of gingival soft tissues into the opened cavity [45]. The use of the GBR method in dental surgery has greatly increased the indications for implant treatments. Barrier membranes are classified as either biodegradable or non-biodegradable.

Table 3: Examples of Non-biodegradable and Biodegradable Barrier Membranes with their raw materials.

<table>
<tr><th rowspan="2">Non-biodegradable Barrier Membranes</th><th colspan="2">Bio-Degradable Barrier Membranes</th></tr>
<tr><th>Natural Polymers</th><th>Synthetic Polymers</th></tr>
<tr><td rowspan="2">Polytetrafluoroethylene (PTFE)</td><td>Collagen</td><td>polylactic acid (PLA)</td></tr>
<tr><td rowspan="2">Chitosan</td><td>polyglycolic acid (PGA)</td></tr>
<tr><td rowspan="2">Titanium (Ti)</td><td>polycaprolactone (PCL)</td></tr>
<tr><td>Alginate</td><td>copolymers (e.g., poly (lactic-co-glycolic acid) (PLGA) and poly (lactide-co-caprolactone)</td></tr>
</table>

Specific criteria must be met, including both biodegradable and non-biodegradable membranes, non-immunogenicity, biocompatibility, void-forming ability, non-toxicity, cell occlusion, tissue integration, and clinical manageability [46]. Non-biodegradable membranes are quite strong and can consistently prevent fibroblast entrance into the region during bone regeneration in order to achieve the required bone formation. In the early 1980s, a polytetrafluoroethylene-based (PTFE) membrane suitable for the GBR technique was developed for the closure of bone defects and a successful application was achieved [48]. Non- biodegradable membranes offer the benefit of retaining their shape and structure during treatment but require a second clinical procedure for removal. The disadvantage of non-biodegradable membranes is that they need a second clinical procedure to take

them, and therefore the risk of infection. Researchers have reported a very high success rate for implant applications treated with PTFE membranes, but early removal of the membrane may be required for reasons such as infection in the surrounding soft tissues [49]. On the other hand, biodegradable barrier membranes can be safely absorbed in the body and therefore do not need a second operation for removal. It is widely used in GBR applications because it does not require a second operation. Most of the biodegradable barrier membrane products available in the medical device industry are derived from animal collagen. As with other animal-derived products, unknown pathogenic material poses a risk of contamination and product quality issues. However, it is difficult to predict the rate of degradation and absorption of membranes derived from collagen. In addition, the enzymatic activity of macrophages and neutrophils can cause rapid disruption of the membrane and loss of barrier function. Considering all these disadvantages, a barrier membrane that will provide all the necessary properties for GBR application is likely to be obtained from a modified chemical, synthetic, biodegradable polymer [50]. Barrier Membrane products in the medical device market are designed to promote tissue regeneration and can be differentiated according to the biodegradability of the base material. The use of biodegradable barrier membranes has gained momentum in GBR studies. Today, various synthetic polymer-based biodegradable barrier membranes are used that do not require a secondary removal procedure, thus preventing possible surgical complications, minimizing patient discomfort and the cost of the application. In biodegradable barrier membranes, the limiting factor is related to the absorption time and the effect of degradation on bone formation [51].

Barrier membranes obtained from synthetic polymers such as poly (lactic acid) (PLA), poly (glycolic acid) (PGA), poly(ε-caprolactone) (PCL), and their copolymers are used frequently due to its properties such as manageability, adjustable biodegradation, processability, and biocompatibility [52-53].

1.6 Clinical advantages of Dental Barrier Membrane Usage

In dental barrier membranes obtained from polymers, formulation, mechanical properties, structural properties, roughness, porosity and pore structure, implantation properties and working time are very important. And because of these critical parameters, the use of all these proven products obtained from synthetic polymers is more common [54]. For barrier membrane products obtained from synthetic polymers, the formulation is important in terms of preventing soft tissue migration and not causing toxicity [55]. Its mechanical properties are important not only for the surgical procedure during the application, but also for maintaining a cavity in order to provide bone regeneration without collapsing under occlusal pressure after the operation. The porosity structure must meet the requirements for cell migration. Working time is also important for patient comfort and surgical success. For implantation, it is important because it will act as a barrier between the epithelial tissue and the graft site [56]. Considering the indications specified in the user guide of many dental barrier membrane products on the market, the clinical benefit of the product in dental surgery is observed. Many products in the medical device market have indicated indications such as sinus lifting operations, bone augmentation, GBR and GTR. The reason why synthetic barrier membranes are more preferred in clinical applications than barrier membranes obtained from natural polymers is because they have eliminated the risk of pathological material. In addition, its use gains importance in applications because the degradation process is predictable, and its biocompatibility has been proven with the awareness of the toxicity of the material [57]. In addition, many clinical studies have proven the success of the GBR technique and the use of appropriate biomaterials in bone regeneration and bone defect augmentation. In addition, dental barrier membranes are biocompatible with the biocompatibility tests that they have to be subjected to for their classification in the ISO 10993-1 standard, as well as in issues such as implantation-induced irritation, toxicity, sensitization in surgical applications.

