DEVELOPMENT OF PRIMARY KERATINOCYTE CELL CULTURE METHOD ON FIBRIN MATRIX

by

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Bachelor's Degree, Metallurgy and Materials Engineering, Yıldız Technical University, 2019

Submitted to the Institute of Biomedical Engineering
in partial fulfillment of the requirements
for the degree of
Master of Science
in
Biomedical Engineering

Boğaziçi University
2022

ACKNOWLEDGMENTS

I would like to thank my advisors, Prof. Dr. Cengizhan Öztürk and Prof. Dr. Ethem Güneren, who has helped and added value to me at every stage of my master's thesis project since the beginning.

I would like to give special thanks to my friend Yusuf Sürücü, who was a partner in the project from the beginning and contributed to developing the project idea. We faced challenges at different stages and overcame them together.

I would like to give sincere thanks to Ebru Kanımdan and Fatmanur Babalı Balıbey, who helped me a lot in my experimental work and contributed with their ideas.

I would like to give sincere thanks to Prof. Dr. Abdurrahim Koçyiğit and Bezmailem Vakıf University Biochemistry Department, who gave me space in their laboratory and allowed me to use their laboratory equipment.

Last but not the least, my deepest gratitude goes to my Mother and other supporters who have always encouraged and paved the way throughout my life. This work is supported by a Bezmialem Vakıf University BAP project.

This thesis is dedicated to my Father Oktay Okatar.

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ABSTRACT

DEVELOPMENT OF PRIMARY KERATINOCYTE CELL CULTURE METHOD ON FIBRIN MATRIX

The use of cultured epithelial grafts in major burn cases is a promising alternative. Keratinocyte cells have the ability to proliferate and form an epithelial layer when suitable conditions are provided and simulate as a dermal substitute. However, this layer, which is produced by the cell culture method, creates difficulties in surgical application due to its fragile structure. In this thesis, a biopolymer fibrin matrix was used as a carrier layer due to the advantages it offers in terms of biocompatibility, non-toxicity, biodegradability, and benefit for cell culture. In order to produce fibrin, fresh frozen plasma (FFP) was obtained from volunteers and with only $CaCl_2$ solution, coagulation of the FFP has been achieved. In the second stage, to create an autologous cell culture method, a primary keratinocyte source is acquired directly from the human skin. Instead of a cell line, skin samples were taken from volunteers who had undergone abdominal panniculectomy surgery, and keratinocyte isolation was performed. After the formation of fibrin and the isolation of keratinocyte cells, keratinocyte cells are seeded on the fibrin matrix surface. The formation of the fibrin matrix scaffold and the feasibility of primary cell culture on this scaffold was examined and a sustainable and accessible method was established. FT-IR analysis was made to observe chemical bonds of polymerized fibrin structure. The success of cell isolation, cell culture, and the effect of fibrin scaffold on cell proliferation was demonstrated in optical microscopy, scanning electron microscopy (SEM), and pan-cytokeratin staining. The interaction of the keratinocyte cells and the feeder layer cells were visualized by SEM. In this thesis, methods of fibrin matrix formation from FFP, keratinocyte cell isolation and culture from a primary source, and keratinocyte culture on fibrin matrix were developed.

Keywords: Keratinocyte Cell, Primary Cell Culture, Fibrin Matrix, Cultured Epithelial Graft

ÖZET

FİBRİN MATRİS ÜZERİNDE PRİMER KERATİNOSİT HÜCRE KÜLTÜR YÖNTEMİNİN GELİŞTİRİLMESİ

Majör yanık vakalarında kültüre epitel greftlerinin kullanımı hayat kurtarıcı bir alternatiftir. Keratinosit hücreleri, uygun koşullar sağlandığında çoğalma, bir epitel tabakası oluşturma ve dermal bir ikame olarak simüle etme yeteneğine sahiptir. Ancak kültür yöntemiyle üretilen bu tabaka frajil yapısı nedeniyle cerrahi uygulamada zorluklar yaratır. Bu çalışmada biyouyumluluk, toksik olmama, biyobozunurluk ve hücre kültürüne uygunluk açısından sunduğu avantajlar nedeniyle taşıyıcı tabaka olarak biyopolimer fibrin matrisi kullanıldı. Fibrin üretmek için gönüllülerden taze donmuş plazma (TDP) alınmış ve $CaCl_2$ solüsyonu ile TDP'nin pıhtılaşması sağlandı. İkinci aşamada, otolog hücre kültürü yöntemi oluşturmak için doğrudan insan derisinden bir primer keratinosit kaynağı elde edildi. Abdominal pannikülektomi ameliyatı geçirmiş gönüllülerden deri örnekleri alınıp keratinosit izolasyonu yapıldı. Fibrin oluşumu ve keratinosit hücrelerinin izolasyonundan sonra, fibrin matris yüzeyine keratinosit hücreleri ekildi. Fibrin matris iskelesinin oluşumu ve bu iskele üzerinde primer hücre kültürünün uygulanabilirliği incelendi ve bir yöntem oluşturuldu. Polimerize fibrin yapısının kimyasal bağlarını gözlemlemek için Fourier Transform Infrared Spektroskopisi (FT-IR) ile analizi yapıldı. Hücre izolasyonunun başarısı, hücre kültürü ve fibrin iskelenin etkisi ışık mikroskobunda, taramalı elektron mikroskobunda (SEM) ve pan-sitokeratin boyama ile gösterildi. Keratinosit kültürünün hücre tutunumu, taramalı elektron mikroskobu (SEM) ile incelendi. Bu tezde insan kanından fibrin matris oluşumu, insan derisinden keratinosit hücre izolasyonu ve kültürü ve fibrin üzerinde keratinosit kültürü yöntemleri geliştirildi.

Anahtar Sözcükler: Keratinosit Hücresi, Primer Hücre Kültürü, Fibrin Matris, Kültüre Epitel Grefti

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LIST OF SYMBOLS

Ca_2Cl	Calcium Chloride
Ca^{+2}	Calcium Ion
g	Gram
cm^2	Centimeter Square
m^2	Meter Square

LIST OF ABBREVIATIONS

FFP	Fresh Frozen Plasma
FT-IR	Fourier Transform Infrared Spectroscopy
SEM	Scanning Electron Microscopy
UV	Ultraviolet
TBSA	Total Body Surface Area
ECM	Extracellular Matrix
CPDA-1	Citrate-Phosphate-Dextrose Adenine-1
PBS	Phosphate Buffered Serum
EDTA	Ethylene Diamine Tetra Acetic Acid
RPM	Round Per Minute
SE	Secondary Electron
PV	Polycythemia Vera

1. INTRODUCTION

1.1 Motivation

Skin burns are an event that can happen to anyone at any time. The epidermis is located in the top layer of the human skin, which is one of three layers of human skin[1]. The Loss of the epidermis makes it difficult to heal skin and can lead people to death. Traditional treatment methods are insufficient and even if the healing occurs, scar tissue develops and the unaesthetic look will remain for a lifetime. Especially in cases where there is insufficient donor area, patients cannot recover. Cultured epithelial graft offers promising results for burn cases. There are many cultured epithelial graft studies in the literature[2],[3],[4].

Scaffolding is made with synthetic or natural polymers to be the carrier layer in cultured cell systems. Such scaffolds require biocompatibility, non-toxicity, and biodegradability [5]. Fibrin, a human-derived and natural polymer, has been used in cultured epithelial graft studies [6]. This bio-polymer can be used in many biomedical applications [7], [8]. Fibrin is formed when fibrinogen monomers coagulate and become polymerized and cross-linked[9].

Keratinocyte cells are a type of cell commonly found in the epidermis. These cells are employed in wound healing and skin regeneration[1]. Keratinocyte cells have the ability to multiply by cell culture when suitable conditions are provided. During cell culture, it is necessary to provide the molecules that keratinocyte cells will need. Appropriate molecules are delivered to the cells with the medium, and some molecules such as epithelial growth factor (EGF) can be delivered using the feeder layer in the same culture medium. Human dermal fibroblast or 3T3 murine fibroblast can be used as the feeder layer. Keratinocyte cells, on the other hand, can be used directly from human origin or by purchasing a primary or frozen isolated cell line.

1.2 Objectives

This thesis, it is aimed to make a cultured epithelial graft with fibrin by producing a fibrin scaffold from human blood and cultivating human-derived primary keratinocyte cells. For this purpose, fresh frozen plasma (FFP) and full-thickness skin samples taken from volunteers were used. The coagulation of fibrinogen was initiated in vitro and analyzed by Fourier-Transform Infrared Spectroscopy (FT-IR) after the fibrin scaffold was formed. Keratinocyte cells isolated from the primary source on the same fibrin scaffold were cultured and analyzed using Scanning Electron Microscopy (SEM) and analyzed by immunocytochemical staining.

Fibrin was preferred as the scaffold due to its high biocompatibility and biodegradability [5]. In addition, it is aimed to provide mechanical strength to the cultured epithelial structure with the mechanical strength of fibrin. On the other hand, it is aimed to introduce a fibrin structure formation method to facilitate the use of autologous cultured epithelial tissue in people with skin burns. The main objectives of this thesis are:

1. Coagulation of FFP using only $CaCl_2$ and production of fibrin scaffold.
2. Separation of epidermis from full-thickness skin sample and then isolation of keratinocyte cells.
3. Culture of keratinocyte cells and characterization of cultured cells.
4. Seeding of keratinocyte cells on the fibrin scaffold and characterization.

2. BACKGROUND

2.1 Skin

Skin is the largest organ of the human body. While it constitutes approximately 16% of a person's body weight, it covers a surface area of approximately 1,8 m^2 on the body and its average thickness is 1-2 mm. It is a metabolically active organ with various vital functions necessary for the maintenance of homeostasis in the body. It acts as a barrier against chemical, physical and biological substances, prevents the loss of water, and helps regulate body temperature [10]. The skin also serves as a sensory organ and provides a surface from which one can perceive their surroundings. In addition, it protects the body from harmful rays with a pigmentation system that absorbs ultraviolet (UV) radiation [11].

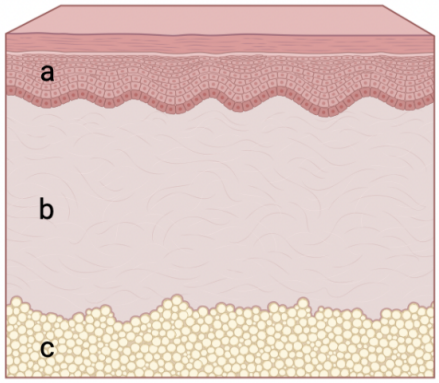

Figure 2.1 Layers of Human Skin: a. Epidermis b. Dermis c. Hypodermis (made at www.biorender.com)

Human skin is typically composed of three main layers as shown in Figure 2.1: the epidermis, dermis, and hypodermis (subcutaneous). The epidermis is the outermost layer of the skin, consisting of a high amount of keratinocyte cells. The dermis, is a layer under the epidermis, mostly containing fibroblast cells, which provides the skin with its structural integrity and elasticity. Between the dermis and epidermis, there is a basal membrane that separates both layers. The hypodermis, the innermost layer

of the skin consists of adipose tissue, which protects the body from mechanical effects. The epidermis consists of 97% keratinocyte cells. Apart from keratinocytes, there are also melanocytes, Langerhans cells, and Merkel cells. The epidermis is divided into four layers by the differentiation of keratinocytes. These layers are from outside to inside: stratum corneum, granular layer, spinosum layer, and basal layer. The main components of the dermis are collagen fibers, elastic fibers, and extrafibril structures made by dermal fibroblasts. Collagen fibers make up about 70% of the dermis. The total amount of collagen in the skin is 80% for collagen type I and 20% for collagen type III [1].

