Toxicological Analysis of a New Fibrin-Derived Dermal Scaffold (Dermoturk); Acellular and Combined with Stem Cells Forms

Fibrinden Türetilmiş Yeni Bir Dermal matriksin (DermoTürk) Aselüler ve Kök Hücreler ile Kombine Formlarının Toksikolojik Analizi

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ABSTRACT

Objective: We aimed to reveal the toxicological analysis of the newly developed fibrin-derived scaffold forms (DermoTurk) before human studies.

Material and Methods: 42 male Albino Wistar rats were used. Two of them were used to produce mesenchymal and epidermal stem cells. Forty rats were divided into five groups, each consisting of 8 rats; the acellular scaffold applied group as Group-1, the mesenchymal stem cells added scaffold used group as Group-2, the MSCs and epidermal stem cells-added scaffold applied group as Group-3, MSCs- and epidermal stem cells-added scaffold applied outbred group as Group-4 and control as Group-5. The changing of laboratory tests in the groups was evaluated five days before application and on the 7th and 40th days. After the autopsy performed on the 40th day of the study, rats' organs and scaffold implanted skin area were evaluated histologically. All the results of the groups were compared. SPSS 22.0 was used for analyses. $p < 0.050$ was accepted as statistically significant.

Results: There were no differences between the groups in terms of laboratory results. Histologically, a mild-grade foreign body reaction against the DermoTurk was found in all groups; this reaction was less in groups 3 and 4 with the richest stem cells.

Conclusion: This study revealed that DermoTurk is safe in rats. It could be an important alternative to skin substitutes, with stem cells or alone. Human studies for clinical efficacy should be carried out.

Key Words: Burn, Fibrin-derived dermal scaffold, Epidermal stem cell, Mesenchymal stem cell, Tissue engineering

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ÖZ

Amaç: Bu çalışmamızda yeni geliştirdiğimiz fibrinden türetilmiş dermal matriksin (DermoTürk) değişik formlarının insan çalışmalarından önce yaptığımız toksikoloji testlerinin sonuçlarını paylaşmayı amaçladık.

Gereç ve Yöntemler: Çalışmada 42 adet erkek Albino-Wistar rat kullanıldı. Bunlardan ikisi mezenkimal ve epidermal kök hücre üretmek için kullanıldı. Kırk tane rat ise her biri 8 denekten oluşan beş gruba ayrıldı; aselüler matriks uygulanan grup (Grup-1), fibrin matriks ile mezenkimal kök hücre (MKH) uygulanan grup (Grup-2), fibrin matriks ile MKH'ler ve epidermal kök hücrelerin (EKH) uygulandığı inbread grup (Grup-3), fibrin matrilks ile MKH'ler ve EKH'lerin uygulandığı outbread grup (Grup-4), kontrol ise Grup-5 olarak belirlendi. Gruplardaki laboratuvar testlerinin sonuçları uygulamadan beş gün önce ve uygulamanın 7. ve 40. günlerinde değerlendirildi. Çalışmanın 40. gününde yapılan otopsinin ardından deneklerin organları ve fibrin matriks implante edilen cilt alanı histolojik olarak değerlendirildi. Grupların tüm sonuçları karşılaştırıldı. İstatistiksel analiz SPSS 22.0 ile yapıldı. $p<0.050$ istatistiksel olarak anlamlı kabul edildi.

Bulgular: Laboratuvar sonuçları açısından gruplar arasında fark yoktu. Histolojik olarak tüm gruplarda DermoTürk'e karşı hafif derecede yabancı cisim reaksiyonu saptandı; bu reaksiyon kök hücrelerin bulunduğu 3. ve 4. gruplarda daha azdı.

Sonuç: Çalışmamız fibrinden türetilen DermoTürk'ün ratlarda güvenli olduğunu göstermiştir. Bu matriks kök hücrelerle veya tek başına diğer deri benzerlerine önemli bir alternatif olabilir. Klinik etkinliliğin gösterilmesi için insan çalışmaları yapılmalıdır.

Anahtar Sözcükler: Yanık, Fibrinden türetilmiş dermal matriks, Epidermal kök hücreler, Mezenkimal kök hücreler, Doku mühendisliği

INTRODUCTION

Despite the significant developments in the treatment methods of burn over the last decades all over the world, it continues to be one of the most important causes of death due to trauma. Still, major skin burns continue to become a health problem that has not been fully solved with the consequences of mortality and morbidity (1, 2). Especially in the case of full-thickness skin burns involving a large part of the body, there is not enough donor, and even if this is achieved, it cannot be reconstructed with all the layers and attachments of the skin. Advances in tissue engineering have increased interest in applying artificial skins obtained by attaching stem cells to different scaffolds (3).

Cellular therapies have emerged as an important development in burn treatment to ensure full-thickness skin formation. The first of these studies was carried out in 1975 when keratinocyte colonies were obtained from human epidermal cells, and it was thought that it could be a good alternative in extensive burns where the autologous skin graft option is insufficient (4). In addition to dermal cells, the recent use of other stem cells, which are essential in regenerative medicine, has revealed a new approach. It is thought that stem cells can provide many therapies for tissue engineering and regenerative medicine thanks to their ability to transform into different cells and their immunomodulatory properties (5).

Mesenchymal stem cells (MSCs) have recently been tested in the treatment of burn wounds as well as in the treatment of many diseases and have been shown to accelerate healing via their capacity to convert into different cells and their paracrine effects as well as their secreted cytokines (TGF-β, IL-10, IL-6), chemokines (CCL2, CCL5, CXCL12), growth factors (VEGF, IGF, FGF, SDF, HGF) (6). MSCs are also used in treating certain immune system diseases due to their immunomodulatory effects. MSCs can be used safely as autologous and allogeneic (7,8).