Chapter 2

Materials and Methods

2.1 Mechanical Characterization of Synthetic Barrier Membrane

Barrier membranes support GBR procedure. While the absence of pores in the first layer prevents fibroblast migration, it is expected to support cell adhesion thanks to the porous structure of the middle and lower layers consisting of polymer fibers. The mechanical property of the membrane is expected to be resistant to surgical application. The degradation of the membrane in direct proportion to the bone regeneration in the damaged area is an important parameter and affects the choice of raw material. In this thesis, Synthetic Barrier Membrane (Ref#PM1520, Bonegraft Biomaterials Co., Turkey) product was used as experimental group and Film Layer was used as control group. Solvent casting method was applied using polylactic acid (PLA) to obtain the film layer that we used as the control group. After the 3-layer fiber structure of the barrier membrane product, the first layer of the film layer, is obtained, a jet spray process is carried out by using the solution obtained by using PLA and chloroform in order to form the 2nd and 3rd layers of the barrier membrane. 3-layer barrier membrane production is carried out by jet spray method. After application, it is dried at 20°C. The final product form is obtained. In this thesis, SEM analysis was applied, and tensile test was applied in order to mechanically characterize the Synthetic Barrier Membrane product.

2.1.1 Scanning Electron Microscopy (SEM) Analysis

A scanning electron microscope (SEM) (Carl Zeiss 300VP, Germany) was operated at 5 kV, and morphology of Synthetic Barrier Membrane was observed at Izmir Katip Celebi University Central Research Laboratory. To limit the extent of sample arcing during SEM observation, the surface of the barrier membranes was coated with a thin coating of gold using an automatic sputter coater (Emitech K550X).

2.1.2 Tensile Test

A universal testing machine having a 500N load cell (Shimadzu AGS-X Model, Japan) was used for applying Tensile Test at Izmir Katip Celebi University Biomechanics Laboratory. The tensile test of the barrier membrane samples was conducted according to the ASTM D638 standard, and the crosshead speed was chosen to be 50 mm/min. For checking repeatability, at least three times, the test was repeated.

2.2 MSC Cultivation and Proliferation

For the osteogenic differentiation study, human bone marrow derived mesenchymal stem cells (hBMSCs) (HMSC-AD-500, CLS cell lines Service, Germany) was used, procured, and cultivated in İzmir Katip Celebi University, Department of Biomedical Engineering, Tissue Engineering and Regenerative Medicine Laboratory. Cells were seeded in cell culture dishes in basal medium containing 250 ng/mL fungizone, Dulbecco's Modified Eagle Medium (DMEM): F12, 100 units/mL penicillin, 10% fetal bovine serum (FBS), 50 µg/mL gentamicin, and 100 µg/mL streptomycin. Cells were incubated at 37°C in the incubator with 5% CO_2. When the culture, whose medium was replaced every two days, reached 90% confluency, it was passaged using 0.25% trypsin/EDTA solution at the proper passed rate.

2.3 Cell Seeding

For cell seeding, passage 3 cells were utilized in the experiments. The Dental Barrier Membrane samples were sterilized by ultraviolet (UV) radiation for 1 h followed by immersion in 70% ethanol for 30 min. and then samples were washed three times with

sterile PBS. After sterilization, samples were conditioned in basal medium for 1 h, and then each sample was seeded with MSC cell suspension (5×10^6 cells/cm^2) within basal medium. The basal medium was changed with osteogenic medium (basal medium supplemented with 100 nM dexamethasone, 10 mM ß-glycerophosphate, 50 µg/mL ascorbic acid) after incubation for 24 h for cell attachment, and for up to 28 days, the cells were cultured in a humidified 5% CO_2 incubator.

2.4 Cell Viability Observation

For evaluating cell viability on well plates Live/dead Cell Viability Assay was used by fluorescent stain and fluorescence microscopy. Double Staining Kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used. In shortly, the dead cells (red fluorescence) and the viable cells (green fluorescence) were studied using a fluorescence microscope after 45 min of incubation in a culture medium supplemented with Calcein AM/DMSO (used for viable cells) and propidium iodide/purified water (used for dead cells). Cell viability is analyzed by IMAGEJ software (National Institutes of Health).