2.1.1 Skin Burn Injuries

Skin burn injuries are an event that can happen to all people at any time. It can cause skin loss and can be caused by many physical and chemical factors. However, burn injuries are mostly caused by direct contact with hot liquid or fire [12]. In heat-induced burns, besides the higher the temperature, the longer the contact and the heat transfer feature are also important. For example, hot water spilled on the clothes may come into contact with the skin for a longer time and cause a deeper burn than without clothes[12]. Skin burns are not only caused by heat. In very cold environments, skin burns can occur as a result of freezing and crystallization of the water in the cells in the skin layer and the death of the cells [13].

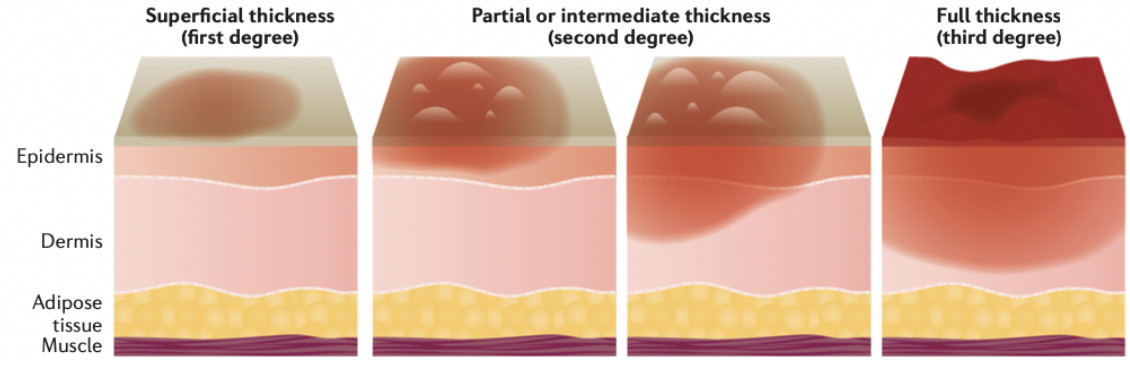

Figure 2.2 Skin Burn Degrees[14]

2.1.2 Skin Burn Evaluation and Degrees

When there is a burn case, it is evaluated in terms of the cause, the depth, and size. The types of skin burns are shown in Figure 2.2. Burns that damage the epidermis in the uppermost layer are classified as first-degree burns. When viewed from the outside, redness is seen and short-term pain is experienced. Second-degree burns go below the epidermis and damage the dermis. It weeps and causes pain. For its treatment, dressing is required and scarring may occur, but it does not require surgery. In deep partial-thickness burns, it is less painful, and blistering is not observed due to the complete destruction of the pain receptors. It requires surgery for its treatment and leaves scars [14].

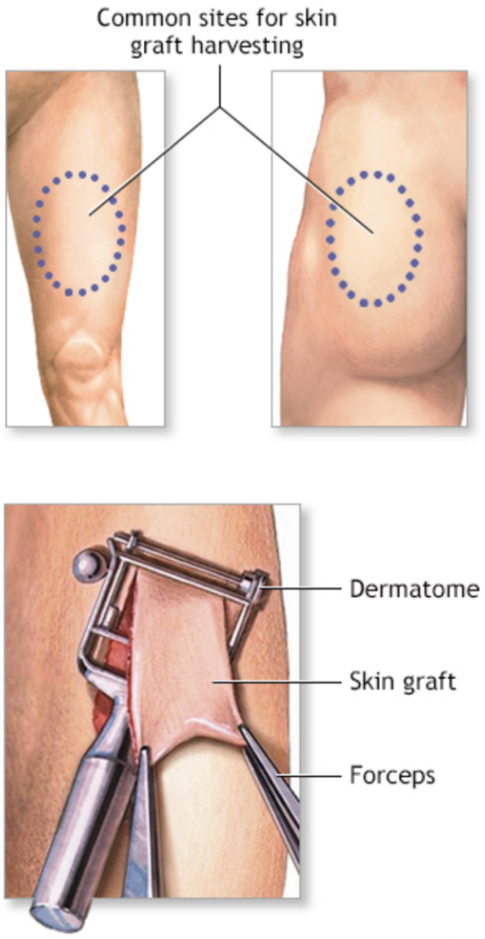

Figure 2.3 Common Sites for Skin Graft Harvesting [15]

In third-degree (full-thickness) burns, cell damage extends to the very bottom

of the dermis. The patient becomes very vulnerable to the risk of infection and surgical patching is necessary. In terms of size, it is expressed as the ratio of total body surface area (TBSA) during evaluation. In terms of size, burns are used in two sizes minor and major. Minor burn is usually expressed when the TBSA is <10%. Major burns are considered >10% TBSA, but in children major burns are if >30% TBSA [14].

2.1.3 Treatment of Skin Burns

First and second-degree burns can be treated without the need for surgery. However, deep partial-thickness and third-degree burns require surgical operations. The most widely used method is autografting in burn surgeries (Figure 2.3). A patch is taken from the healthy (donor site) parts of the patient with split thickness grafting, and the burned areas are closed. Thus, the risk of infection and pain can be reduced and early recovery can be achieved. Split-thickness skin grafts are taken from the undamaged areas of the patient using the dermatome tool. If the burn surface area is larger than the donor surface area, the split-thickness skin graft taken has meshed and its area is enlarged (Figure2.4). As in Figure 2.5 can be seen that healing with autologous split-thickness grafting is good but all the damaged areas could not be covered and scar tissue has occurred. If the burn area is too large and the mesh is stretched too much, allografts taken from a cadaver are used to temporarily cover it [16]. The reasons for the failure of the autograft method are due to donor site insufficiency, rupture, or infection [17]. In cases where time is needed for it to heal itself, the open burn wound can be closed temporarily with allograft or xenograft [2].

Artificial skin substitutes can be used in cases where there is insufficient donor area [2]. These artificial skins made with biological or synthetic materials provide temporary or permanent closure of burn wounds [3],[4]. Skin substitutes provide re-epithelialization and in deep partial thickness skin burns as well as the barrier feature that closes the wound [19]. Cultured epithelial autograft can be used in areas with very large burns. A cultured epithelial autograft can be obtained by isolating and multiplying keratinocytes from a piece of skin provided by the patient. Besides being

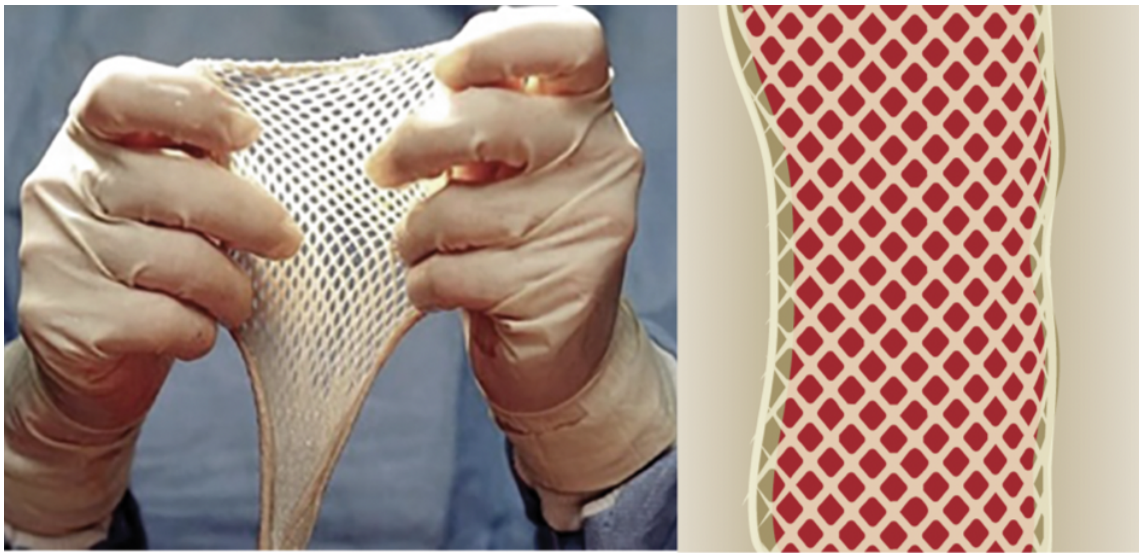

Figure 2.4 Split Thickness Skin Graft Meshing and Increasing the Cover Area [18]

ideal for large-area burns, it has some disadvantages. These include fragility, cost, difficulty in manufacturing, and difficulty in implementation [20].

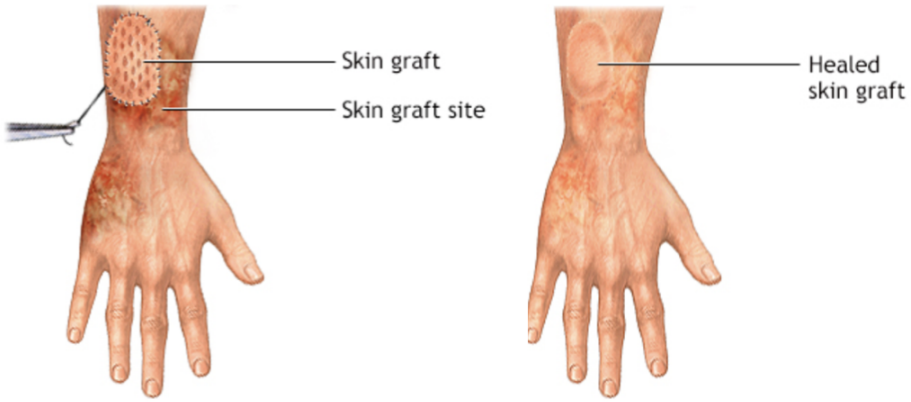

Figure 2.5 Healing of Skin Burn With Skin Graft and Scar Formation After Healing [15]

2.2 Skin Tissue Engineering

Tissue engineering is a multidisciplinary field and consists of three main components. Tissue scaffolds, cell culture, and biochemical molecules are key elements of tissue engineering. Today, many tissues are produced with a tissue engineering

approach and there are studies for the translation of tissue engineering products for humans' benefit. The first tissue-engineered product offered for the benefit of humans is tissue-engineered skin or cultured epithelial autografts [21]. There are many available products for skin healing as shown in Table 2.1.

Table 2.1
Available Commercial Skin Tissue Engineering Products[14]

Company/Brand Name	Type of Graft	Country
Epicel (Vericel)	Cultured epithelial autologous skin grafts	USA
ReCell (Avita Medical)	Autologous cell suspension	USA
MySkin (Regenerys)	Autologous cell suspension	UK
SkinGun (RenovaCare)	Autologous epidermal and stem cell suspension	USA
Keraheal	Autologous cultured cell suspension and fibrin glue	South Korea
Integra	Collagen matrix and silicone layer for temporary cover	USA
OASIS Wound Matrix	Derived from the porcine intestine for temporary cover	USA
Matriderm (MedSkin)	Collagen and elastin for temporary cover	Germany
NovoSorb BTM	Biodegradable polyurethane foam for temporary cover	Australia
MatriStem	Derived from porcine for temporary cover	USA
Alloderm	Decellularized human cadaver for temporary cover	Ireland
SUPRATHEL	Synthetic polymers blend for temporary cover	Germany

Skin tissue grafts produced by skin tissue engineering approaches have been developed to close the wound permanently or temporarily, creating a mechanical barrier against infections and fluid loss [22]. In 1975, Howard Green and his collaborator James Rheinwald started a new era in keratinocyte cell culture techniques [23]. 3T3 mouse fibroblasts were exposed to radiation to create a feeder layer on which keratinocytes were to be cultivated. An epithelial layer was formed by using keratinocyte cells. With the help of this technique, it was possible to obtain the desired amount of cultured epithelial autografts (CEA) based on a small biopsy sample taken from a patient. As a result of 3-week cultivation of cells obtained from a skin biopsy sample of 1 to 5 cm^2, an epithelial cell layer can be obtained and will cover up to 70% of the total body surface [24]. Subsequently, Cuono et al. (1986) [25] used epidermal autografts in combination with dermal allografts, adding a new dimension to the technique. This was followed by a new skin equivalent developed at Bell laboratories. The dermal layer formed by keratinocyte cultivation on a collagen matrix containing fibroblast cells was completed with an epidermal conjugate, creating a bilayer model [26].