Although treatment methods using cell suspension alone are simple and reliable for minor burns, they have disadvantages such as prolonged operation time, high cost, and insufficient for extensive burns (9). In addition, the fact that tissue engineering products containing combined fibroblasts contain growth factors such as VEGF, PDGF, IGF-I, TNF, and TNF-β has paved the way for the development of tissue engineering products obtained with synthetic matrices in which human dermal cells are seeded. In animal experiments conducted with these products, an acceleration in the healing of burn wounds, a decrease in infection, and a decrease in the development of hypertrophic scars were observed (10).

Different types of natural polymers can be obtained from various sources for tissue engineering applications for regenerative medicine. Collagen and hyaluronic acid from animal sources and agarose and alginate from algae are among the examples that can be considered. The common feature of all these polymers is that they are soluble in buffer solutions and can be degraded under in-vivo conditions. Collagen, hyaluronic acid, and elastin are essential for skin regeneration (11). The fact that these effective biomaterials are not of human origin appears as an obstacle. At this point, the use of human-derived fibrin stands out. Various studies have demonstrated the regenerative capacity of the fibrin matrix. Full-thickness skin biopsy samples taken after applying the fibrin matrix showed that the fibrin matrix stimulates various cellular changes in the skin. The main changes have been reported as fibroblast activation, new collagen deposits, increased angiogenesis, and stimulation of subepidermal adipocytes (12). Fibrin matrix can have a chemotactic effect on many cell types, such as endothelial cells, epidermal cells, and keratinocytes, thanks to the various growth factors it contains, such as PDGF, TGF-β, VEGF, EGF, and IGF. In addition, these factors can trigger the production of collagen and fibronectin and accelerate angiogenesis (13). These properties allow fibrin-based structures to be combined with skin cells or as fillers or graft holders.

Our group is working on an effective tissue engineering product for the treatment of deep burns, considering the positive effects of stem cells and fibrin matrix on skin regeneration. This study aims to reveal the necessary toxicological data before moving

on to human studies by examining different versions of the product we designed.

MATERIALS and METHODS

Ethics committee approval required for the study was obtained from Diskapi Yildirim Beyazit Training and Research Hospital Experimental Animals Local Ethics Committee (Decision No. 01.03.2012/2). The study was conducted in the Experimental Animals Laboratory of Diskapi Yildirim Beyazit Training and Research Hospital. Forty-two male Albino Wistar rats weighed 250-300 grams were used. Two rats were used to obtain tissue samples for use in the production of stem cells. The other 40 were used in experimental groups. One group was outbred (Group 4), and the remaining four groups were composed of inbred. The subjects were fed in an ad libitum style during the experiment with sufficient standard feed and water.

Collecting tissue samples for cell production

The groin area of one inbred rat, to which general anesthesia was applied by administering ketamine hydrochloride (35mg/kg intramuscularly) and xylazine hydrochloride (5mg/kg intramuscularly), was shaved, and this area was cleaned with 10% polyvinylpyrrolidone iodine solution (Polyod ®, Drogsan, Türkiye). An incision was made in the groin, and 1 cm of fat tissue was removed for MSC production. Then, 20 hair follicles were removed with the FUE (Follicular Unit Extraction) technique using the microsurgical motor. The tissue samples were placed in Ringer's Lactate solution containing 1% Penicillin, Streptomycin, and Amphotericin B 50 mcg/mL. Tissues were transferred to Acıbadem Labcell Istanbul laboratory on the same day in a transport bag at 2-8 ˚C temperature.

Culture of Adipose Tissue-Derived Mesenchymal Stem Cell

After the mechanical dissection of the fat tissues by splitting with scalpel, 25% collagenase (Sigma Aldrich, C6885) was added and incubated in a 37 ^{0}C medium. After incubation, PBS was added to the tube (Biological Industry, BI02-023-1A) and the resulting mixture was centrifuged at 800 G for 5 minutes to decompose the cells. The cells obtained were cultured within T-150 flasks of DMEM LG (Biological Industries, 01-050-1A) containing 1% antibiotic (Biological Industries, BI03-031-1B) and 10% fetal calf serum (FBS) (Biological Industries, 01-050-1A) with 5% CO_2, 7% O_2 and 37 ^{0}C medium. The medium was changed every 3 days and the cells were passaged when the cells covered 70% of the flask base. The cells were removed from the flask surface using trypsin (Biological Industries BI03-054-1B) and 0.5 ml of FBS was added to neutralize the enzyme. These cells were collected and transferred to a tube and added with PBS (Biological Industrires BI02-023-1A) and centrifuged at 400 G for 10 min to wash out. Once the washing procedure was repeated, the cells were trypsin free and the cells were resuspended in the same medium and under the same conditions. The medium was replaced for every 3 days with the 70% of the flask base, the passage was repeated and the cells were replicated. After second passaging, cells were removed with trypsin to be implanted into the fibrin scaffold, and after washing procedure, a sample was taken for quality control tests and flow-analysis analysis (Figure 1: A).

Preparation of epidermal stem cell suspension

The hair follicles were taken into a tube containing 25% collagenase (Sigma Aldrich, C6885) and incubated in a 37 ^{0}C medium for half an hour. After incubation, the cells were centrifuged for 5 minutes at 800 G by adding PBS (Biological Industries, BI02-023-1A). After centrifugation, cells deposited in the lower part of the tube were cultured within T-150 flasks of DMEM LG (Biological Industries, 01-050-1A) containing 1% antibiotic (Biological Industries, BI03-031-1B) and 10% fetal calf serum (FBS) (Biological Industries, 01-050-1A) with 5% CO_2, 7% O_2, and 37^{0}C medium. The medium was changed every three days, and the cells were passaged when they covered 70% of the flask base. Passages were carried out as described in the MSCs passages. After the second passaging, the sample was collected from this passage for quality control and flow analysis (Figure 1:B).