2.5 Osteogenic differentiation of MSC on Dental Barrier Membrane

Cell-seeded dental barrier membranes were rinsed with usage of PBS and lysed with 10 mM Tris supplemented with 0.2% triton in PBS at every time point (7, 14, 21, and 28 days). The lysed samples were used to determine DNA content, Calcium content and ALP activity. The samples' double-stranded DNA content, Calcium content and ALP activity were determined using the DNA Quantifcation Kit (Sigma Aldrich, St. Louis, MO, USA), QuantiChrom Calcium Assay (Bioassay Systems, Hayward, CA, USA) and QuantiChrom ALP assay (Bioassay Systems, Hayward, CA, USA). In brief, on lysed samples bisBenzimide H 33258 Solution were made and given. Fluorescence was measured by utilizing a spectrophotometer (BioTek, Winooski, VT, USA) at an excited wavelength of 360 nm, and emission wavelength of 460 nm at room temperature. ALP activity was assessed by p-nitrophenylphosphate (pNPP) in alkaline solution at 405 nm by utilizing the ALP Assay. To begin, 50 µL of lysed sample was

mixed with 200 μL of total reaction volume in a 96-well plate before adding assay bufer, 5 mM magnesium acetate, and 10 mM pNPP. On a multiplate reader (BioTek, Winooski, VT, USA), optical density (OD) was measured at the start (t=0) and after 4 minutes (t=4 min) in 405 nm. By combining 50 μL of suspension with 150 μL of working solution, the calcium content of the dental barrier membranes was determined. The OD at 612 nm was correlated to the equivalent amount of Ca^{2+} using a calibration curve plotted with reference calcium solutions after incubation. The calcium content measured at each time point during the experiments was used to calculate the total mineralized deposit of each sample. The measured ALP activities and calcium contents were normalized to cell numbers at each time point by dividing by the DNA contents.

2.6 Statistical Analysis

All experimental data was statistically evaluated by using two-way ANOVA (SPSS 12.0, SPSS GmbH, Germany) and the Student-Newman-Keuls method as a post hoc test. Using p values less than 0.05, considerable differences between groups were established. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Chapter 3

Results and Discussions

3.1 Mechanical Characterization of Synthetic Barrier Membrane

The Synthetic Dental Barrier Membrane product (Ref#PM1520, Bonegraft Biomaterials Co., Turkey) is a double-layer fiber structure with bioresorbable polylactide (PLA) as the main raw material. It is known that the barrier membrane product is produced with the jet-spraying method, and the product is presented to the medical device market as sterile by gamma sterilization method. Synthetic Dental Barrier Membrane is uniquely structured bioresorbable PLA membrane designed to be used in many applications within GTR and GBR procedures. The polymer of the membrane is metabolite lactic acid and is degraded to Carbon Dioxide (CO_2) and Dihydrogen Monoxide (H_2O). The basic polymer has a long history of safe medical use.

3.1.1 Scanning Electron Microscopy (SEM) analysis

Since cell adhesion and proliferation are targeted in biomaterials, the surface assets of the biomaterials are very important [58]. For instance, the sensitivity of osteoblasts to surface roughness is known from literature [59]. Moreover, the basis for a successful TE product is linked to highly successful mimicry of the extra cellular matrix (ECM). The extracellular matrix is a fibrous network layer with a form and biological properties that promote cell migration, adhesion, proliferation, and differentiation [60]. For this reason, the success of biomaterials is directly proportional to the success of ECM mimicking. Especially if a successful tissue regeneration is aimed, the surface

roughness of the material, ECM structure similarity and mechanical properties are important [61].