Figure 2.6 Human Skin Substitutes and Available Commercial Brands[27]

In other studies, Yannas and Burke created a new skin conjugate with a silicone cover over a dermal conjugate containing collagen (from bovine) and chondroitin-6-sulfate (derived from shark cartilage). Successful results were obtained by using the new product in the clinic under the name of Integra Regeneration Template [28],[29]. There are many different types of brands used as human skin substitutes (Figure 2.6). Unfortunately, a skin tissue engineering product that fully meets the complex structure of normal human skin is not yet available. However, many skin conjugates are known to partially replace skin tissue [30].

2.2.1 Skin Tissue Engineering Scaffolds

A combination of cells, biocompatible materials, and cell signals are required for effective tissue healing and skin regeneration. Today, biomimetic scaffolds, called tissue scaffolds, are produced using various biocompatible materials and different techniques and are used in many treatments. Especially in chronic wounds, in cases where there is no or very slow healing, it is aimed to stimulate the wound in various ways to gain healing potential and at the same time reduce the risk of infection by covering the open

wound. There are numerous studies and products, produced from cellular, cell-free, drug-loaded/unloaded, natural, or synthetic polymers. Natural dermal matrices such as AlloDerm, which are widely used in clinical applications, have a human-derived ECM structure. Tissue scaffolds produced with natural materials such as alginate, fibrin, and collagen that mimic the ECM structure are also used. Dermal matrices allow wound closure and rapid formation of resident cell infiltration and granulation tissue [31].

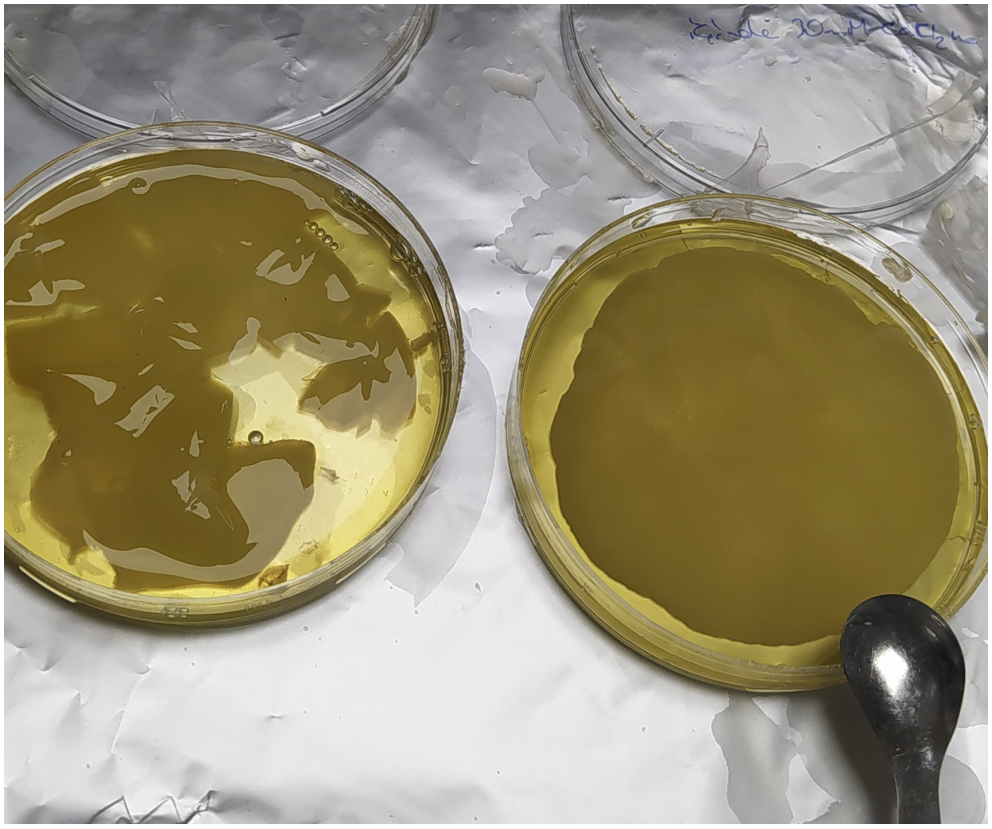

Figure 2.7 Fibrin Scaffold Samples Inside Petri Dish

Synthetic, cell-free scaffolds such as Integra act as scaffolds for cell proliferation, provide an external barrier to the wound, and allow site remodeling. While the collagen is found in the appropriate order in the newly formed tissue, the lack of other dermal elements such as elastic fibers and nerves is observed. Biomimetic hydrogels are seen as a promising alternative for dermal regeneration. These hydrogels are supported by cells and can accommodate cell-based treatments, and they keep the wound area moist with their high water retention properties [32]. One example of a hydrogel scaffold has been shown in Figure 2.7 which is used in this thesis.

The addition of other factors such as cells, growth factors, and biological agents to bio-mimetic scaffolds alters the micro-environment of the damaged area and promotes wound closure. Intensive research is carried out in this area to find the structure and composition of the most suitable material, the molecules released into the damaged area and the cell types used [33].

2.2.1.1 Scaffold Materials. The biomaterials used in skin tissue engineering are expected to protect the wound against infections by forming an external barrier, biocompatible, biodegradable, highly porous, accelerate the healing process, and be cost-effective [34]. Considering these features, the use of natural polymeric materials as scaffolding becomes important. Natural materials are biologically active and promote cell adhesion and growth. In addition, the biodegradability of natural materials allows cells in the matrix or cells from surrounding tissues to replace the dissolved scaffold by producing their own ECM [35]. While the skin tissue mostly contains keratinocytes and fibroblast cells, it also contains glycosaminoglycans such as collagen, elastin, fibronectin, laminin, hyaluronic acid, controlin-6-sulfate, dermatan sulfate, heparan sulfate. Since natural polymers are generally preferred in the production of cell scaffolds, various tissue engineering studies are carried out by using ECM elements [36]. The use of decellularized scaffolds as natural materials is also common. These scaffolds are ECMs obtained by removing cells from tissues using various chemicals to prevent immune response formation [37].

2.3 Fibrin

Fibrin is a biopolymer composed of fibrinogen monomers. In blood plasma, fibrinogen proteins are dispersed and start polymerization only when the coagulation cascade starts. With the effect of thrombin and Ca^{2+}, it takes the form of fibrin and insoluble clot forms in the blood. This clot is a material that is effective in wound healing and skin regeneration because of its mechanical properties [38]. Fibrin has been used as a biopolymer scaffold in tissue engineering studies due to its porosity,

biodegradability, elasticity, and biocompatibility [5]. The cells show the ability to bind to the fibrin scaffold. This binding occurs as a result of the interaction of alpha-C regions in the fibrin structure and fibronectin [9].

2.3.1 Structure of Fibrinogen

Fibrinogen is a 45nm long blood plasma protein that is a building block of fibrin. It is a glycoprotein found in human blood plasma at a concentration of 1.5-3mg/L. It consists of two pairs of A-alpha, two pairs of B-beta, and two pairs of gamma chains linked by 29 disulfide bonds. The A-alpha, B-beta, and gamma chains consist of 610, 461, and 411 amino acids, respectively, and their molecular weights are 67, 55, and 47.5 kD. These fibrinogen chains, each of which is encoded by a separate gene, are connected to the central E region, also called the central nodule, with spiral structures. The E region forms the origin of the N terminal ends of the 6 chains that make up this helical structure. The helical structure has a D region at both ends, and the C-terminal ends have individually folded gamma- and beta- nodules. The D and E regions play an important role in the regulation of fibrinogen, its polymerization to fibrin, the formation of crosslinks, and platelet interaction. The C terminal end of the A-alpha chain is called alpha-C. The crystal structure and attachment points of fibrinogen are shown in Figure 2.8 [39] [40] [41].

2.3.2 Mechanism of Fibrin Polymerization and Cross-linking

In the central region of the fibrinogen molecule (site E), there are two polymerization sites (knob A and B) protected by fibrinopeptides A and B. With the presence of thrombin, the fibrinopeptide structures are separated from the amino-terminal regions of the A-alpha and B-beta chains of fibrinogen, and the fibrinogen molecule turns into a fibrin monomer. The holes a and b (polymerization sites) on the two outer D regions are complementary and bond with the knob A and B in the central E region. Monomeric fibrin molecules self-assemble via polymerization sites to form rod-shaped

fibrin fibers. In Figure 2.9 the polymerization process has been shown [9].

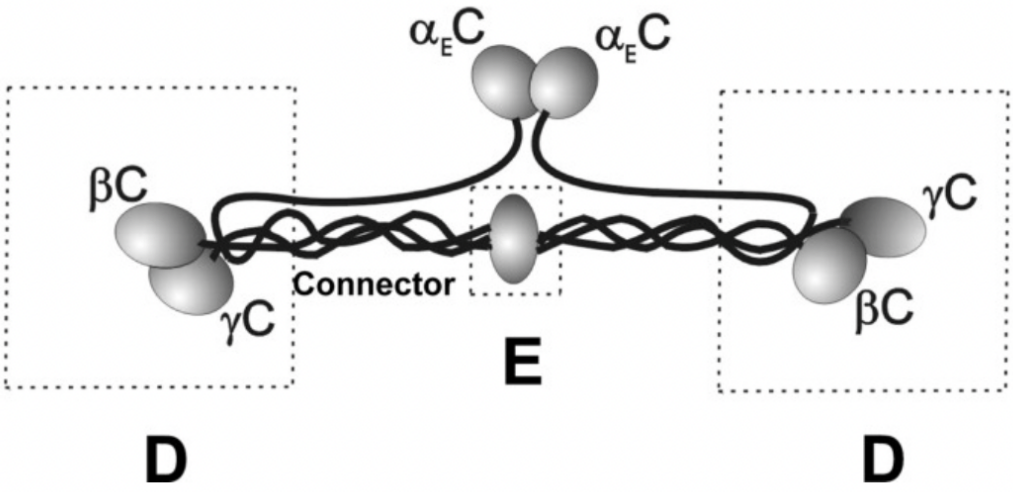

Figure 2.8 Scheme of a Fibrinogen Structure [42]

Cross-linking of the fibrin structure is mediated by the fibrin stabilizing factor (FXIII), and FXIII is a transglutaminase catalyst. FXIII; has a tetrameric (A2B2) structure consisting of two A and B subunits. With the separation of the A and B subunits, FXIII becomes active [43]. The A subunit has catalytic properties. The separation of the two subunits from each other takes place in the physiological process in which thrombin and Ca^{2+} follow each other. First, it causes hydrolysis of the peptide bond between the Arg37 and Gly38 residues on the N-terminus of the thrombin A subunit, resulting in the release of the activation peptide. Then, with the presence of Ca^{2+} in the environment, the B subunits are separated from the structure and the FXIII-A2 dimer is enzymatically activated (Gly38-FXIII-A2*). During this conformational change, the masking of the cysteine active site on the A subunit is prevented and it is allowed to react with its substrates. Increasing the amount of Ca^{2+} alone, without requiring a proteolytic pooling for the activation of cellular FXIII (cFXIII), converts the structure to the active form [44] [45].

