Cell Therapy and Investigation of Angiogenesis of Fibroblasts with Collagen Hydrogel on the Healing of Diabetic Wounds

Short Title: Investigation of Angiogenesis

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Abstract
INTRODUCTION: A diabetic ulcer is a common disease in diabetic patients. Due to antibiotic resistance, new therapeutic alternatives are being considered in diabetic foot patients to reduce complications and mortality. This study aimed to evaluate the effect of collagen hydrogel on wound healing process in diabetic rats.

METHODS: Diabetic wounds were induced with streptozotocin in all 42 male Wistar rats. The rats were divided into four groups: (a) treated with fibroblast cells, (b) collagen hydrogel, (c) collagen cultured with fibroblast cells, and (d) control group. Microscopic and histological (H&E staining and Mason trichrome staining), measurement of wound surface with Image J, skin density and thickness by the ultrasound probe, and skin elasticity with cutometer tool was used to evaluate the wound healing in days 1, 14, and 21 after the treatment.

RESULTS: The results showed that the treatment of diabetic wounds with fibroblast cells cultured in collagen hydrogel greatly reduces inflammatory responses in the skin tissue and significantly accelerates the healing process. Also, 21 days after the start of treatment, skin elasticity, thickness and density were higher in the collagen + fibroblast group than in the control group.

CONCLUSION: Also, the results of the present study show that diabetic wound dressing can significantly reduce the inflammatory phase in the wound healing process by increasing the speed of collagen synthesis, skin density and elasticity, and angiogenesis.

Keywords: Fibroblast, Collagen hydrogel, Burn wound healing, Diabetic rat

1. Introduction
A diabetic ulcer is a common disease in diabetic patients. Between 15 and 25 percent of people with diabetes develop diabetic foot ulcers. The 5-year mortality rate for diabetic wounds that require reputation varies between 39 and 80 percent. In addition, new therapeutic alternatives are being considered in diabetic patients. There has been a lot of research in recent years on the effects of collagen hydrogels and fibroblasts, but research in this area is still of interest to scientists. Stem cells can proliferate and are transformed into different cell types1-3. Collagen hydrogel is used for a wide range of skin diseases by increasing blood flow4-6. Huang reported that stem cells can heal skin wounds by increasing the thickness and elasticity of the skin. Other research has shown that hydrogels cultured with stem cells help rapid healing of diabetic skin ulcers through angiogenesis and collagen deposition7,4,7. In contrast, recent research has shown that mesenchymal stem cells interfere with the healing of diabetic wounds. Despite significant findings regarding the positive effects of collagen hydrogels7,4,7, the results of some studies in this area are disappointing9. There is conclusive evidence of the effects of collagen hydrogels on the healing of diabetic skin ulcers, which clearly indicates wound healing after the use of fibroblasts derived from foreskin and collagen.
hydrogels. Based on this and considering that, common treatments are very complex. In this regard, the use of collagen hydrogels cultured with fibroblasts derived from circumcised skin may be effective. In this study, the effects of collagen hydrogel were investigated using cell culture and histology methods. The results of this study can be used in the design of treatment methods to heal skin wounds.

2. Materials and Methods

All the study procedures were carried out according to the local guidelines for the care of laboratory animals of the Faculty of Pharmacy and Pharmaceutical Sciences, Tehran Medical Sciences, the Islamic Azad University, Iran (IR.IAU.PS.REC.1399.148).

2.1. Preparation of Collagen Hydrogels

40 mg of bovine collagen (Biomaterials Company) was dissolved in a Falcon tube containing 25 mL of deionized sterile water. 2 mL of PBS buffer and 8 mL of HEPES buffer were added to the Falcon tube and placed at room temperature for one day. The obtained hydrogel was dewatered using an acetone solution and sterilized by UV light of the UV irradiation. The morphology of the hydrogel was examined and photographed using the Olympus BX61 Research Slide Scanner microscope.