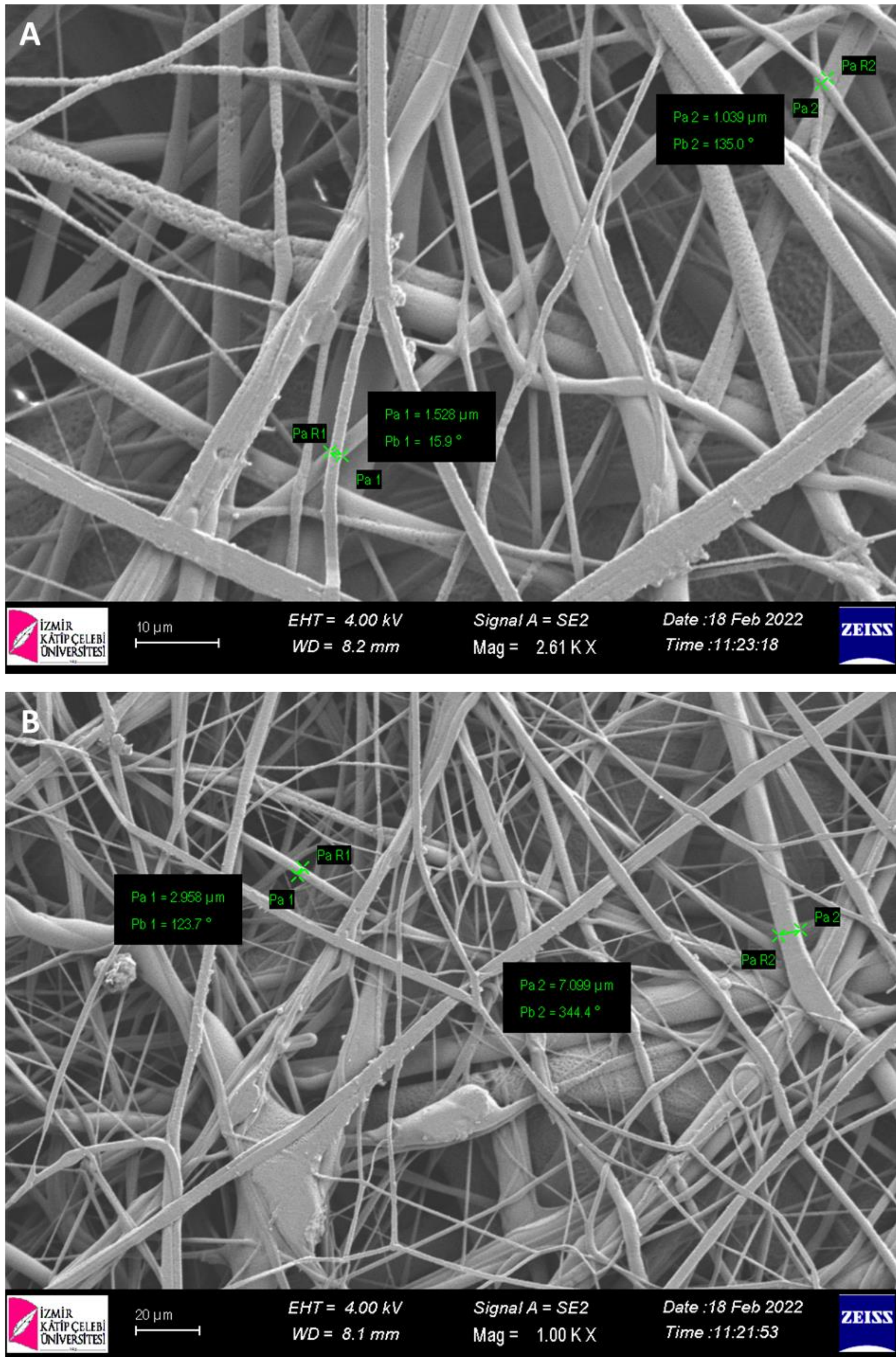

Figure 3: SEM images of Synthetic Barrier Membranes. (A) represent 10 µm SEM image, (B) represent 20 µm SEM image.

One part of the Synthetic Barrier Membrane product is the film layer, and the other side is the fibrous PLA layer. While the film layer part prevents epithelial cell migration, it targets bone stem cell proliferation, adhesion, and differentiation on the fibrous structure. As can be seen in the figure 3, SEM images of the dental barrier membrane product in different scales, there is a random, and homogenous fibrous structure in the product. Moreover, there is an interconnected fibrous structure, these morphologic properties positively effects cell behaviors, cell attachment, proliferation, and differentiation properties. Furthermore, the observed porous structure from the figure 3, supports cell adhesion, proliferation, differentiation, and cell-cell interaction, as it provides an ECM-like structure. As mentioned before, in order for a biomaterial to be of high quality and effective, it must have porous structure, high porosity, surface activity in addition to its biocompatibility and biodegradability properties. The randomized and homogeneous distribution of the fibrous structure created provides a suitable environment to stem cells must be able to self-renew and differentiate in order to be used for aimed regeneration.