Fibrin polymer is an ideal substrate for fibrin stabilizing factor (FXIII). FXIII; Activated by thrombin and Ca^{2+}, it transforms into FXIIIa form, by performing fibrin cross-links, it forms gamma dimers and alpha polymers. Crosslinking between alpha chains occurs more slowly. Cross-linking occurs between multiple glutamines and

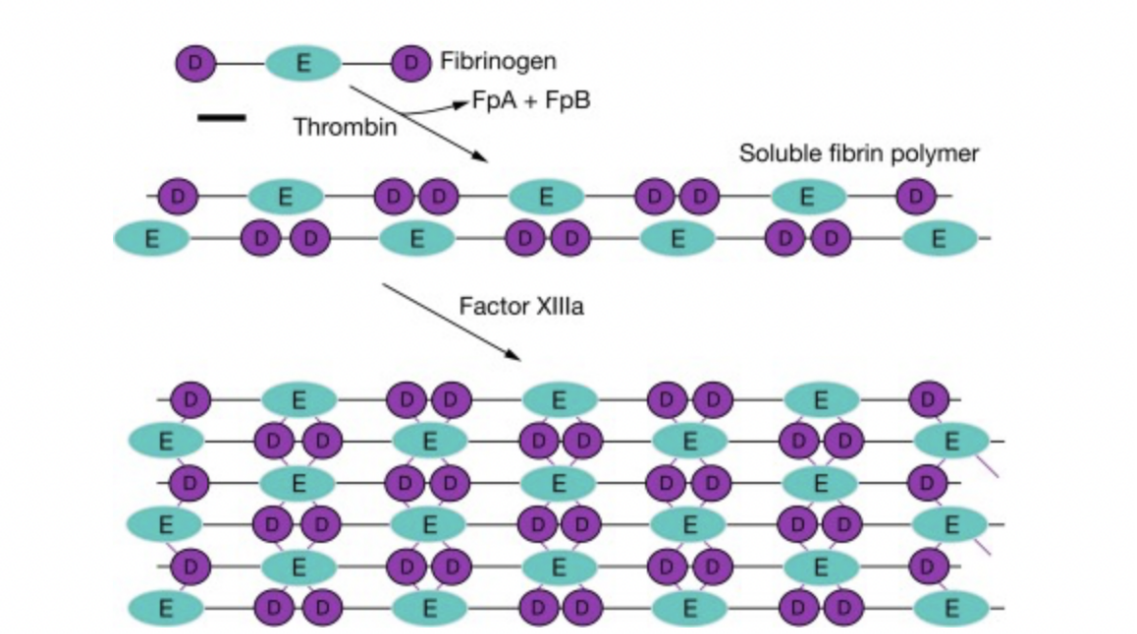

Figure 2.9 Fibrin Polymerization Diagram [46]

lysines, resulting in alpha polymers [9]. It is known that the resulting cross-links provide stability to the fibrin structure, contribute to clot stiffness, and provide strength and resistance to fibrinolysis [40][47].

2.3.3 Using Fibrin in Biomedical Engineering

Fibrin polymer can be used alone or in combination with different natural or synthetic materials, providing a scaffolding environment for the cells, and can be used in the regeneration of different tissues such as fat, bone, heart, cartilage, liver, nerve, eye, skin, and tendons, and effective results are obtained [7] [8]. Fibrin clots with longitudinally aligned fibrin fibers can be used as scaffolds for axonal development in traumatic peripheral nerve injuries [48]. Geer et al. [49] used fibrin for skin engineering and studied the growth and migration of keratinocyte cells to establish an in vitro model for re-epithelialization of scar tissue. In addition, Carriel et al. [6], using a scaffold obtained with fibrin-agarose, suggested the potential of this fibrin to regenerate natural skin tissue in the long term in vivo.

Figure 2.10 Fibrin Sealant Product of Tisseel [50]

Fibrin is injectable (Figure 2.10), biodegradable, and supports skin tissue regeneration and wound healing. Fibrin is known to stimulate the growth of fibroblast cells in vitro and in vivo. For these reasons, there are various cellular and noncellular fibrin-based tissue engineering products and clinical studies on the market [51] [52]. In addition, fibrin fillings formed by mixing fibrinogen and thrombin are widely used to stop bleeding [53]. Various fibrin filling products are also available in the market and they have different mechanical and hemostatic properties [54].

3. MATERIALS AND METHODS

Keratinocyte cell isolation and cell culture method has been modified from the "Classical Human Epidermal Keratinocyte Cell Culture" study by Rasmussen et. al. [55]

3.1 Fibrin Matrix Production

1. Obtaining FFP and making it ready for use: Blood samples are taken from volunteers who suffer from Polistemia Vera. These patients are treated by phlebotomied blood regularly and their blood is taken into blood bags containing CPDA-1. The blood sampling process was made by expert technicians at the Bezmialem Vakif University blood center under the ethical committee's approval (number 04/85 date:02.03.2021:). In order to separate the plasma from the whole blood, a blood-bag centrifugation device has been used. This plasma contains 13 coagulation factors such as factor I, factor II, factor III, factor IV, factor V, factor VI, factor VII, factor VIII, factor IX, factor X, factor XI, factor XII, factor, factor XIII, and prothrombins. If these factors are at room temperature, they are going to be destroyed over time. Because of this situation, the plasma has to be frozen to -86 $^{\circ}C$ just after the centrifugation process. In total, 12 FFPs were prepared in deep freezers. Before using FFP bags which are at -86 $^{\circ}C$, they had been thawed at 36 $^{\circ}C$ water bath for 20 minutes. After thawing, it was brought to the laboratory within 30 minutes for use.

2. Solution Preparation: In order to start the matrix formation and coagulation of FFP, a solution should be prepared to initiate the coagulation cascade. This solution should contain enough calcium to counteract the effect of the calcium binder CPDA-1 solution. In order to determine the optimum amount of $CaCl_2$, 3 different concentrations of $CaCl_2$ solutions were prepared and tested on plasma. Solutions containing 210 mM, 420 mM, and 630 mM $CaCl_2$ dihydrate concentrations were prepared, respectively, and it was calculated that a 1 mL solution should be used for 20 mL plasma.

$CaCl_2.4H_2O$ M:110 daltons (Sigma Aldrich) were put into 40 mL distilled water by weighing 924 mg, 1848 mg, and 2772 mg $CaCl_2$, respectively, using a high precise weighter. These solutions were mixed for 30 minutes in a magnetic stirrer and made ready for use.

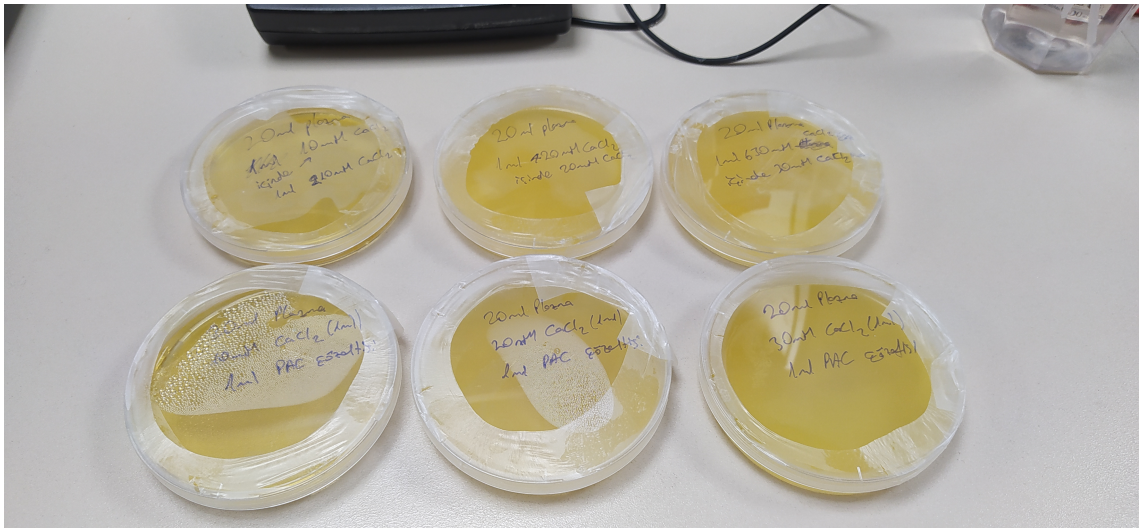

Figure 3.1 Fibrin Scaffold Preparation with 3 different $CaCl_2$ concentrations. Each petri dish contains 20 mL of blood plasma and 1 mL of clotting solution. For 3 different concentrations of clotting solution, 2 Petri dishes were prepared. The samples were kept at 37 $°C$ in an incubator overnight.

3. Fibrin matrix formation: 20 mL of plasmas which were at room temperature are placed in 6 different 100 mm plastic petri dishes. The coagulation cascade was started in petri dishes by taking 1 mL of $CaCl_2$ solutions from three different concentrations prepared previously. Thus, 3 samples were prepared from each solution concentration. After combining the solution and plasma in the petri dish, it was mixed by gently shaking. Petri dishes were wrapped with parafilm and left in the incubator at 37 $°C$ overnight under atmospheric conditions (Figure 3.1). As fibrin is a hydrogel, the serum of plasma shouldn't evaporate during the formation of fibrin in the incubator. The parafilm protects the evaporation.

4. Testing of samples: Samples are left in the incubator overnight in order to obtain the fibrin structure. The samples were first visually inspected. In the coagulated samples, it was easily seen that the entire petri dish was solid in a single phase. The samples which were prepared with 210 mM solution were in the liquid phase and it was observed that coagulation never started. It was observed in all samples that coagulation

occurred properly in 420 mM and 630 mM samples. In the manual examination (Figure 3.2), it was determined that the structure was self-protecting and suitable for manual manipulation. It was noted that the fibrin scaffold, which was separated from the petri dish, released the water and decreased in size.

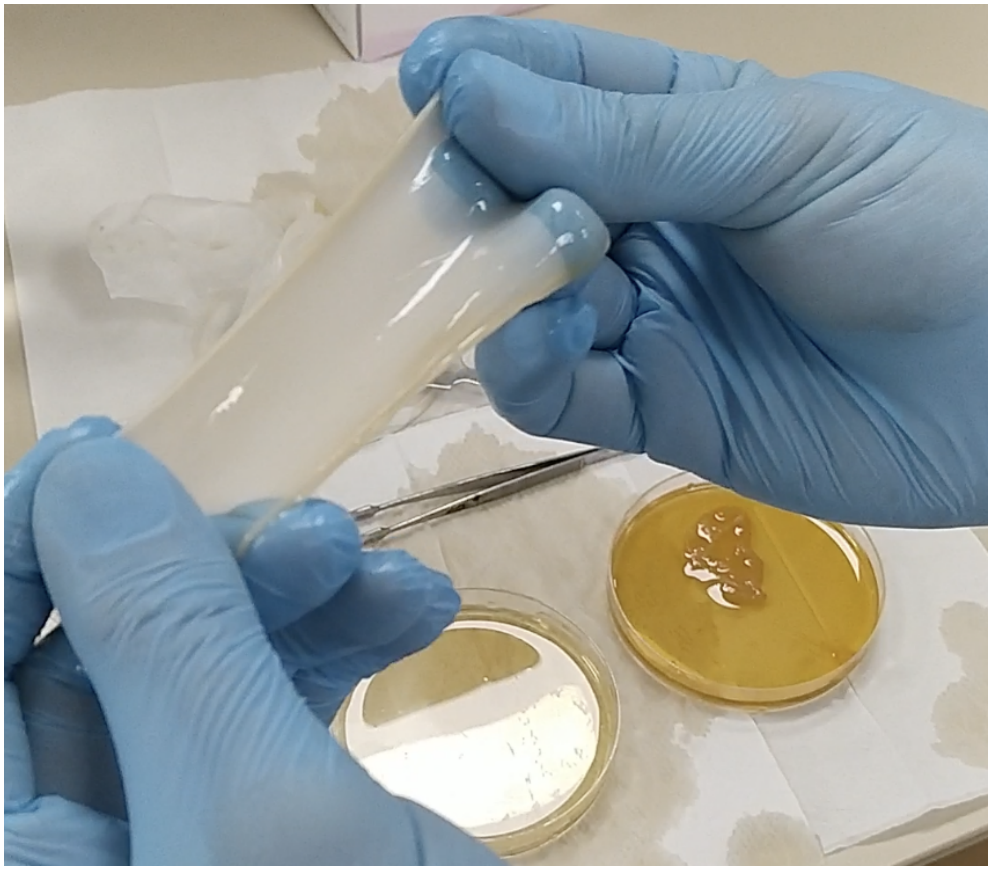

Figure 3.2 Evaluation of Fibrin Matrix in terms of Handelability and Mechanical Rigidity