2.2. Isolation of Fibroblast Cells

First, the foreskin of a human infant was collected and stored using HEPES buffer at 4 °C. The skin sample was cut into small pieces employing surgical forceps in a bacteriological petri dish and washed to prevent microbial contamination in a Falcon tube containing 10 mL of 70% alcohol. In the next step, the dermis was separated from the epidermis and incubated with dispase enzyme and HEPES buffer for 24 hours. Dermal samples were then incubated with collagenase (Sigma-Aldrich, USA) at 0.1% for 2 hours. The contents of the dish were transferred to a 50 mL Falcon tube containing 5 mL of DMEM-HG culture medium (GIBCO, USA) and containing 10% FBS and pipetted. The contents of the Falcon were passed through a mesh filter with 70 μm pores to separate the cells from the tissue fragments. The cells were centrifuged for 5 min at 200 g at 14 °C. The supernatant was drained and was added serum-free DMEM-HG culture medium to 5 mL of cell sediment. Finally, the cells were centrifuged at 200 g at 14 °C for 5 min.

2.3. Fibroblast Cells Characterization and Count

Immunocytochemical staining was performed to confirm the identification of isolated fibroblast cells. To stain the nucleus, the slide was immersed in hematoxylin for 1 minute and then rinsed with water. Dehydration and clarification were performed by placing slides in 50, 70, and 96% alcohol. Cells were counted by Fisher Scientific hemocytometer and Olympus IX 70 light microscope. PI-Acridine staining was applied to evaluate cell viability according to the manufacturer's instructions. Cells were cultured, isolated, washed, and incubated with annexin-V and 7-AAD PE grafted in annexin-V (BD Biosciences) binding buffer for 15 minutes. Finally, the percentage of living and dead cells was examined in the Beckman Coulter Navios flow cytometer.

2.4. Culture of Fibroblasts in Collagen Hydrogel

First, the hydrogel was prepared on the desired plate. After reaching the desired density, fibroblasts were trypsinized and transferred to hydrogels. The cells containing hydrogel were placed in an incubator and observed daily under a microscope. Fibroblast cell culture was observed in hydrogels using an inverted microscope (Olympus, IX 70) and a scanning electron microscope (SEM, Hitachi, S-3000 N, Japan). After 14 days, collagen disks cultured with fibroblast cells were used for wound dressing.

2.5. Hydrogel Cytotoxicity Assessment

To measure cell viability, $1 \times 10^5$ fibroblast cells were cultured with RPMI medium in a 96-well plate. After 24 hours of incubation, the cells with hydrogel were treated. After 48 hours of exposure of the hydrogel to the cells, 20 μL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) solution was added (0.05 mg/well) to the plates in the dark. After 5 hours of incubation of the plates, 100 microliters of DMSO solution were added to each well, and after 10 minutes of stirring, the resulting color intensity was measured by the Elisa Reader at 570 nm. After culturing with glutaraldehyde and drying in room air, sectioning and then coating with gold, the cultured cells were subjected to SEM imaging.

2.6. Animal Experiments

42 male Wistar rats were purchased weighing 200 to 220 g from Pasteur Institute (Tehran, Iran). Animals were kept in metal cages with access to water and food. Rats were exposed to 12 hours of darkness and 12 hours of light at 23°C for one week in special conditions in terms of light and temperature. The rats were divided into 4 groups (6 in each group): (a) control group (normal saline intake group), (b) fibroblast treated cells (fibroblast group), (c) collagen hydrogel treated (collagen group), and (d) collagen cell treated group + fibroblast group (collagen + Fibroblasts). Diabetes was induced by injecting a dose of 50 streptozotocin (STZ, 40 mg/kg, Aladdin) intraperitoneally in rats. Symptoms of diabetes, such as binge drinking and urinary incontinence, appeared in rats three days after the injection. Glucose levels in the blood sample taken from the tail were measured using a
glucometer 4 days after the injection. Rats with a blood glucose level greater than 246 mg/dl were considered diabetic rats. The animals were anesthetized with 2 mg of intraperitoneal injection of ketamine, and 0.2 mg of xylazine was completely rubbed on the back of the animal. The wound was created using a sterile punch. The dressings were fixed on the wound using Vaseline gauze and transparent adhesive tape. Images of the wound area were captured on days 7, 14, and 21 after the wound using a digital camera (S9+, Samsung, South Korea). Image J was measured in the mm scale.

2.7. Histological Examination
Animals were exterminated by spinal method on days 7, 14, and 21, and the skin tissue including the wound area and the surrounding skin area was removed by 2 cm and placed in 10% formalin solution. Fixed samples in 10% formalin were stained with H&E (Thermo Fisher Scientific Co., Ltd., Shanghai, China) dyes for tissue analysis and Mason trichrome for cellular changes. Fixed textured slides were photographed using a digital camera (Olympus IX70).