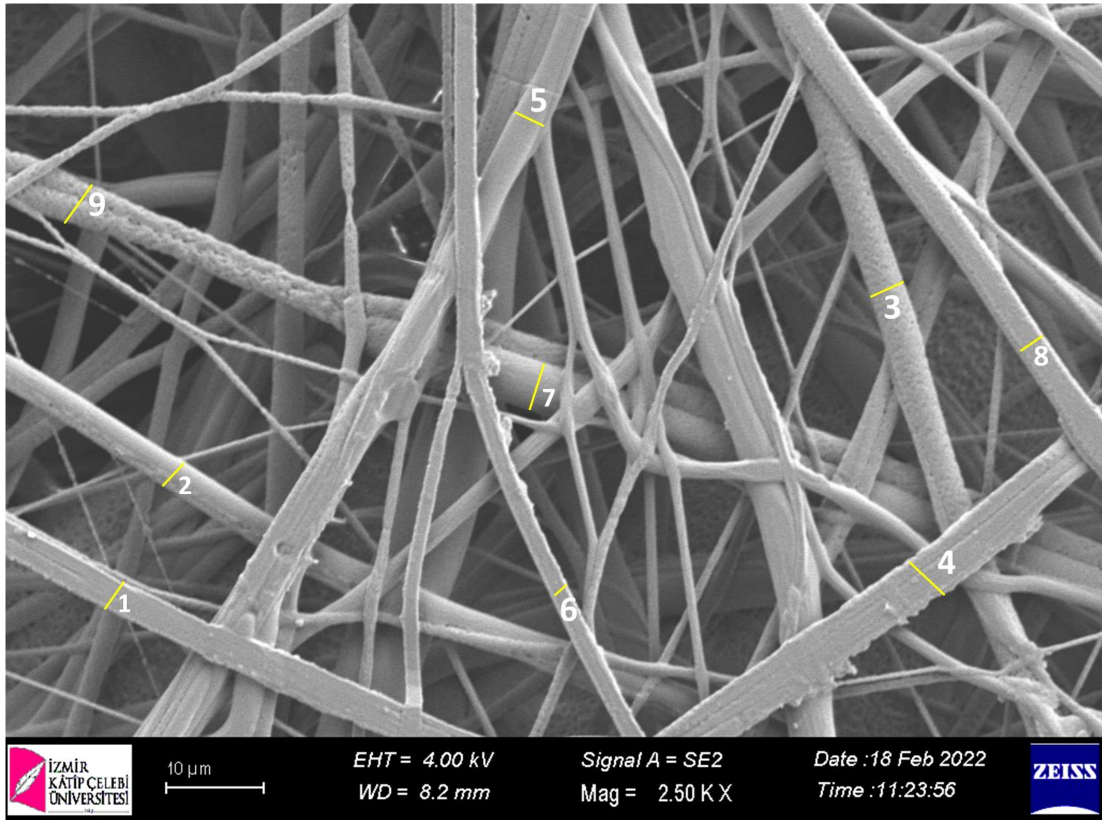

Figure 4: SEM images of Synthetic Barrier Membranes which is marked parts for diameter measurement by imageJ.

Table 4: Measurement of fiber diameter of Dental Barrier Membrane by İmageJ.

Sample	Measurement Number	Fiber Diameter (µm)
Synthetic Barrier Membrane (Ref#PM1520, Bonegraft Biomaterials Co., Turkey)	1	3.257
	2	3.362
	3	3.638
	4	4.744
	5	3.503
	6	2.072
	7	4.953
	8	3.274
	9	4.260

According to the fiber diameter measurements in Table 4, an average of 3.6 µm diameter fibers and a homogeneous fiber structure were observed. The homogeneous fiber structure creates an environment advantageous to cell adhesion, proliferation, and differentiation by mimicking the ECM. These properties provide a positive effect on the barrier membrane product, such as cell adhesion and proliferation, as well as supporting regeneration in the created cavity.

3.1.2 Tensile Test

Aimed at a successful barrier membrane application in the GBR/GTR technique, the barrier membrane must be biocompatible so that it does not cause an inflammatory effect in the implanted area, have decomposition properties suitable for new tissue construction, have mechanical and physical properties suitable on behalf of surgical application and implantation, prevent collapse and it is expected to have the strength to act as a barrier and to have the properties to act as a barrier in a way that prevents the migration of cells I do not want to be prevented from migrating [62]. For proper surgical handling and implantation, barrier membrane has a good mechanical strength. For evaluation mechanical strength value of barrier membrane tensile strength was applied.

Table 5: Tensile Strength results of Synthetic Barrier Membrane.

Sample	Tensile Strength (MPa)
Synthetic Barrier Membrane (Ref#PM1520, Bonegraft Biomaterials Co., Turkey)	1.63710
	2.04724
	1.57579

According to the tensile strength values of barrier membrane as shown in Table 5, an average of 1.75337 MPa was measured. The mechanical results of the Synthetic Barrier Membrane show that the product has proper tensile strength that allows successfully surgical application. Thanks to the fibrous structure of the barrier membrane product, it supports bone tissue regeneration as well as preventing collapse after application and optimum mechanical strength during application. With the tensile test, the mechanical strength of the product was found to be appropriate to the tensile strength value given for biodegradable barrier membrane products in the literature [63].