3.1.1 Keratinocyte Cell Isolation

Skin samples were taken from the operating room of the Plastic Surgery Department of Bezimalem Vakif University (Figure 3.3). Excess skin to be disposed of from patients who had undergone abdominal panniculectomy surgery was brought to the laboratory under sterile conditions with the patient's permission and Bezmialem Vakif University ethical committee permission (number: 15/314 date: 08.09.2020). The skin sample of approximately 5*5cm was taken into a sterile sample container in the operating room and saline solution was added to cover the entire sample. It was not exposed

to any special temperature while being brought to the laboratory and was transported in room conditions. In order to maintain the sterile condition, the sample container was opened in a laminar flow cabinet.

Figure 3.3 Skin Samples Delivering From Operation Room

The sample was first washed with PBS (phosphate buffered-serum) and the excess fat layer remaining on the skin was scraped off with the help of a scalpel. It was then cut into small pieces of 5 mm. The reason for cutting it into small pieces is to accelerate the treatment with collagenase type 1 (Thermo Fisher) more kinetically. It was placed in a 100 mm petri dish so that the epidermis layer remained on top, and 6 mL of collagenase was added to it. This Petri dish with samples and collagenase was kept at $+4\ ^{\circ}C$ for 24 hours and then kept in an incubator at $37\ ^{\circ}C$ for 1 hour (Figure 3.4). With this process, the basal membrane between the epidermis and dermis was digested and the two layers were separated.

With the help of a forceps (Figure 3.5), the epidermis was separated and taken to a different petri dish, and washed with PBS. These pieces were cut to mm sizes with a scalpel, but care was taken to ensure that the scalpel was vertical while cutting. In order not to damage the cells, horizontal movements as if breaking or tearing were avoided. The keratinocyte cells that we want to isolate are in the epidermis, and for this reason, the culture process was continued with only the epidermis layer.

Figure 3.4 Collagenase type 1 treatment process to skin samples. These sample kept at 4 $^{\circ}C$ for 24 hours

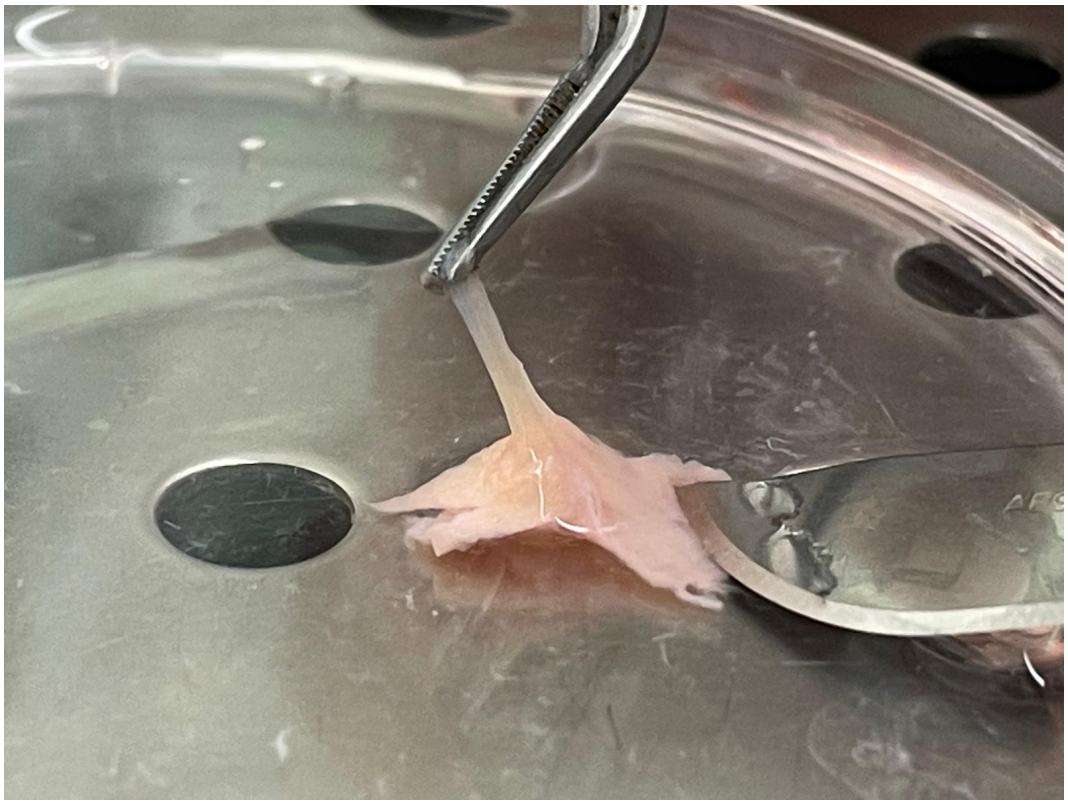

Figure 3.5 Epidermis and dermis separation after collagenase type 1 Treatment

Mechanically smalled epidermis pieces were also treated with trypsin for isolation of keratinocyte cells. 3 mL of trypsin was added to these cells and left in the incubator for 10 minutes to separate the cells from each other. 3 mL of medium was added to the mixture containing trypsin and cells and trypsin activation was blocked, and the falcon was taken into a tube and centrifuged at 1500 rpm for 5 minutes so that

the pellet (Figure 3.6) that occurred at the bottom was suitable for cell culture.

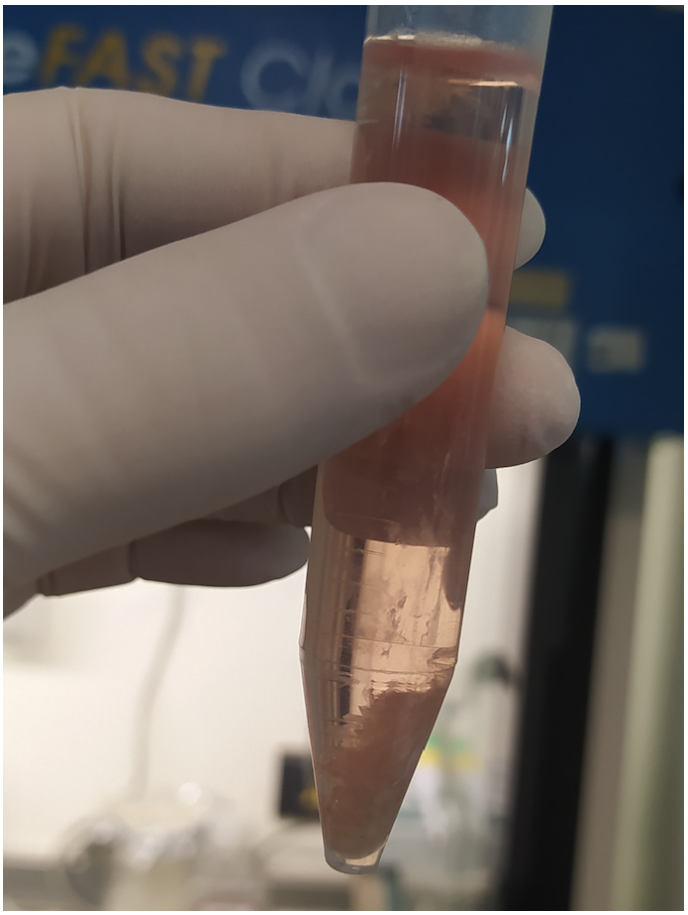

Figure 3.6 Separated Epidermis Layers at the Bottom of Falcon Tube

3.1.2 CCD 1072 Human Dermal Fibroblast Cell Line Cell Culture

CCD 1072Sk (ATCC, USA) human dermal fibroblast cell line was used as a feeder layer in co-culture. These cells help the growth and proliferation of keratinocyte cells by making extracellular secretions such as epithelial growth factors. In the flasks in which the keratinocyte cells will be cultivated, this cell line was confluent beforehand and it was provided to cover the entire surface. Before seeding the keratinocyte cells, the dividing properties of these cells must be stopped. Otherwise, it will continue to multiply by dominating the keratinocyte cells. As shown in Figure 3.7, gamma radiation (BVU, gamma radiation center) was applied to stop cells from dividing while continuing to live. The method has been followed by Mujaj et. al.[56] and used 25 Gy which is equal to 2500 rad for two times.

Figure 3.7 Gamma Radiation Treatment to CCD 1072 Cell Line With Two Doses of 2500 rad

3.1.3 Keratinocyte Cell Culture

Keratinocyte cells, approximately $5x10^5$ cells were seeded on a 25 cm^2 (Figure 3.8) flask with CCD 1072 feeder layer. In each passage, the feeder layer is pre-prepared in the flask. In the primary cell source of the epidermis, there are other cells besides keratinocytes, and we do not want those cells to proliferate. Only keratinocyte cells were fed by creating a selective medium for it.

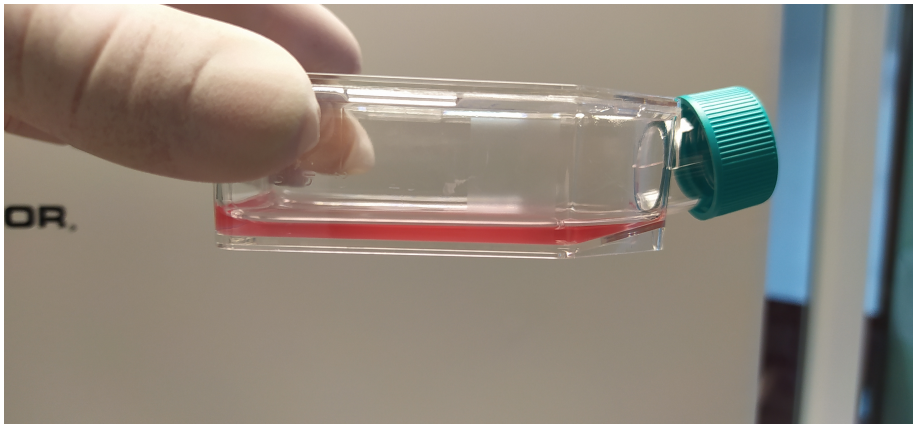

Figure 3.8 Culture Flask of Keratinocyte Cells

The medium consists of %72 Ham's F-12(D6421 Sigma Aldrich),%24 DMEM (D5796 Sigma Aldrich), and %4 other chemicals. Other chemicals concentrations are as follows: 0,4μg/mL hydrocortisone(Sigma Aldrich) solved in HEPES (Sigma Aldrich), 5μg/mL insulin (I2643 Sigma Aldrich) solved in HCl, 21,2 ng/mL isoproterenol (Vem

Ilac, Turkey) solved in HCl, 24 μg/mL adenine (A2121 Biomatik) solved in HCl, 10 ng/mL epidermal growth factor (AF100-15 Peperotech) solved in HEPES and 50 μg/mL penicillin (Capricorn Sci) and added to the culture medium. The medium in the culture flask was changed every 3 days. When the keratinocyte cells reached sufficient confluence, the cells were taken into a laminar cabinet for passage and the medium was aspirated. Thus, the medium which contains trypsin inhibitor FBS will be removed and the excessive medium will be washed with PBS.