Flow cytometric analysis

Specific monoclonal antibodies were used as CD34, CD45, CD90, HLA-DR, CD105, and CD73 cell surface antigens for the mesenchymal stem cells, and CD200, CD34, CD45, CD271, CD29 (ß1 integrin), and Nestin cell surface antigens for the epidermal stem cells. The cells were taken up into the test tube, and 10 µl of the fluorescent isothiocyanate (FITC), phycoerythrin (PE), and alloficocyanine (APC) conjugated monoclonal antibodies and isotype controls were added, then was incubated for 45 minutes at room temperature in a light-protected environment. After incubation, the cells were resuspended in 400 µl wash solution and analyzed by the FACSDiva ® program in the BD FacsCanto-II flow meter (Figure 2).

Differentiation tests of mesenchymal stem cells

Separated mesenchymal stem cells were cultured with chondrocytes (ThermoFisher Scientific, StemPro™ Chondrogenesis Differentiation Kit A1007101), fat (ThermoFisher Scientific, StemPro™ Adipogenesis Differentiation Kit, A1007001) and bone (ThermoFisher Scientific, StemPro™ Osteogenesis Differentiation Kit A1007201) mediums for evaluation of differentiation capacities. A staining procedure was performed on the 14th day of culture for chondrocyte and fat differentiation and on the 21st day for bone differentiation. For the determination of chondrocyte differentiation, toluidine (Fisher Scientific, 92-31-9), for bone differentiation, alizarin-Red (Sigma Aldrich, Alizarin-Red Staining Solution, TMS-008), and for fat differentiation, oil-Red (Sigma Aldrich, O0625-25g) dyes were used (Figure 1: D,E,F).

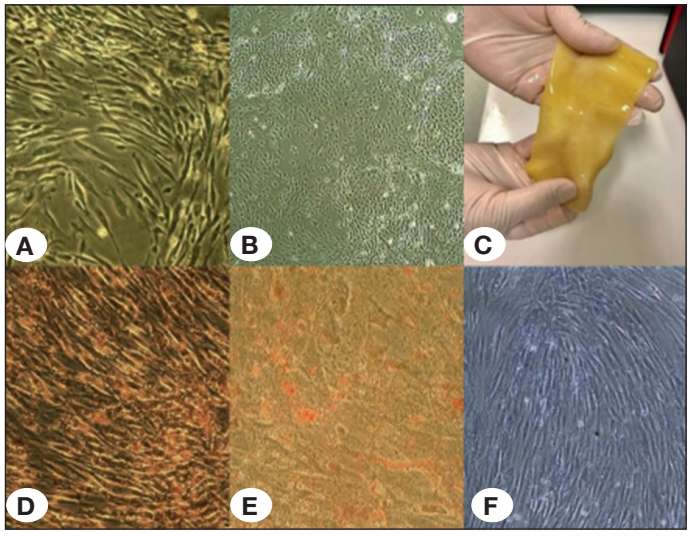

Figure 1: **A);** Appearances of the mesenchymal stem cells in culture medium at the end of the second passage (10X), **B);** Invert microscopic views of the epidermal stem cells in culture medium at the end of second passages (5X), **C);** The view of DermoTurk 00 at the end of the production. In D, E, F, the picture of the mesenchymal stem cells' differentiation tests are seen. **D);** The fat cells were shown by staining with Oilred, **E);** bone cells with Alizarin and **F);** cartilage cells with Toluidine.

Marking the cells

MSCs and ESCs were labeled with the Q-Dot technique to distinguish them from the rat's cells. Before Q-Dot labeling, the cells were sampled for flow cytometric analysis to measure labeling efficiency. Components A and B were shaken gently in the Qtracker Cell Labeling Kit (ThermoFisher Scientific Q Tracker® 605 Cell Labeling Kit). For every $1x10^6$ cell to be labeled, 10 µl of components A and B were added into a tube and incubated for 5 minutes at room temperature. Subsequently, 0.2 ml of fresh medium was added for every $1x10^6$ cells, and the mixture was vortexed for 30 seconds, then cells were counted. At the end of the one-hour incubation period, fresh medium was added to the cells and centrifuged at 400 G for 10 minutes to wash out. This process was repeated, and the samples were taken for flow cytometric analysis. Marked and unmarked cells were analyzed in flow cytometry, and the marking rate was determined.

All tissue samples were stained with DAPI (4′,6-diamidino-2-phenylindole) and then examined with a fluorescent microscope to track Q-Dot positive cells in the tissue.

Preparation of Fibrin Matrix (DermoTurk)

Five frozen human cryoprecipitates, were thawed and then distributed equally into 50 ml conical tubes. Portioned cryoprecipitates were irradiated at 25 kgy and sterilized by the gamma irradiation. Sterile cryoprecipitate was distributed as 10 ml into each of the six-well plate wells. Calcium Gluconate Levulinate (Calcium Picken 10% ampoule, Adeka Pharmaceutical Company, Türkiye) was added as 10% of the amount of cryoprecipitate into each well, and 1% of Tranexamic acid (Transamine® 10% Ampoul, Actavis Pharmaceutical Company, Türkiye) was added and incubated for 37 hours. It was incubated for approximately 5 minutes until it solidified in an environment with a temperature of 100 °C. The matrix thus obtained was treated for stabilization. After an hour of stabilization, the fibrin matrix (DermoTurk) was ready for application (Figure 1: C).

Preparation of fibrin matrices containing MSCs and epidermal stem cells

Q-Dot marked cells were planted in the fibrin matrix at $3x10^6$ cell/cm^2 in the Petri dishes (TPP, 93060). In group 1, no cells were added to the fibrin matrix. In group 2, only MSCs were added to the fibrin matrix. In group 3, MSCs and ESCs were added to the fibrin matrix and applied to inbred rats. In Group 4, a fibrin matrix loaded with MSCs and ESCs was applied to outbred rats. Phenol red-free DMEM LG (Biological Industries, 01-050-1A) containing 1% antibiotics (Biological Industries, BI03-031-1B) and 10% FBS (Biological Industries, 01-050-1A) was added. In groups 3 and 4, the height of the medium was reduced to 1 mm on the 6^{th} day. Thus, air contact was achieved by reducing the height of the medium, and keratinocyte differentiation was induced with 20% O_2 pressure.