3.2 Cell Viability Observation

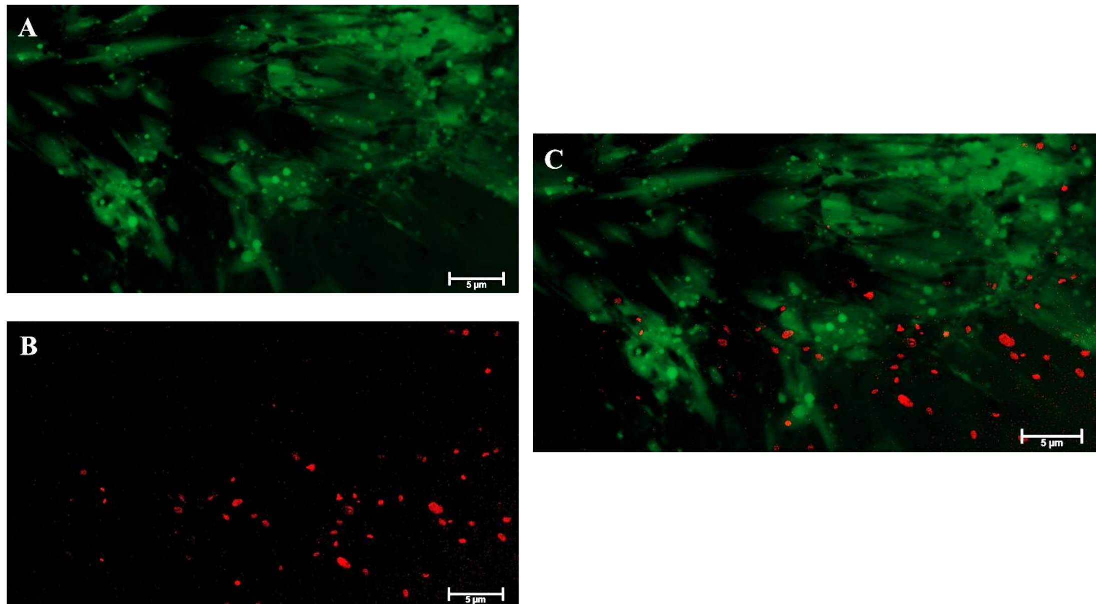

Figure 5: Application of Live & Dead Viability/Cytotoxicity assay on dental barrier membranes. A) Live cells' view, B) Dead cells' view, C) Combined image of Live and Dead cells.

Figure 5 represents the formation of living and dead cells. Cells stained with red show dead cells, and cells stained with green show live cells. Separate screenshots of live and dead cells were taken, and two images were combined to give color intensity of living & dead cells.