Trypsin + EDTA (Sigma Aldrich) solution was heated to 37 $^{\circ}C$ and transferred 1 mL of the solution into the flask and kept in a 5% CO_2 incubator at 37 $^{\circ}C$ for approximately 5 minutes, and the cells were expected to remove from the flask surface. The inhibition of the trypsin enzyme was achieved by adding a medium. Suspended cells were taken into the falcon tube and centrifuged at 1360 RPM for 5 minutes to allow the cells to form pellets at the bottom of the centrifuge tube. The supernatant formed as a result of centrifugation was removed and the pellet remaining at the bottom was suspended with some nutrient medium and divided into 2 new cell culture flasks, and the culture process was continued by adding medium to them.

3.1.4 Seeding Keratiocytes to Fibrin

The previously prepared fibrin matrix was washed with PBS and the remaining liquid from the serum was cleared. The cell suspension was homogeneously distributed over the washed fibrin matrix in the petri dish as shown in Figure 3.9. The medium must be at such a height that the cells do not interfere with oxygen and carbon dioxide exchange. The medium was added to the surface of the fibrin layer, which was approximately 5-6 mm thick, in such a way that it overflowed approximately 1 mm. During the seeding of keratinocytes on the fibrin, CCD cell line culture was not performed as a feeder layer previously. Since the CCD cells remaining in the suspension of the cell mixture have not died yet, the old feeder layer feature was used. The medium of cell culture made on fibrin was also changed after 3 days.

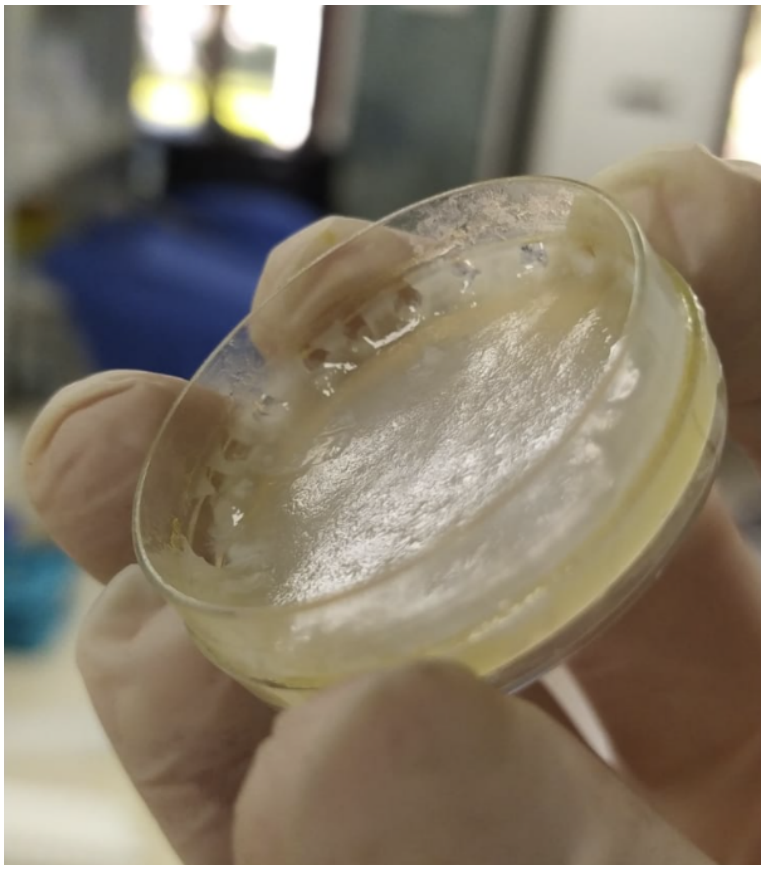

Figure 3.9 Seeded Keratinocyte Cells on Fibrin Matrix

3.1.5 Characterizations Techniques

3.1.5.1 FT-IR Analysis. Fourier transform infrared spectrophotometer is a spectroscopy device that evaluates the bonds of molecular compounds. The fibrin matrix, which was prepared without cells and whose coagulation process was completed, was frozen at -86 $°C$ for one hour and dried in a lyophilizer for one hour. FTIR spectroscopy was performed for the structural characterization and chemical bonding of the produced fibrin matrix. FTIR spectra were evaluated in the wavelength range of 2000-1000 cm^{-1}. The results were compared with previous studies [57].

3.1.5.2 Scanning Electron Microscopy. Scanning electron microscopy provides morphological visualization of the surface of the samples at the submicron level. In this thesis, samples were prepared to monitor cells and their interaction with their environment, and samples were examined in a Secondary Electron (SE) imaging device

Figure 3.10 Preparation for Pan-cytokeratin Immunocytochemical Staining

(Coxem, South Korea). Approximately 2000 cells have been used. Since the samples will be observed under a vacuum, the fixation process should be carried out and the water should be evaporated. To examine the interaction of keratinocyte cells and feeder layer cells, the culture medium was aspirated and treated with ethanol (%70) at -20 $^{\circ}C$ for 1 hour [58].

3.1.5.3 Pan-cytokeratin Immunocytochemical Staining. Pan-cytokeratin, an immunocytochemistry stain, was used for keratinocyte cell characterization. (Figure 3.10). Approximately 2000 cells have been used. In order to fix the cells, the sample, from which the medium was taken, was first kept in Acetone at -20 $^{\circ}C$ for 10 minutes, then it was kept for 3 minutes with Triton X-100 (0.3% w/v) to be permeabilized. Then, it was kept in a solution containing 5% BSA in PBS for 30 minutes at room temperature. Anti-Cytokeratin, Pan [AE-1/AE-3] (Biogenex, The Netherlands) anti-

body was diluted at 1:20, and 50 um was added into 1 mL medium and incubated for 1 hour at room temperature. Adhesion and labeling of keratinocyte-specific proteins were achieved with the primary antibody. Primary antibodies also need to be stained with a fluorescent dye with the secondary antibody. To give this ability glow under a fluorescent microscope, Goat Anti-Mouse was stained with IgG (H+L) (Alexa Fluor 488, Invitrogen). Alexa Fluor 488 was added to PBS at a rate of 2 ug/mL and it was allowed to interact with the cells for 1 hour. Finally, the sample was rinsed by washing with PBS [59].

4. RESULTS

4.0.1 FT-IR Analysis

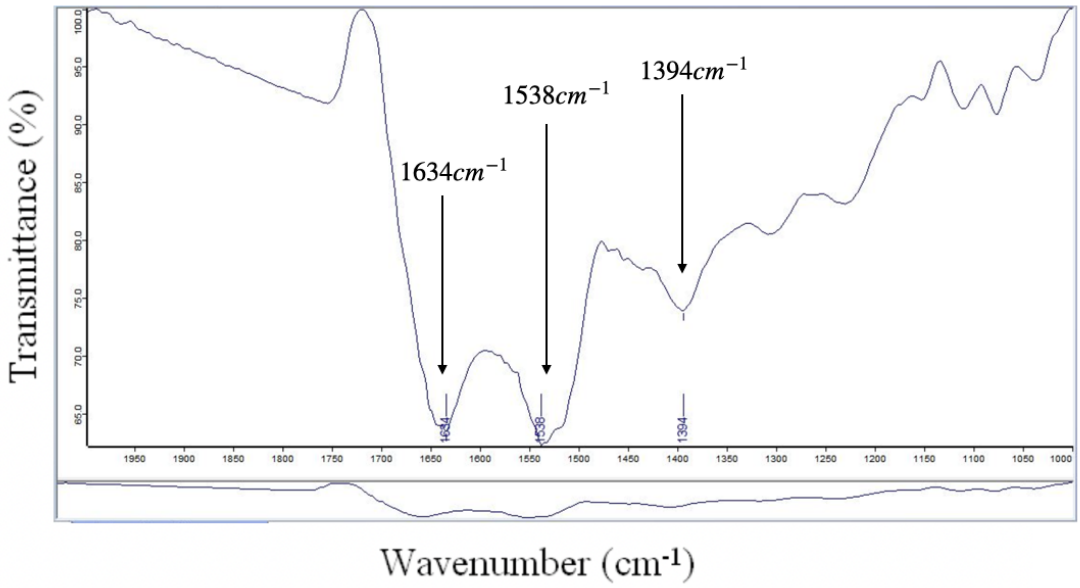

Figure 4.1 FT-IR transmittance spectrum of fibrin matrix. The amide bonds are shown with the arrows and the identified gaps are 1634 cm^{-1}, 1538 cm^{-1}, and 1394 cm^{-1}.

According to Bramanti et. al study [57] "Determination of Secondary Structure of Normal Fibrin from Human Peripheral Blood" the fibrin matrix is composed of about 30% alpha-helix and this structure has characteristic amide I (1653 cm^{-1}) and amide III (1284 cm^{-1} and 1312 cm^{-1}) bonds 40% beta-sheets have characteristic amide I (1625 cm^{-1}) and amide III (1231 cm^{-1}) bonds. The rest of the fibrin matrix is composed of 30% turns and these turns have characteristic amide I (1696 cm^{-1}, 1680 cm^{-1}, 1675 cm^{-1}) and amide III (1249 cm^{-1}) bonds. As these spectra are known as the fibrin matrix's characteristic bonds and transmittance-wavelength in FT-IR, our results are compared accordingly.

The bonds of the fibrin matrix which is produced in this thesis are shown in Figure 4.1. Since the produced spectrum and transmittance-wavelength graphs using the FTIR are given the peaks to be evaluated are those extending downwards. The stretching vibrations of carbonyl (C=O) stretching groups in the 1600-1700 cm^{-1} bandgap

(amide I) were observed in the FTIR spectra of the fibrin matrix, N-H bending in the 1500-1600 cm-1 bandgap (amide II), and N–H vibrations in the bandgap of 1200-1400 cm^{-1} (amide III) [60] [61]. Amide groups are known as bonds that bind amino acids together [62]. As a result of the peaks formed, we can see that polymerization takes place and that amide I, amide II, and amide III bonds are the result of the formation of cross-linking.

4.0.2 Optical Microscopy Images

The optical microscope has been used frequently in terms of imaging practicality and providing basic information about cell culture. Figures 4.2 A and B show the presence of only the CCD 1072 cell line seeded on the Flask. The image represented by A is the image of CCD cells after 3 days after seeding. It is clearly seen that it is attached to the surface of the flask by showing a branched structure. The CCD cells that have reached sufficient confluence and completely covered the surface at the end of the 14th day after exposure to gamma rays can be seen in the image represented by B. In the images represented in Figures 4.2 C and D, it is shown that the attachment of keratinocyte cells after seeding on the CCD cell layer on the 3rd day.

It is seen that the branched cells at the base are the feeder layer and in the areas indicated by the arrows, is seen keratinocyte cells are gathered as colonies. In the image represented by Figures 4.2 E, it is seen that at the end of the 7th day, colonies of keratinocyte cells condense and continue to cover the surface. In the image represented in Figure 4.2 F, we can see that at the end of the 14th day, keratinocyte colonies proliferate on the surface of the flask and begin to cover the surface. It was noted that the feeder layer cells were removed from the surface of the flask over time and were discarded while the medium was changed. It is seen that there are no feeder layer cells in the E and F images.