Monitoring glucose consumption and lactic acid production

To show that the cells planted in the fibrin matrix were alive and active, samples were taken from the fibrin matrix culture medium at 0, 4, 8, and 24 hours, and glucose and lactic acid measurements were made with the ADVIA 1800 Chemistry System. Thus, glucose consumption and lactic acid production rates were obtained.

Transport of the products

All prepared fibrin matrix forms were washed three times with Ringer's Lactate solution and put into Petri dishes containing Ringer's Lactate. Finally, the products were transported at 2-8 0C in heat-sealed carrying cases, delivered to the application center within 6 hours, and applied within 24 hours.

The design of the groups

In the study, the effectiveness and toxicological effects of only fibrin matrix, MSC-loaded fibrin matrix, ESC-loaded fibrin matrix, and MSC+ESH-loaded fibrin matrices were examined. At the beginning of the study power analysis was done by a statistical expert. According to this analysis, subjects were divided into 5 groups, each consisting of 8 rats. The groups were designed as shown below

1. Group 1 (Acellular fibrin matrix scaffold): Scaffold applied without cells (DermoTurk 00).
2. Group 2 (MSCs-added fibrin matrix scaffold) (DermoTurk 01).

3. Group 3 (MSCs- and ESCs-added fibrin matrix scaffold): Inbred group in which MSCs and ESCs added (DermoTurk 02).
4. Group 4 (Outbred group): DermoTurk 02 is applied allogeneically.
5. Group 5 (Control group): The subjects were opened with 1 cm incision on their backs and closed without any procedure.

Laboratory tests

Five days before the scaffold implantation, one cc of blood was taken from the tail veins, and basal laboratory values were obtained. Similar to previous studies, 7th-day laboratory results were examined for acute effects, and 40th-day laboratory results and autopsy findings were reviewed for long-term outcomes. Therefore, blood was retaken on the 7th, and 40th days after the procedure. On the 40th day, 0.5 cc cerebrospinal fluid (CSF) was obtained. On the same day, rats were sacrificed, and samples were taken for pathological examination from the skin area where the matrix was applied and the brain, lung, heart, liver, spleen, kidney, pancreas, small intestine, and testicle.

Hemoglobin (Hb), hematocrit (Hct), white blood cell (WBC), platelet count (Plts), sodium (Na^+), potassium (K^+), calcium (Ca^{+2}), chlorine (Cl), glucose (Glu), albumin (Alb), alanine aminotransferase (ALT), creatine (Cre), alkaline phosphatase (ALP) were examined from the venous blood, and Na, Cl, glucose and protein levels from CSF samples. Results are summarized in Table I.

Histopathological examination

Following the autopsy performed on the 40th day of the study, the skin where the scaffolds were implanted was evaluated histopathologically regarding inflammation, granulation, foreign body reaction, and the status of implanted cells. The samples taken from the brain, lung, heart, liver, spleen, kidney, pancreas, small intestine, and testis were examined, and the groups were compared with the control group.

Application of DermoTurk series

Under general anesthesia (described above), the dorsum of the rats was shaved and cleaned with 10% polyvinylpyrrolidone iodine. Then, a 1 cm long vertical midline incision was made. The prepared products were implanted under the skin of the rats according to the created groups. In the rats in the control group, a 1 cm long vertical incision was made and closed without inserting anything inside. The incisions were sutured with 3/0 Prolene (Propylene ®, Doğsan, Turkey). Wound dressing was made daily with 10% polyvinylpyrrolidone iodine solution for a week (Figure 3: A).

Statistical analysis

Statistical analysis was performed using the IBM Statistical Package for the Social Sciences, version 22.0 (SPSS Inc., Armonk, NY, IBM Corp., USA). Categorical variables were displayed as numbers and percentages, and numerical variables were expressed as mean and standard deviation or median (IQR-Interquartile Range). The Shapiro-Wilk test was used to examine whether the data were normally distributed. Hgb, Hct, Plt, WBC, Na^+, Albumin, Calcium, and Glucose values obtained on days 0.7, and 40 were normally distributed, while the other variables were not normally distributed. Differences between groups were examined with one-way analysis of variance (ANOVA) for those with normal distribution. When a difference was found, Dunnett's t-test was applied to determine the source of the difference. For variables that were not normally distributed, the Kruskall-Wallis test and the Bonferroni test were used as post-hoc tests. $p < 0.050$ level was accepted as statistically significant.

RESULTS

Flow cytometric analysis of ESCs performed with the FACSDiva ® program, CD200, CD29 (β1 Integrin), CD271, K15, and Nestin antibodies were found to be positive, and CD34 and CD45 antibodies were negative (Figure 2: A,B,C,D,E). In viability tests, it was determined that the cells were 90% alive and 90% stained with Q-Dot. The viability of cells in MSC-loaded fibrin matrix and MSC&ESC-loaded fibrin matrices was analyzed. Following cell transplantation, glucose, and lactic acid rates were determined in the samples taken into the culture medium at intervals for 24 hours. According to glucose consumption and lactic acid production, cells planted in the fibrin matrix were shown to be alive and active.