2.8. Biometric Analysis
The thickness and density of the skin were assessed at the wound site using 75 MHz ultrasound imaging (digital ultrasound imaging system DUB Skin Scanner 75, tpm taberna pro medicum GmbH, Germany) on days 7, 14, and 21. Skin elasticity (net elasticity (R2), gross elasticity (R5), and post-recovery deformation (R7)) were examined using a catheter (Courage & Khazaka electronic GmbH, Germany) on days 7, 14, and 21 after the treatment in the area of the wound.

2.9. Data Analysis
Data were analyzed using SPSS statistical analysis software. One-way ANOVA and then the Tukey method applied for comparisons between groups. In the statistical study, the significance level was considered \( p < 0.05 \).

3. Results
3.1. Microstructure and Morphology of Collagen
The color of the collagen hydrogel was clear, and the results showed that UV light was sufficient for 1 hour to sterilize the collagen and hydrogel samples (Figure 1A). Observation of the microstructure of the collagen samples showed a relatively rough surface with a spongy composition and appeared smooth and uniform (Figure 1B).

![A](image1A.png) ![B](image1B.png)

Figure 1. Digital images of (A) the hydrogel samples and (B) microstructure of the collagen (Scale in nm).

3.2. Isolated Cell Viability and Cell Count
The results showed that a high percentage of fibroblasts were alive on the day of isolation, a small number of them died, and this method was suitable for isolation.
3.3. Macroscopic Observation and Wound Area Measurement
The results showed that the wound section in the group receiving fibroblasts and collagen cells in comparison with the control group and groups receiving each factor alone had the highest improvement on days 0, 7, 14, and 21 (Figure 3). Also, with time, the amount of wound shell thickness in this group was more than that in other groups, and the wound diameter decreased more and, therefore, was faster the wound healing process.

3.4. Microscopic Examination of the Wound
The results showed that the neutrophil count reached its maximum on the third day after wounding and then decreased. This rate was almost the same in all 4 days of the study. Therefore, it can be concluded that all 4 groups
do not stimulate the immune system and cause an inflammatory reaction and increase the number of neutrophils at the wound site. At 21 days after the treatment, the number of macrophages decreased in all groups, and in the group treated with collagen, the hydrogel was significantly lower than in the other groups. Epithelial tissue was observed on the 7th day after the treatment and increased for 14 days. On the 7th day, angiogenesis in the collagen hydrogel group was significantly less than in that the 3 experimental groups(Figure 4).

Figure 4. Histological sections of harvested wound area specimens on days 7, 14, and 21 in the control and experimental groups.

3.5. Biometric Analysis
The results showed that the thickness of skin (epidermis + dermis) in the groups receiving fibroblast stem cells, the group receiving collagen hydrogel, and the group receiving fibroblast stem cells + collagen hydrogel increased significantly compared to the control group ($p < 0.001$). Also, the amount of skin thickness in the group receiving fibroblast stem cells + collagen hydrogel was significantly different from the group receiving fibroblast stem cells ($p < 0.05$). Finally, in the group receiving fibroblasts + collagen hydrogel stem cells, a significant difference was observed with $p < 0.01$ compared to the group receiving collagen hydrogel(Figure 5).
Figure 5. Data obtained from measuring the effects of fibroblast cells and collagen hydrogels in the studied groups on skin thickness in the healing of diabetic wounds in an animal model.

The percentage of skin density in the group receiving fibroblast stem cells and fibroblast stem cells + collagen hydrogel significantly increased compared to the control group \((p < 0.001, p < 0.05)\). Finally, the percentage of skin density in the group receiving fibroblast stem cells + collagen hydrogel significantly increased compared to the groups receiving fibroblast cells and collagen hydrogel \((p < 0.001, \text{Figure 6})\).

Gross skin elasticity \((R2)\) significantly increased in the group receiving fibroblasts + collagen hydrogel compared to the control group and the group receiving collage hydrogel \((p < 0.01)\). Also, the net skin elasticity \((R5)\) increased significantly in the group receiving fibroblast stem cells and fibroblast stem cells + collagen hydrogel compared to the control group \((p < 0.01, p < 0.001)\). The rate \((R7)\) of skin recovery in the group receiving fibroblast stem cells and fibroblast stem cells + collagen hydrogel increased significantly compared to the control group \((p < 0.001)\) (Figures 7, 8, and 9).