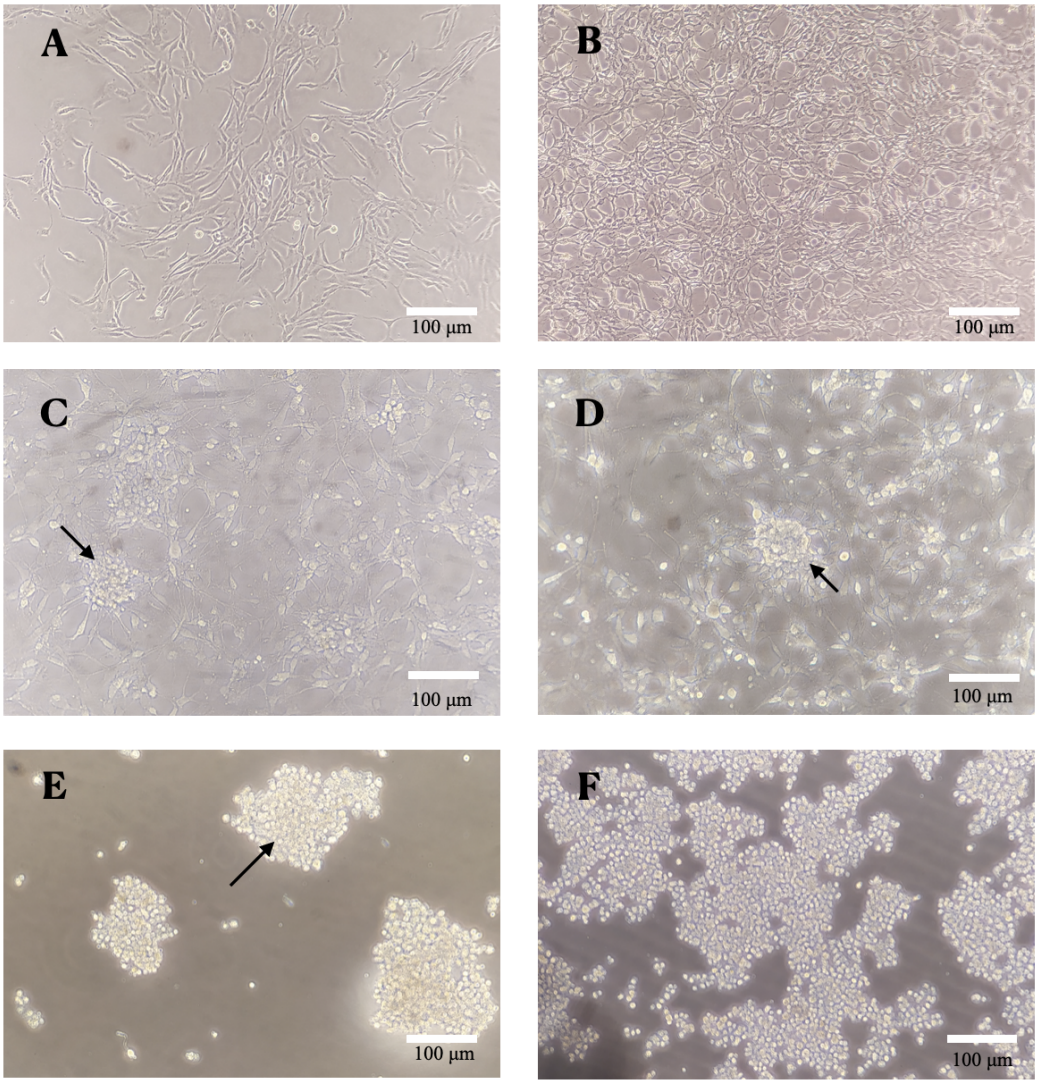

Figure 4.2 Optical microscopy images. In this Figure A) CCD 1072 human dermal fibroblast cell line after seeded on flask. B) Confluent and gamma radiation treated CCD 1072 human dermal fibroblast cells. These cells have branched and needle-like structures. C-D) Seeded keratinocyte cells on the feeder layer and the keratinocyte cells have a round-shaped structure and make colonies. The small arrows show the colonized keratinocyte cells. E) After several times changing the medium, it can be seen that the feeder layer cells have been removed and discarded. Colonized structures are keratinocyte cells. F) Keratinocyte cells colonies were covered in the cell culture flask and it is nearly %70 confluent.

4.0.3 Scanning Electron Microscopy (SEM) Images

The interaction of fibroblast cells and keratinocyte cells is shown in Figure 4.3. It was observed that CCD 1072 cells were attached to the ground and keratinocyte cells were located on these branched feeder layer cells. In Figure 4.3, the SEM images of two different locations from top to bottom at x500, x1000, and x2000, respectively, show that spherical cells are keratinocyte cells and branched cells are CCD.

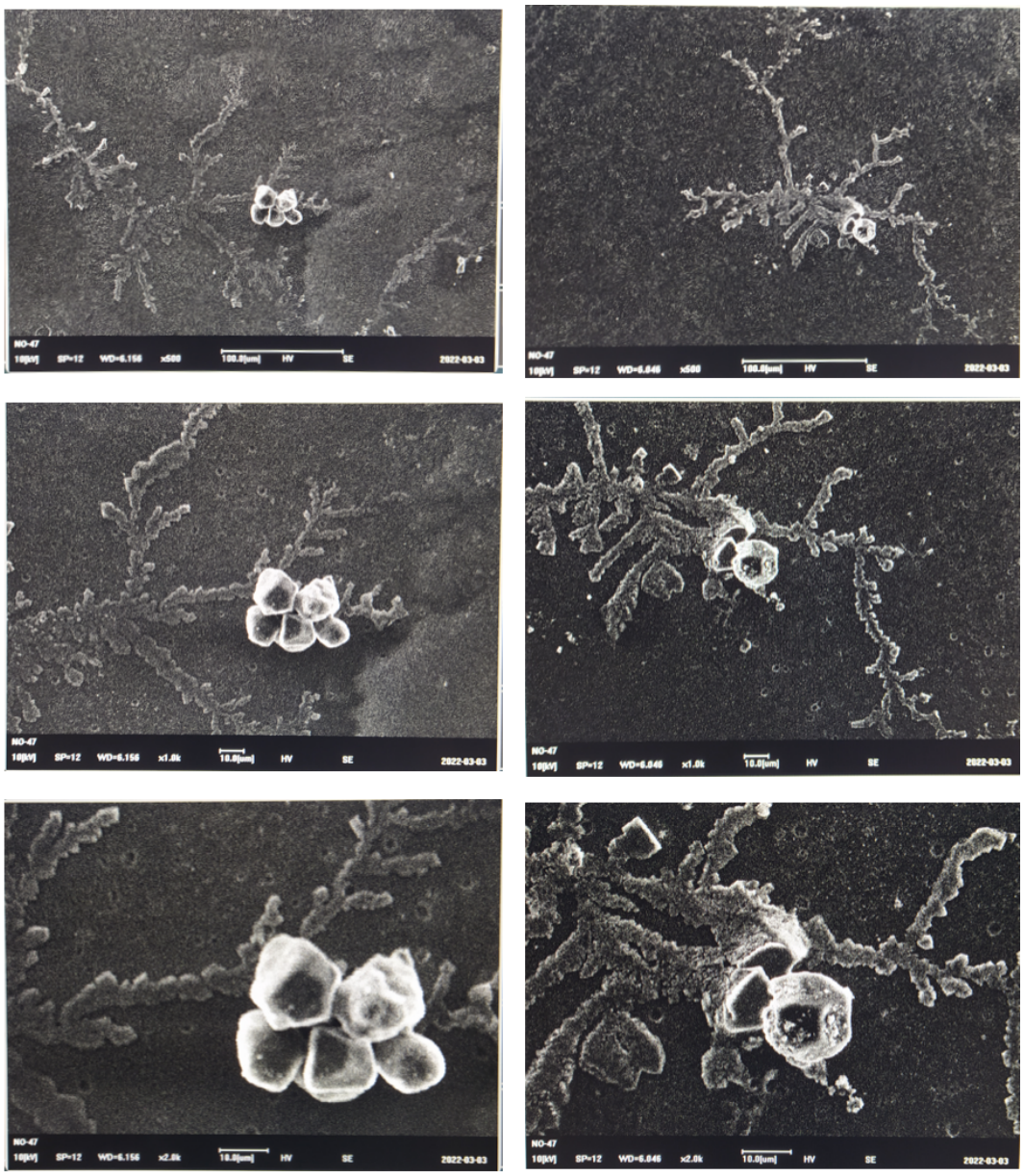

Figure 4.3 SEM images of keratinocyte cells (round shape) and CCD 1072 (branched) feeder layer human fibroblast cells. In these SEM images, it can be clearly seen that keratinocyte cells have close interaction with fibroblast feeder layer cells. Keratinocyte cells are proliferated specifically near fibroblast cells.

Figure 4.4 shows the results of the fibrin matrix SEM on which keratinocyte cells were seeded. The light gray parts at the base are the fibrin matrix, and the dark spots like the shadow on it are keratinocyte cells. Images were taken from two different locations at x500, x1000, and x2000 magnifications, respectively. It is estimated that the sample preparation to be drawn in SEM slightly deforms the structure of the cells during drying. For this reason, the cells could not be clearly visualized, but their location and distribution show that the cells adhere and multiply on the fibrin.

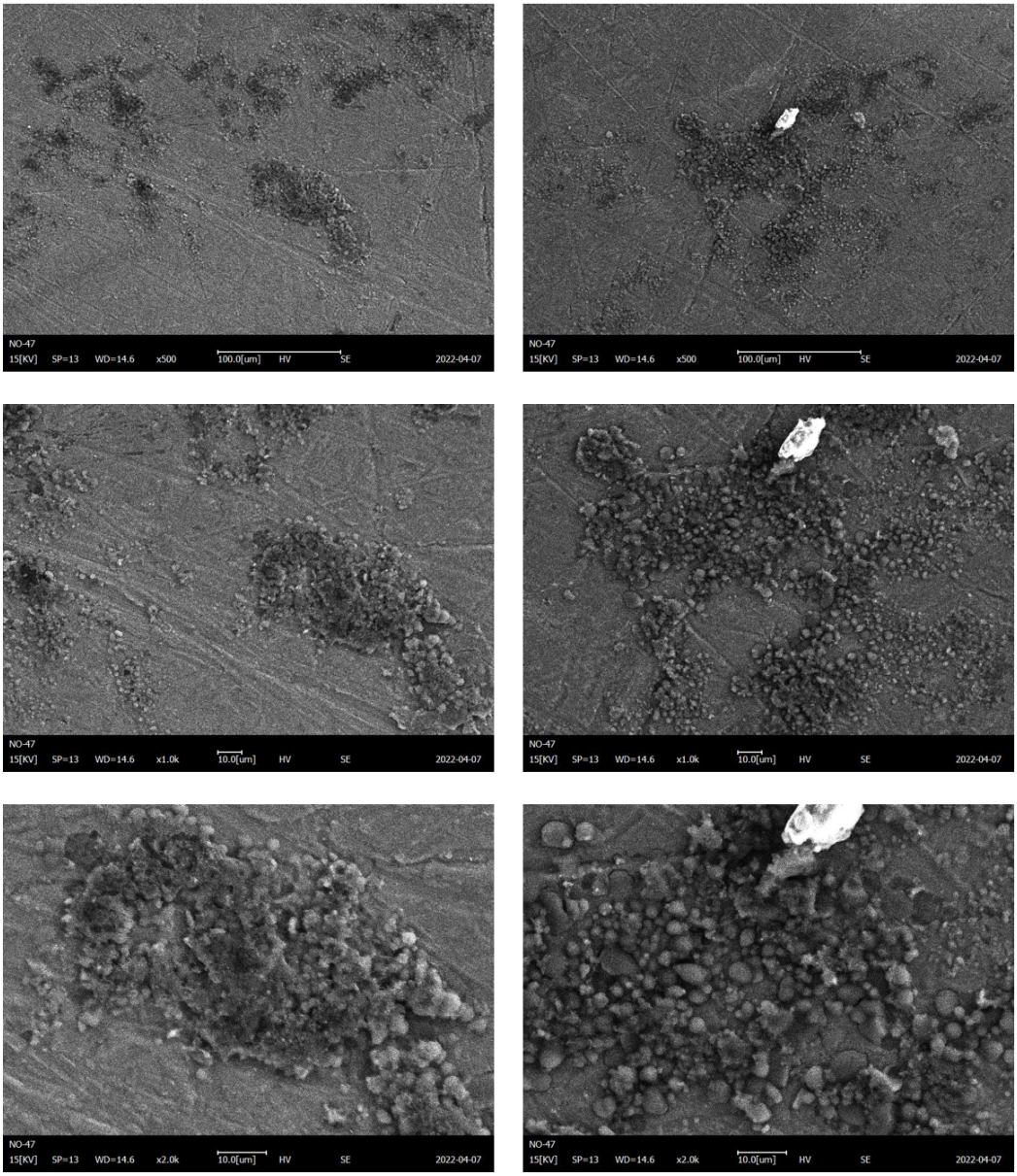

Figure 4.4 SEM images of keratinocyte cells on fibrin matrix. The light grey areas (bottom layer) are fibrin matrix and the dark grey areas are keratinocyte cells. The keratinocyte cells have attached to the fibrin surface and continued proliferation. The images represent x500, x1000, and x2000 magnification of two different views.