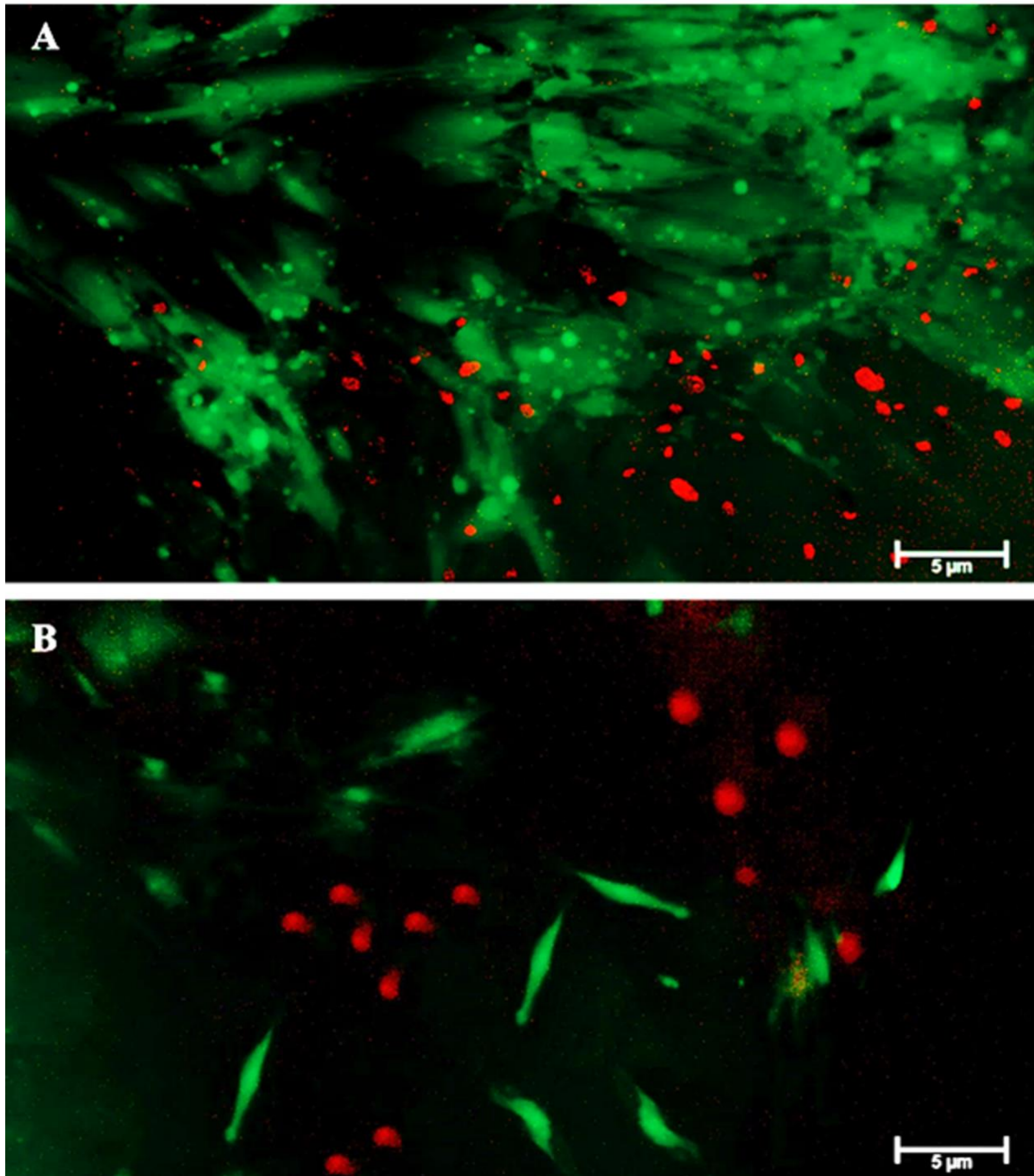

Figure 6: Combined viability images of cells on a 10x magnification scale. A) Combined viability image of the experimental group, which is fibrous barrier membrane 5mm x 5mm sample, B) Combined viability image of the control group which is film layer 5mm x5mm sample**.**

As observed in Figure 6, the viability of the cells shown in green in the experimental, barrier membrane group was considerably higher than in the control, film layer group, and the red color was quite low. However, in the control group, because there is a film layer and there is no fiber structure, it is seen that both the green cells that appear green are less, and the cell adhesion is low. These findings reveal that cell viability and adhesion were superior in the experimental group due to the fiber structure since the green color intensity in the experimental group was higher than that of the control group. Based on these, we can say that dental barrier membranes in this study can provide a good microenvironment for hMSCs seeded in vitro. As a result of mechanical tests and cell viability studies, it is supported that the surgical application of the Synthetic Barrier Membrane in the dental surgery field has a positive effect compared to the use of non-biodegradable products. The use of biodegradable barrier membranes is increasing nowadays because they do not require a secondary operation. With the raw materials, PLA, and production methods used, the product is biologically harmlessly degraded in the body by providing the necessary mechanical and biological expectations within the body. It has been observed that the expected mechanical properties of the study and the cell work done, and the cell adhesion are a product that has positive effects thanks to the fiber structures.

3.3 Osteogenic differentiation of MSC on Dental Barrier Membrane

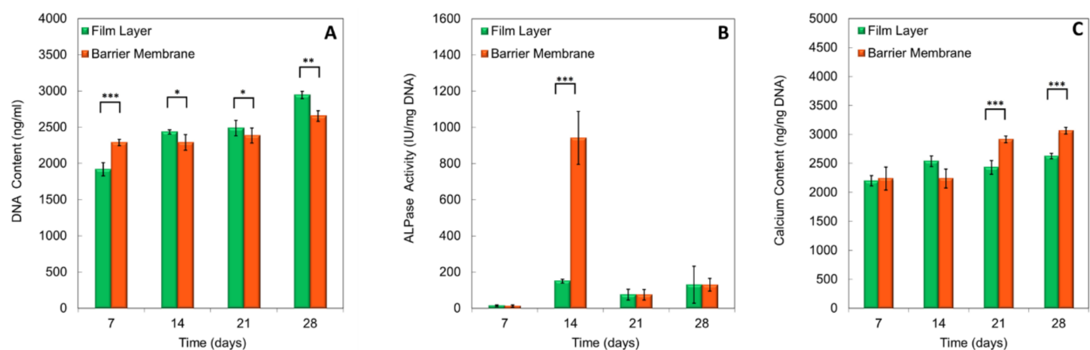

Figure 7: DNA content (A) ALP activity (B) and calcium content (C) of human bone marrow stem cells (hMSCs) seeded on Synthetic Dental Barrier Membrane and incubated in osteogenic medium for up to 28 days.