Figure 6. Data obtained from measuring the effects of fibroblast cells and collagen hydrogels in the studied groups on the percentage of skin density in the healing of diabetic wounds in the animal model.
Figure 7. Data from the measurement of the effects of fibroblast cells and collagen hydrogels in the studied groups on the amount of gross skin tension (R2) in the healing of diabetic wounds in an animal model.

Figure 8. Data obtained from measuring the effects of fibroblast cells and collagen hydrogels in wounds in an animal model the studied groups on the amount of skin elasticity (R5: net elasticity) in the healing of diabetic.
The results obtained from ultrasound imaging showed that the wound thickness increased on day 21 in all groups compared to days 7 and 14, which increased primarily in the group receiving fibroblasts + collagen hydrogel and then in the collagen group had the highest amount (Figure 10).

* Significance compared to the control group, ¥ Significance relative to the group receiving fibroblast cell, φ Significance relative to the group receiving collagen hydrogel (***: $p < 0.001$, ¥¥¥ $p < 0.001$, ψψψ $p < 0.001$).
Figure 10. Ultrasound imaging for measurement of density and thickness of wound area skin using a 75 MHz ultrasound probe on days 7, 14, and 21 after the treatment.

4. Discussion

Although many studies have shown that collagen hydrogels and fibroblast cells are effective in wound healing, the effect of cultured collagen hydrogels with circumcised fibroblasts on healing skin wounds is still challenging. It is the most interesting research topic. Based on the present study, using histological and microscopic methods, the effects of collagen hydrogel transplantation cultured with circumcised skin fibroblasts have been investigated to show that transplantation of cultured collagen hydrogels with fibroblast cells can heal the wound at the right time and by what mechanisms it does this repair. According to the results, collagen hydrogel did not show significant cytotoxic effects on neonatal foreskin-derived fibroblast cells and is a bio-safe substance for use for wound healing. The results showed that the wound cross-section had the highest healing rate in the group receiving fibroblasts and collagen simultaneously. Also, over time, the thickness of the wound shell in this group was more than that in other groups, and the wound diameter decreased more and the wound healing process was faster. Histological studies with two types of H&E and Mason Tricom showed that the number of macrophages decreased on day 21 in all 4 groups. This amount was significantly lower in the cellular hydrogel group than the other 3 groups. The epithelial formation was observed on the seventh day and increased until the 14th day. On days 7, 14, and 21, the percentage of epithelial formation in the cellular hydrogel group was always significantly higher than the other three groups. On day 7, angiogenesis in the cellular hydrogel group was significantly lower than the other three groups. According to these results, Bai et al. (2020), showed that diabetic wounds treated with bone marrow stem cells (BM-MSCs) cultured in chitosan hydrogel compared with the control group completely healed the wound 15 days after treatment. Shen et al. (2015), showed that the wounds of diabetic rats in the collagen-treated group accelerated wound healing compared with the control group and achieved complete healing on the 18th day after the treatment. In a study, Shi et al. (2016) examined the effects of fat-derived stem cells on skin ulcers in male diabetic rats and reported that stem cell transplantation significantly reduced ulcers on day 15. In another study, Pomatto et al. (2021) examined the effects of fat-derived mesenchymal cells on skin ulcers in diabetic rats and showed that fat-derived mesenchymal cells repair wounds by increasing angiogenesis. Still, in another study of the effects of fat-derived stem cells on skin wounds using flow cytometry and Western blotting on DBW mice, Pak et al. (2021) reported that fat-derived stem cells (ADSCs) maximize signaling. Paracrine and angiogenesis via the PI3K/AKT pathway provide synergistic effects of wound healing. Nuschke (2014) examined the effects of mesenchymal stem cells (MSCs) by MTT and immunohistochemistry. The results of the study showed that mesenchymal stem cells through tissue collagen deposition can reduce tissue inflammation and induce angiogenesis and wound healing.