Keratinocyte cells cultured for 14 days were seeded on the fibrin. Therefore, CCD 1072 cells are not visible in SEM images.

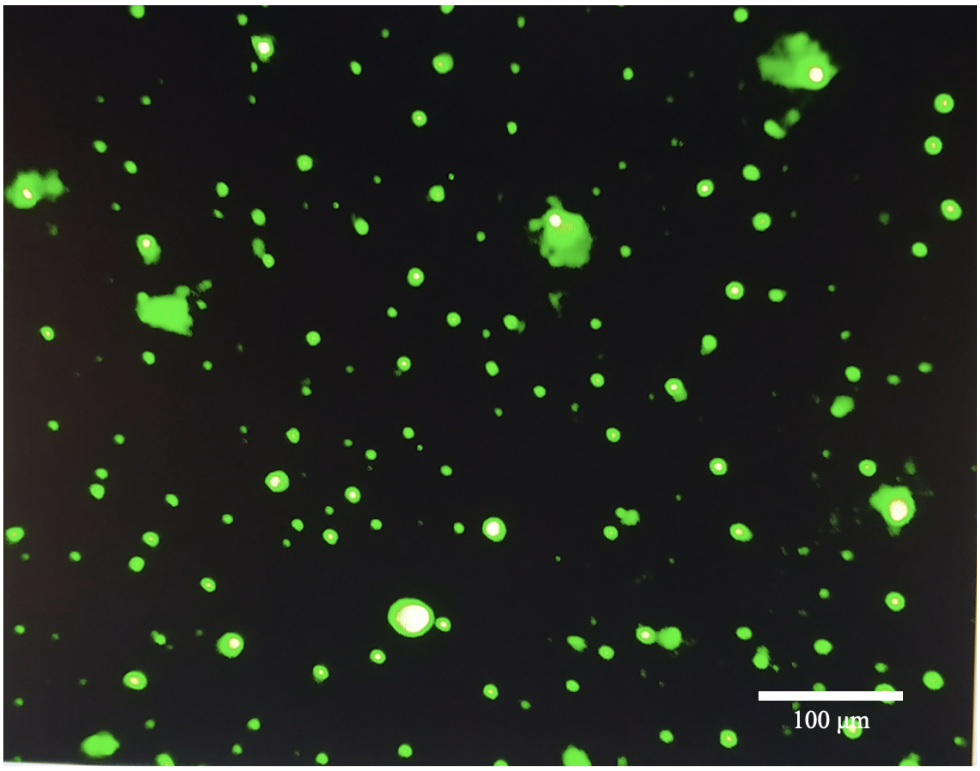

Figure 4.5 Pan-cytokeratin and alexa fluor 488 anti-mouse IgG staining results. Green areas show the keratin deposition and there are no branched green areas which mean round-shaped cells are keratinocyte cells.

4.0.4 Pan-cytokeratin Immunocytochemical Staining

In previous imaging techniques, branched fibroblast cells and colonized keratinocyte cells were visualized. However, to be certain that the round-shaped cells were keratinocyte cells, they had to be proved by immunocytochemical staining that would stain the keratinocyte. The pan-cytokeratin antibody is a specific antibody that stains deposited keratin molecules. Keratinocyte cells make keratin deposition around the cell in their environment. In Figure 4.5, the fluorescent microscope image found that it is clearly seen that the round-shaped cells glowing green are keratinocytes. In fact, although there were branched fibroblast cells on the same sample, only the round ones were stained.

5. DISCUSSION AND CONCLUSIONS

The production of fibrin was provided by blood plasma taken directly from human beings. Polycythemia Vera (PV) patients phlebotomied regularly to control excessive erythrocyte accumulation; collected blood units are not suitable for transfusion and are discarded as medical waste. The fibrin matrices produced in this thesis could be successfully made fibrin with the blood of PV patients. It is known that patients use low-dose blood thinner aspirin for treatment[63]. However, this low-dose aspirin did not prevent fibrin formation. Whole blood taken from volunteers is converted into fresh frozen plasma, ensuring that the factors in it do not degrade. In other existing studies, it is known that thrombin is added to initiate coagulation[64]. However, thrombin is not a sustainable method in fibrin production in terms of both availability and price. There are some examples where $CaCl_2$ is used as an alternative[65]. With this method, the factors in the plasma are activated with $CaCl_2$. In this case, the factors in the blood must be preserved, and this is achieved with fresh frozen plasma. The triggering of the coagulation cascade to be initiated for fibrin formation was successfully achieved with $CaCl_2$. It has been understood that the fibrin formation can be done using only $CaCl_2$, the fresh frozen plasma production method is successful and $CaCl_2$ is a sustainable agent.

Normally, when the cells are confluent in the flask and removed as a layer, they have a fragile structure, and application to burn patients is hard. For this reason, there are difficulties in terms of handle-ability during the application to burn patients[66]. In order to avoid this problem, some biodegradable supporting materials are used[67]. In this thesis, a fibrin matrix was used as a carrier layer for cells and its usability in surgical practice was observed in terms of flexibility and mechanical strength in the manual examination.

All of the plasma was coagulated without isolating fibrinogen. A separate process and device are required for fibrinogen isolation. The starting point of this thesis

was to reveal the fastest and cheapest method. For this reason, it was aimed to form a matrix without isolating fibrinogen, and it was done successfully. A comparison of the matrix produced in this thesis and the matrix produced with isolated fibrinogen will be compared in future studies.

The fibrin matrix, besides being a support material, plays an important role in the proliferation and adhesion of cells. Another advantage of the cells used as feeder layer is that it creates an extracellular matrix for keratinocyte cells [68]. The fibrin matrix provides an ideal ECM for the keratinocyte cells seeded on it, thanks to its porous and nano-sized fibrous structure. With electron microscope images, it has been proven that cells proliferate on the fibrin matrix and attach to it. In ongoing studies, it should be investigated to what extent the fibrin matrix and cell layer preserve cell viability while being transported to the operating room or what conditions affect it. In addition to the advantages that fibrin provides to cells during cell culture, it should be investigated whether it provides an advantage to cells after cell culture.

Skin samples were obtained from abdominal panniculectomy patients. In this type of skin surgery, the skin of the patients is normally discarded as medical waste. Thus, it is a very common type of surgery in plastic surgery, hundreds of square centimeters of healthy skin samples can be extracted daily from a medium-density operating room. Since our goal is the production of autologous grafts, the cultured epidermal graft produced will actually be available to the patients who donate skin. The use of cultured epidermal graft for different patients is to be investigated in further studies or it can be classified in terms of tissue compatibility. If the allogeneic application can be used, it will be very beneficial in terms of use. Since sustainability is a point we focus on in this thesis, this type of cell source could prove that our thesis can be commercialized in the future.

There are many different kinds of cells in the primary skin sample. The cell type we want to culture is only keratinocyte cells. For example, fibroblast cells from the skin sample can inhibit the growth of keratinocyte cells, as they proliferate faster than keratinocyte cells during cell culture. The cells of the primary sample taken

must be isolated. A flow cytometry device is normally used for cell isolation [69]. In this thesis, instead of using flow cytometry, cell isolation was performed by creating a selective medium. Flow cytometry increases isolation costs, and requires trained personnel, and expensive equipment. The sample was first separated from the dermis and epidermis by collagenase type 1. Thus, fibroblast cells, which are concentrated in the dermis, were not cultured as much as possible. Epidermal separation via collagenase facilitates selective tissue digestion and cell isolation. Afterward, the prepared medium was prepared only for the proliferation of keratinocyte cells and fibroblasts and a high dose of adenine concentration in the media suppresses contaminant fibroblasts.

Normally 3T3 Murine fibroblast is used as the feeder layer [23]. In our experiments, CCD 1072 human dermal fibroblast was used instead of the 3T3 cell line. The CCD 1072 cell line is a type of cancerous cell, but it has since been observed that it disappears towards the end with displacements and only keratinocyte cells remain. In future studies, it can be examined whether fibroblasts taken from the patient can be used instead of animal-derived or cancerous fibroblasts as a feeder layer and their division can be stopped. In this thesis, the feeder layer stopping is done with gamma rays. There are some chemicals as an alternative to gamma rays. For the stopping process of the feeder layer, a chemical or gamma radiation comparison can be made.

In the optic microscope and SEM images, the adhesion of keratinocyte cells on the branched fibroblast cell line was clearly seen. This proves that co-culturing with fibroblasts benefits keratinocyte cells from fibroblasts during survival or proliferation. Since we were trying to isolate keratinocytes from the primary source, we had to make sure that the cells with round-shaped attached to fibroblasts were keratinocytes. It was proved by fluorescence microscopy with pan-cytokeratin antibody staining that cells attached to fibroblasts were keratinocytes.

Our thesis results indicate successful isolation and cultivation of primary keratinocytes over a single staged manufactured fibrin scaffold. A comparison of the methods used during fibrin production, cell isolation, and culture with other existing methods will be done in future studies. It is desired to test the success of this method,

which was revealed in the next study, in vivo. For this, animal experiments will be planned and it is aimed to repeat the same primary cell culture method for animals. If successful, it is desired to continue with clinical trials. While designing animal and human experiments, plastic surgeons will participate in the thesis with the aim of using it as a final product.

In the United States and in some European Countries, such cultured epidermal grafts are commercially offered for the benefit of burn patients. Epicel Company, which has been producing epidermal grafts for culture for many years, has created treatments for hundreds of people. Epicel is FDA approved and currently only offers treatment for burns of 30% or more [70]. The reason for this limitation is that it is required to be used in cases where there is insufficient donor area and in cases of life-threatening situations. By increasing the reliability and success rate of epidermal grafts produced by cell culture, it can also be used in burns of 30% or less. It can be used not only for life-threatening but also for the treatment of minor burns to improve the aesthetic appearance. In order for this to happen, the use of products of animal origin should be avoided as much as possible and solutions should be found.

This thesis is aimed to be commercialized and translated in the long term so that it does not remain in the laboratory and benefits humanity. For this, applications will be made for entrepreneurship and incubation center supports.

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