The differentiation of hMSCs into the osteoblastic pedigree is a complicated procedure that contains adhesion, differentiation, creation, maturation, and mineralization of hMSCs [64]. The most essential boundaries that could be utilized to assess osteogenic differentiation are ALP activity, adequate cell growth, Calcium content. Osteogenic differentiation of hMSCs on the Synthetic Dental Barrier Membrane product was determined by measuring DNA amount, ALP activity and calcium content up to 28 days. In this study, the evaluated ALP activity value and the calcium content were normalized by dividing the DNA content at each time point. As seen in Figure 7, a decrease in the DNA content is observed for 28 days. On the day 7, while the experimental group value was 2288.41±41.9 ng/ml, the control group value was 1919.822±91.4 ng/ml. On the day 14, the experimental group value was 2288.41±108.36 ng/ml, the control group value was 2432.64±28.85 ng/ml. On the day 21, the experimental group value was 2384.56±104. 01 ng/ml, the control group value was 2487.12±106.68 ng/ml. On the day 28, the experimental group value was 2653.79±72.17 ng/ml, the control group value was 2942.25±49.96 ng/ml. The considerable difference between the experimental group and the control group was observed on the day 7. While osteogenic differentiation increases, a decrease in the amount of DNA can be observed. This situation has also been observed in previous studies in the literature [65]. While proliferation and differentiation increase, the amount of DNA might decrease. The positive effect of fiber structure on proliferation and differentiation also shows the difference in DNA amount between the experimental and control groups.

ALP activity of the experimental group was 12.35±5.79 IU/mg DNA and control group value was 13.61±4.44 IU/mg DNA on the day 7. On the day 14, experimental group was 941.21±145.71 IU/mg DNA and control group value was 150.15±10.97 IU/mg DNA. On the day 21, experimental group was 74.34±29.38 IU/mg DNA and control group value was 74.34±30.10 IU/mg DNA. On the day 28, experimental group was 129.66±34.52 IU/mg DNA and control group value was 129.66±102.20 IU/mg DNA. ALP activity increased significantly on the 14. time point, and then started to decrease at 21., and 28. time point due to the long incubation period and mineralization. Besides, it was determined that the ALP activity of the cells on the synthetic dental barrier membrane was higher than that on the control group with a film layer, indicating the positive effect of the fibrous polymer structure of the barrier membrane on osteogenic

differentiation. In the studies conducted by previous researchers, it was seen that ALP activity reached the highest level in 14. time point, and decreased in the following time points, and results supporting osteogenic differentiation were obtained [64,65].

Calcium content is a hallmark of the maturation phase, and for this reason, Calcium content in hMSCs is expected to increase [66]. The synthetic barrier membrane experimental group and control group calcium content at time point 7, was 2238.90±196.74 ng Ca/ng DNA, and 2200.16±90.20 ng Ca/ng DNA respectively. At time point 14, experimental group and control group calcium content was 2238.90±163.16 ng Ca/ng DNA, and 2538.56±91.80 ng Ca/ng DNA, respectively. At time point 21, experimental group and control group calcium content was 2912.86±57.25 ng Ca/ng DNA, and 2430.80±119.44 ng Ca/ng DNA, respectively. At time point 28, experimental group and control group calcium content was 3066.66±58.36 ng Ca/ng DNA, and 2624.90±45.86 ng Ca/ng DNA, respectively. The synthetic barrier membrane experimental group seeded with hMSCs showed a considerable increase in calcium content (ng Ca/ng DNA) at time points 21., and 28. in the incubation period. In studies conducted by previous researchers, a meaningful increase was observed in the amount of calcium content at the 21., and 28. time points [67]. The results of this study showed that the amount of calcium reached the highest level on the 28. time point in the experimental group, which indicates that the fibrous barrier membrane contributes to the maturation of hMSCs. As a result of osteogenic differentiation studies, considering the measurement values of the experimental and control groups, the fiber structure was found to have a positive effect on hMSC proliferation and maturation, as well as a significant increase in calcium content, and peak value of the ALP activity at 14. time point.

Chapter 4

Conclusion

In conclusion it has been observed that the characterization tests before cell seeding on the synthetic dental barrier membrane are suitable for surgical application and cell adhesion. hMSCs cultivation on the synthetic biodegradable barrier membrane product was successfully carried out. Cell viability analysis and osteogenic differentiation studies were performed at the specified time points. With the cellular viability study, the physical property of the dental barrier membrane product with homogeneous fiber distribution positively affected cell adhesion, proliferation, and viability compared to the film layer which does not have fibrous structure. With the osteogenic differentiation study, it was observed that the synthetic barrier membrane product had a positive effect on hMSCs proliferation, and maturation. It is thought that synthetically obtained biodegradable biomaterials will shed light on studies that are open to development in the future, as they promote bone formation during the implantation process and do not necessitate a secondary surgical procedure.

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