Flow cytometric analysis of MSCs produced from the adipose tissue of rats, performed with the FACSDiva ® program on the BD Facs Canto-II flow cytometry device, CD73, CD90,

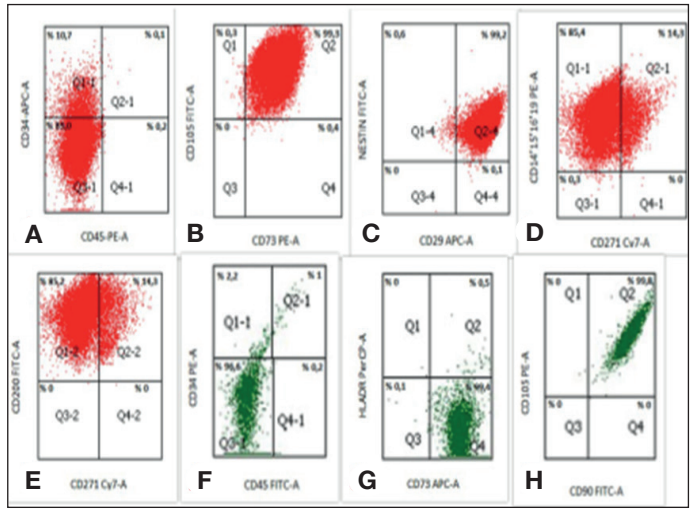

Figure 2: Histogram images showing surface antigens of epidermal stem cells and mesenchymal stem cells in flow cytometry analysis. **A, B, C, D, E);** Surface antigens belonging to epidermal stem cells including CD29 (β1 integrin), CD14, CD15, CD16, CD19, CD73, CD105, CD200, CD271 and Nestin were found positive whereas CD34 and CD 45 were negative. **F, G, H);** In mesenchymal stem cells, CD73, CD90 and CD105 surface antigens were positive whereas CD34, CD45 and HLADR surface antigens were negative.

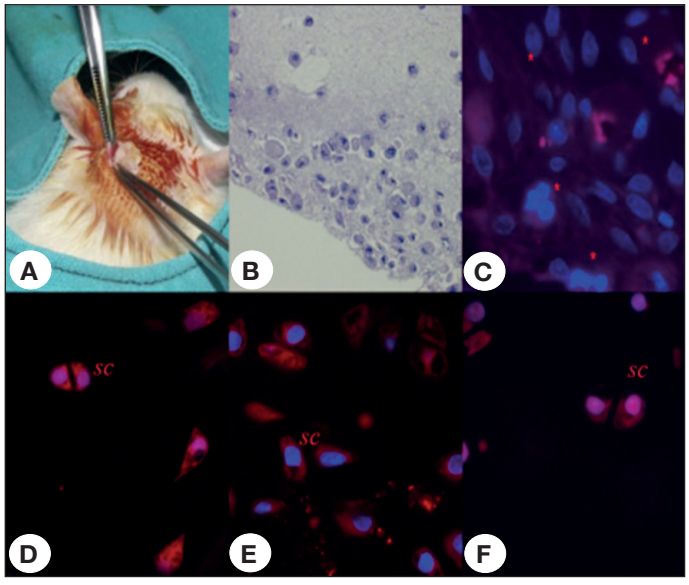

Figure 3: **A);** Implantation of the biomatrix subcutaneously to the rats' skin. **B);** Appearance of the stem cells (sc) in H&E staining within DermoTurk 02 (X20) in the sample taken immediately before application. **C, D, E, F);** In the immunofluorescence examination, it was observed that the stem cells located in the subcutaneous biomatrix were still alive at the day of 40th and were colonized there. **C);** Qdot positive cell (MSC) is not observed in the samples taken from rats in Group 1. The cells seen (*) are their own cells. Q-dot positive stem cells (sc) were seen in **D);** rats in Group 2, **E);** rats in Group 3 and **F);** rats in Group 4. Q-dot positive cells are marked with arrow.

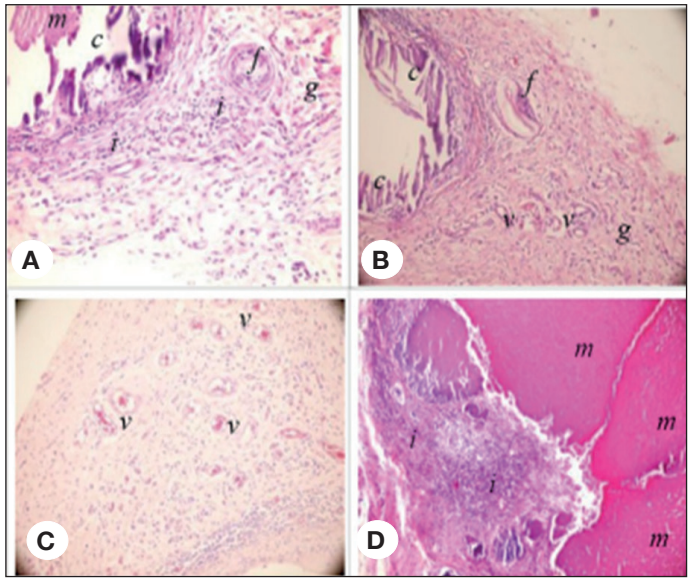

Figure 4: In the samples taken on the 40th day of the application, an increase in inflammation, granulation tissue formation, and foreign body reaction were observed in the vicinity of the biomatrix. **A, B);** Around matrix (m), calcification foci (c), diffuse inflammatory cell deposition, granulation formation (g) and foreign body reaction (f) were observed. **C);** More neovascularization (v) is observed in the groups in which the matrix given by epidermal stem cells and mesenchymal stem cells (DermoTurk 02) is applied. This indicates an increased recovery process. **D);** A more intense inflammatory cell increase (i) was observed around the non-cellular matrix (m).