In another study, Kaisang et al. (2017) examined the effects of collagen hydrogels cultured with fat-derived stem cells on wounds by histological methods on diabetic rats. Their findings showed that collagen hydrogels cultured with fat-derived stem cells improve and optimize stem cell function in order to increase diabetic wound healing. In addition, they investigated the effects of hydrogels cultured with bone marrow mesenchymal stem cells (BMSCs) on the wounds of diabetic rats by histological and immunohistochemical methods and reported that this treatment involved granulation, angiogenesis, and extracellular matrix secretion and showed that rapid epithelialization helps heal diabetic skin ulcers. In a study, da Cunha et al. (2014) examined the effects of collagen and alginate hydrogels cultured on fibroblast cells by flow cytometry and RT-PCR. Immunohistochemical results confirmed that cultures of encapsulated skin fibroblasts enhance the morphology of various cells and that this biomaterial can regulate wound healing progress. In another study, Yu et al. (2018) examined the effects of fat-derived stem cells (ASC) on immunohistochemical methods in mouse skin wounds, and their results showed that stem cells could reduce macrophage uptake and increase paracrine by collagen deposition in order to heal wounds. Uysal et al. (2014) studied the effects of mesenchymal stem cells (MSCs), bone marrow-derived stem cells (BSCs), and fat-derived stem cells (ASCs) on histological and immunohistochemical methods on skin lesions in mice Male Fisher field, and their results showed that the fastest wound healing rate was observed by mesenchymal stem cells while increasing the rate of epithelialization through angiogenesis. In a study, Rodriguez et al. (2015) examined the effects of fat-derived stem cells (ASC) on immunohistochemical methods on skin wounds in nude mice, and their results showed that stem cells could repair wounds through blood perfusion. The results showed that the concomitant recipient group of fibroblasts and collagen cells with amazing ability increases the thickness and percentage of skin density in the wound area. Also, only the fibroblast and collagen cell receiving group significantly increased the gross skin elasticity (R2) in the wound area. Regarding the results of the study of net skin elasticity (R5) and recovery process (R7), all the studied groups, except for the collagen receiving group alone, have significant incremental changes. According to these findings, a study by Kittana et al. (2018) showed that different concentrations of carbon nanotubes increase the thickness of the epidermis in the wound area. In a study, Luna et al. (2021) examined the effects of bone marrow mesenchymal cells on skin wounds by...
histological and Western blotting methods on diabetic rats. The results showed that bone marrow mesenchymal cells inhibit wound healing and are a treatment option for wounds in people with diabetes. In the study, Huang et al. (2021) examined the effects of collagen hydrogel cultured with umbilical cord stem cells (SCF) on diabetic wounds by histological methods on diabetic rats and found that collagen hydrogel is a desirable scaffold. And hydrogel-loaded stem cell factor as a dressing is a promising treatment for diabetic tissue regeneration. In a study, Williams et al. (2020) examined the effects of collagen hydrogels on wounds with histological and immunohistochemical methods in non-diabetic rats. The results showed that collagen hydrogel-treated wounds showed significant improvement compared to controls. In contrast, Qiu et al. (2007) studied the effects of fibroblast cells on wound collagen production in diabetic rats, and based on the results presented in this study, transplanted fibroblast cells at the wound site did not significantly increase collagen production.

In terms of the mechanism of action of fibroblasts and collagen hydrogels on the healing of diabetic wounds, it seems that these cells increase the number of fibroblasts and consequently increase the secretion of these cells. Increased secretion leads to an increase in collagen and interstitial matrix, followed by epithelial cells that have a high ability to migrate to the granular tissue and can block the wound opening in less time and cause the wound to heal.

5. Conclusion
The results of the present study showed that the bonding of collagen hydrogels with fibroblasts can increase the thickness, density, and elasticity of the skin in the wound area by increasing collagen synthesis. This dressing also increases angiogenesis, stimulates macrophages, and accelerates epithelial formation. The present study has limitations in measuring growth factors in wound tissue as well as the expression of regenerative genes involved in skin repair in skin cells and the epidermis. The results of this study can be used to clarify the association between collagen hydrogel and fibroblast cells with diabetic ulcers, as well as the management and prevention of diabetic skin ulcers. However, more research is needed to examine the process of wound healing through other types of hydrogels on other stem cells and to determine how the bonding of collagen hydrogels to fibroblasts affects wound healing.

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7. Conflict of interests
The authors state that there are no conflicts of interest regarding the publication of this article.

Ethical approval: All applicable international, national, or institutional guidelines for the care and use of animals were followed.
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