Table I: Comparison of laboratory values of blood samples taken from tail veins of subjects 5 days before starting of the study

Variables	Group 1	Group 2	Group 3	Group 4	Group (Control)	Reference values	F	X^2	p
Hgb*	14.4±0.5	14.2±0.6	14.3±1.1	14.4±0.8	13.6±1.2	11-19.2	1.233	-	0.315‡
Htc*	44.9±2.1	47.4±6.7	44.9±3.9	45.4±2.1	42.3±5.1	39.6-52.5	1.400	-	0.254‡
Plt*	790000±137721	734000±300802	776375±218406	939875±133087	816625±111871	500-1.3x10^6	1.292	-	0.292‡
WBC*	8751±1703	7577±1766	10994±2127	6736±2581	8983±2488	6-18x1000	4.453	-	0.005‡
Na*	141.8±2.9	141.8±1.9	144.3±2.9	143.3±2.8	142.5±2.0	140-150	1.409	-	0.251‡
Alb*	3.3±0.2	3.4±0.1	3.3±0.2	3.2±0.1	3.3±0.2	3.8-4.8	0.862	-	0.496‡
Ca*	10.2±0.3	10.3±0.4	9.8±0.2	9.7±0.3	10.1±0.5	8-13	3.590	-	0.015‡
Glu*	303.3±49.7	321.1±65.2	249.9±38.7	324.1±54.4	260.3±51.6	50-160	3.460	-	0.017‡
K†	5.45 (0.50)	5.70 (1.63)	5.00 (0.68)	5.60 (0.93)	4.90 (1.35)	4.3-5.6	-	7.597	0.108§
Cl†	101.00 (3.75)	102.50 (5.25)	100.50 (3.75)	102.50 (6.50)	99.50 (4.50)	95-115	-	7.980	0.092§
Alt†	54.00 (14.25)	56.00 (24.75)	61.50 (21.75)	72.00 (11.75)	68.50 (24.75)	35-80	-	13.890	0.008§
Cre†	0.46 (0.07)	0.52 (0.10)	0.47 (0.10)	0.50 (0.05)	0.45 (0.13)	0.5-1	-	5.730	0.220§
Alp†	169.00 (57.25)	156.00 (22.25)	184.50 (80.00)	203.00 (86.75)	205.00 (120.75)	62-230	-	4.129	0.389§

*****$\bar{x}$±SD, †Median (IQR), ‡***One-way ANOVA test was used;*** *the Dunnett t test was used for post-hoc comparisons,* §***Kruskal-Wallis test was used;*** *the Mann-Whitney test with Bonferroni correction was used for post-hoc comparisons.*

and CD105 antibodies were found to be positive, and HLA-DR, CD45, and CD34 antibodies were negative (Figure 2: F, G, H). In viability tests, it was determined that the cells were 95% alive and 92% stained with Q-Dot. It was observed that MSCs could differentiate into chondrocytes, fat cells, and bone cells in the appropriate environment. At the end of the differentiation analysis, fat cells were stained with Oil red, bone cells with Alizarin, and cartilage cells with Toluidine (Figure 1: D, E, F).

Laboratory results

Biochemical values in all groups (5 days before the start of the study, 7th, 40th days) were evaluated. The results of the study

groups (group 1,2,3, and 4) were compared with the control group (Group 5) and then compared each other.

The biochemical values of 5th day before procedures and their comparisons within groups were shown in Table I. According to this, there was no difference between all groups in terms of Hgb,Htc, Plt, Na^{+}, Alb, K^{+}, Cl^{-}, Cre and ALP levels. However, there were statistically significant differences between the groups in terms of WBC, Ca^{+2}, Glucose and ALT values

Table II: Biochemical values obtained from rats on the 7th day of the study

Variables	Group 1	Group 2	Group 3	Group 4	Group 5 (Control)	Reference values	F	X^2	p	group number‖	p
Hgb*	14.1±0.5	13.8±0.3	13.9±1.3	13.9±0.5	13.0±1.3	11-19.2	1.840	-	0.144‡	–	–
Htc*	44.2±2.6	43.9±1.1	44.6±3.7	43.1±2.9	41.7±5.1	39.6-52.5	0.901	-	0.474‡	–	–
Plt*	814625±60469	817000±105287	677000±121604	838429±113979	778875±75971	500-1.3x10⁶	3.367	-	0.020‡	–	–
WBC*	9033.8±2535.0	7553.8±1111.5	11755.0±2740.7	7128.6±945.1	7790.0±1706.1	6-18x1000	7.335	-	<0.001‡	3	0.001‡
Na*	141.8±1.4	141.0±1.3	141.6±1.8	138.7±1.4	140.6±0.9	140-150	5.740	-	<0.001‡	4	0.039‡
Alb*	3.5±0.2	3.5±0.1	3.4±0.2	3.2±0.1	3.4±0.2	3.8-4.8	1.970	-	0.121‡	–	–
Ca*	10.5±0.4	10.7±0.3	9.9±0.4	9.5±0.2	10.2±0.5	8-13	12.697	-	<0.001‡	4	0.002‡
Glu*	N/A	N/A	N/A	N/A	N/A	50-160	N/A	-	N/A	–	–
K†	4.80 (1.05)	4.70 (0.38)	4.90 (1.78)	5.60 (0.90)	5.50 (0.55)	4.3-5.6		14.614	0.006§	2	0.016§
Cl†	102.00 (3.00)	101.50 (2.00)	102.00 (1.50)	100.00 (2.00)	100.00 (3.50)	95-115		8.517	0.074§	–	–
ALT†	49.50 (14.75)	56.50 (15.25)	62.00 (13.25)	71.00 (9.00)	72.50 (35.00)	35-80		15.044	0.005§	1	0.008§
Cre†	0.42 (0.05)	0.40 (0.06)	0.39 (0.15)	0.39 (0.02)	0.40 (0.06)	0.5-1		3.752	0.441§	–	–
ALP†	123.00 (52.50)	129.50 (34.25)	179.50 (60.75)	219.00 (74.00)	190.00 (63.25)	62-230		11.202	0.024§	–	–

*$\bar{x}$±SD, †Median (IQR), ‡**Oneway ANOVA test was used;** *the Dunnett t test was used for post-hoc comparisons,* §**Kruskal-Wallis test was use;** *the Mann-Whitney test with Bonferroni correction was used for post-hoc comparisons.* ‖ *Group compared whit the control group.*

Table III: Biochemical values obtained from rats on the 40th day of the study

Variables	Group 1	Group 2	Group 3	Group 4	Group 5 (Control)	Reference values	F	X^2	p	group number‖	p
Hgb*	14.4±1.1	14.6±1.2	16.5±1.9	14.5±1.4	13.4±2.0	11-19.2	3.567	-	0.016‡	3	0.003‡
Htc*	45.8±2.9	47.9±5.1	54.1±6.3	49.1±4.8	45.6±7.0	39.6-52.5	2.716	-	0.047‡	3	0.022‡
Plt*	696125±110988	624625±197373	588333±83691	703857±257882	723000±330419	500-1.3x10⁶	0.475	-	0.754‡	–	–
WBC*	9022.5±3078.6	5882.5±2104.5	7713.3±2799.7	6551.4±1569.1	5665.0±1578.6	6-18x1000	2.906	-	0.037‡	1	0.022‡
Na*	141.1±2.2	142.5±0.8	141.5±2.6	142.7±4.3	142.6±1.4	140-150	0.650	-	0.631‡	–	–
Alb*	3.6±0.2	3.6±0.2	3.6±0.2	3.4±0.1	3.3±0.3	3.8-4.8	3.903	-	0.011‡	1. 2	<0.050‡
Ca*	N/A	N/A	N/A	N/A	N/A	8-13	N/A	-	N/A	N/A	N/A
Glu*	278.3±62.0	288.0±91.2	269.0±36.2	275.0±101.1	215.8±84.3	50-160	1.036	-	0.404‡	–	–
K†	5.10 (3.00)	8.40 (2.35)	7.30 (4.00)	7.35 (2.55)	5.20 (3.05)	4.3-5.6	-	8.138	0.087§	–	–
Cl†	100.00 (3.00)	101.00 (1.50)	101.50 (2.50)	103.00 (2.50)	102.00 (2.75)	95-115	-	6.613	0.158§	–	–
Alt†	68.00 (44.00)	109.00 (100.25)	87.50 (51.50)	114.00(251.50)	94.50 (58.00)	35-80	-	5.371	0.251§	–	-
Cre†	0.44 (0.10)	0.50 (0.09)	0.47 (0.17)	0.49 (0.14)	0.47 (0.12)	0.5-1	-	2.412	0.661§	–	–
ALP†	103.00 (52.00)	108.00 (56.75)	126.00 (29.75)	167.00 (46.00)	183.00 (71.00)	62-230	-	14.283	0.006§	1. 2	<0.05§

*$\bar{x}$±SD, †Median (IQR), ‡**Oneway ANOVA test was used;** *the Dunnett t test was used for post-hoc comparisons,* §**Kruskal-Wallis test was used;** *the Mann-Whitney test with Bonferroni correction was used for post-hoc comparisons,* ‖ *Group compared whit the control group.*

Table IV: Biochemical values of the rats cerebrospinal fluids on the 40th day of the study

Variables	Group 1	Group 2	Group 3	Group 4	Group 5 (Control)	F	x^2	p
Cl*	124.6±1.7	123.3±2.3	122.0±1.4	123.6±2.8	123.9±2.6	1.277	-	0.299‡
Na†	156.0 (4.0)	155.5 (1.8)	154.5 (3.0)	156.0 (1.0)	156.0 (2.8)	-	4.456	0.348§
Glu†	86.5 (11.0)	81.0 (11.5)	83.5 (7.5)	85.0 (10.0)	84.5 (24.5)	-	1.255	0.869§
Protein†	42.5 (4.6)	42.6 (12.4)	38.3 (8.0)	41.0 (4.0)	40.6 (10.1)	-	3.780	0.437§

**$\bar{x}$±SD, †Median (IQR), ‡Oneway ANOVA test was used, the Dunnett t test was used for post-hoc comparisons, §Kruskal-Wallis test was used, the Mann-Whitney test with Bonferroni correction was used for post-hoc comparisons.*

Tablo V: Histological examination results of rats

Parameter	Group 1	Group 2	Group 3	Group 4	Group 5 (Control)
Inflammation and granulation rates Available/n	8/8	4/8	6/8	2/8	0/8
Distribution according to the degree of inflammatory reaction					
0	1	4	1	6	8
1	3	1	3	1	–
2	2	2	2	–	–
3	2	1	2	1	–
Distribution according to the degree of granulation reaction					
0	1	4	1	6	8
1	1	3	4	–	–
2	3	1	2	1	–
3	3	–	1	1	–

(p=0.005, p=0.015, p=0.017 and p=0.008 respectively) (Table I). While the change in these laboratory values is statistically significant, the change in values is within the limits of normal references laboratory values and is clinically meaningless. These increases were attributed to the sudden increase in sympathetic activity, which resulted from acute stress.

Results of post-procedure 7th day's were shown in Table II. Compared with the control group, ALT values of group 1 (p=0.008) and the K^+ values of Group 2 were lower (p=0.016). The WBC counts of Group 3 were higher than the control group (p=0.001). Both Na+ values (p=0.039) and Ca^{+2} values (p=0.002) of Group 4 were lower than the control group. While the change in these laboratory values is statistically significant, the change in values is within the limits of normal references laboratory values and is clinically meaningless. In terms of other values, there were no statistical differences between all the other groups and the control group. When compared among themselves, there were statistically significant differences between the groups regarding Plt, WBC, Na^+, Ca^{+2}, K^+, ALT and ALP values. However, all values except for Na^+ values of Group 4 were within normal references values. The Na^+ values of Group 4 were less than the reference values.

Results of the 40th day were shown in Table III. Compared with the control group; there were statistical differences in term of WBC, Alb, and ALP values of Group 1 (p=0.022, p<0.050, p<0.050 respectively); Alb and ALP values of Group 2 (p<0.050, p<0.050 respectively) and Hgb and Htc values of Group 3 (p=0.003, p=0.022 respectively). However, the values of all groups were within acceptable limits.

The results of CSF on the 40th day were shown in Table IV. In the statistical analysis there were no statistically significant differences between the groups and the control group.

Histopathological results

MSC&ESC-loaded fibrin matrix was examined macroscopically (Figure 1: C) and microscopically with Hematoxylin&Eosin staining (Figure 3: B). The fibrin matrix maintained its integrity, and the transplanted cells maintained viability.

At the end of the study, the most typical finding in the histopathological examination was that there was a mild-grade foreign body reaction in almost all groups against the scaffolds, but this reaction was observed to be less in the stem cell-rich groups (groups 3 and 4). Increased inflammation, granulation formation, and foreign body reactions were observed in the vicinity of the biomatrix (Figure 4: A,B,C,D). It was more pronounced in allogeneic group (group 4) (Table V). In the immunofluorescence, the stem cells in the fibrin matrices implanted under the skin were still live on the 40th day and to be colonized here (Figure 3: D,E,F). No histopathological changes were observed in the other tissues. The results of the histological examination are summarized in Table V.

DISCUSSION

The ultimate goal of tissue engineering for artificial skin is to produce a self-renewable, functional skin similar to all layers of the skin and all skin appendages, including hair follicles, sweat glands, and neurosensory structures. Because conventional treatments do not produce the desired results in treating burns, scientists use tissue engineering techniques to make artificial skin. In this sense, one of the first milestones was carried out in 1981 by O'Connor et al.(14). They used cultured epithelial autografts (CEA) obtained by the proliferation of autologous keratinocytes in the laboratory on an excessively burned child (14). However, it is not preferred because the preparation of keratinocyte culture takes a long time; during this time, the risk

of infection increases, and the produced skin is very fragile; the success rate in third-degree burns is low, is an expensive method, the skin does not appear in the desired appearance (15).

The effectiveness of many cell types has been examined in tissue engineering applications. Studies have shown that MSCs positively affect wound healing and tissue regeneration thanks to their secreted trophic factors. Adipose tissue-derived MSCs have stem cell-specific surface markers CD90, CD105, CD73, CD44, and CD166 and low expression of CD45 and CD34. They have a collagen production capacity and are genetically and morphologically more stable in long-term cultures, with low senescence and high proliferation capacity (16).

Various materials have been used as scaffolds in the production of artificial skin. However, the desired result has not been obtained. Allograft skin grafts from cadavers or volunteers have long provided temporary coverage in burn patients. The most significant disadvantage is rejection due to the immunogenic structures. Another significant disadvantage is the risk of transport of some infections from the donor to the recipient (17). While many studies have been conducted with different scaffold samples in many centers, most scaffold structures studied are synthetic biomaterials, and some are allogeneic cadaveric. For these reasons, we believe that a fibrin-derived scaffold is a considerable alternative for skin loss conditions such as full-thickness burns because it can be easily, quickly, and autologously prepared.

Sclafani applied a platelet-rich plasma-derived fibrin matrix to the skin for the first time in 2010. After the application to 15 patients with deep nasolabial folds, it was reported that it stimulated fibroblasts thanks to the growth factors contained in the fibrin matrix, ensured collagen deposition in the dermal-subdermal connections, and statistically significant decreases were observed in wrinkle scores at the end of 12 weeks (18). In another study examining the effectiveness and safety of fibrin matrix application for aesthetic purposes for treating facial depressions, nasolabial folds, and scars, Sclafani followed 50 patients for ten months. He reported that the swelling in all patients disappeared within five days at most, only minimal bruising was observed, and most patients were satisfied with the treatment. It was reported that the fibrin matrix was safe and effective (19).

Although the fibrin-derived matrices have been shown to be more effective in the treatment of full thickness burns than the control group, this effect was not found to be sufficient. However, it is highly probable that fibrin matrices are more effective after being combined with stem cells because these cells increase their efficacy (20).

Gentile et al. applied a fibrin matrix in a mixture with adipose-derived stromal vascular fraction cells to patients with burn sequelae and post-traumatic scars. They compared them with the group in which they used only adipose-derived stromal vascular fraction cells. It was observed that the reconstruction rate was 69% in the fibrin matrix with the Adipose-derived stromal vascular fraction cells application group, and this rate was 39% in the adipose-derived stromal vascular fraction cells only group (21). Autologous limbal cells were seeded on fibrin structures and applied to the corneas of 152 patients with ocular burns for various reasons. The study showed that severely damaged human cornea could be successfully treated using limbal cells combined with a fibrin-derivated matrix (22). Considering all these results, our group believes that the effectiveness of a fibrin-based matrix can be increased by supporting it with autologous stem cells. A completely autologous skin substitute would be more effective.

In this study, we aimed to evaluate DermoTurk, an acellular matrix we developed, and its cellular forms in terms of toxicity before preclinical studies. Although no systemic toxicity was found in the cellular and acellular fibrin matrix forms, pathological examination revealed foreign body reaction, inflammation, and granulation in all forms in the subcutaneous area where it was applied. The most likely reason for this was thought to be excessive membrane phospholipids from the cryoprecipitate used to prepare the fibrin matrix. We considered the most likely reason of this to be the dense membrane phospholipids from the xenogeneic platelet-rich plasma used to prepare DermoTurk (23). Therefore, depending on the applied matrix, an acceptable rate of inflammation, granulation, and foreign body reaction localized to the applied area was considered reasonable. However, it is noteworthy that this reaction is less in the cellular forms of the product, especially in biomatrices richer in stem cells. We think the most likely reason for this is the immunosuppressive effect of the MSCs and skin stem cells (24).

One of the critical findings of this study is that on the 40th day after implantation, allogeneic stem cells can maintain their presence under the skin. Previous studies have shown that allogeneic MSCs can continue to be functional in the host tissue for five weeks without tissue adherence, with a small number of long-term host tissues (25,26). This finding is vital in developing fully human-derived skin substitute biomaterials in patients with sudden skin requirements.

In conclusion, the toxicological examination of DermoTurk forms revealed they are safe. Therefore, it can be considered to be used in the development of new products. It is thought that clinical efficacy studies of DermoTurk forms should be conducted.

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