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ORIGINAL ARTICLE

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

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ABSTRACT

Objectives: A diabetic ulcer is a common disease in patients with diabetes. Because of 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 the wound-healing process in diabetic rats.

Materials and 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) a control group. Microscopic and histological (hematoxylin and eosin staining and Mason trichrome staining), measurement of wound surface with image J, skin density and thickness by the ultrasound probe, and skin elasticity with cytometer tool were used to evaluate wound healing at days 14 and 21 after the treatment.

Results: The results showed that treating diabetic wounds with fibroblasts cultured in collagen hydrogel greatly reduces inflammatory responses in the skin tissue and significantly accelerates the healing process. In addition, 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: In addition, 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.

Key words: Fibroblast, collagen hydrogel, burn wound healing, diabetic rat

INTRODUCTION

A diabetic ulcer is a common disease in patients with diabetes. Between 15% and 25% of people with diabetes develop diabetic foot ulcers. The 5-year mortality rate for diabetic wounds that require resuscitation varies between 39% and 80%. In addition, new therapeutic alternatives are being considered in patients with diabetes. There has been much 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 types.[1-3] Collagen hydrogel is used for several skin diseases by increasing blood flow.[4-6] Huang et al.[7] reported that stem cells heal skin wounds by increasing the thickness and elasticity of the skin. Other research has shown that hydrogels cultured with stem cells facilitate rapid healing of diabetic skin ulcers through angiogenesis and collagen deposition.[1,4,8] In contrast, recent research has shown that mesenchymal stem cells (MSCs) interfere with healing diabetic wounds. Despite significant findings regarding the positive effects of collagen hydrogels,[8,9] the results of some studies in this area could be better.[10] 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 and collagen hydrogels. Based on this and considering that

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common treatments are very complex,[1,11] 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.

MATERIALS AND METHODS

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

Preparation of collagen hydrogels

Bovine collagen (40 mg, Biomaterials Company) was dissolved in a Falcon tube containing 25 mL of deionized sterile water. Two milliliters of the phosphate-buffered saline buffer and 8 mL of the 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 ultraviolet (UV) irradiation. The morphology of the hydrogel was examined and photographed using an Olympus BX61 Research Slide Scanner Microscope.[12]

Isolation of fibroblasts

First, the foreskin of a human infant was collected and stored in a HEPES buffer at 4 °C. The skin sample was cut into small pieces using 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 disease enzyme and the HEPES buffer for 24 h. Dermal samples were then incubated with collagenase (Sigma-Aldrich, USA) at 0.1% for 2 h. The contents of the dish were transferred to a 50 mL Falcon tube containing 5 mL of Dulbecco's modified Eagle medium (DMEM)-high glucose (HG) culture medium (GIBCO, USA) and 10% FBS and piritaged. 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 and 14 °C. The supernatant was drained and serum-free DMEM-HG culture medium was added to 5 mL of cell sediment. Finally, the cells were centrifuged at 200 g at 14 °C for 5 min.[13,14]

Fibroblasts characterization and count

Immunocytochemical staining was performed to confirm the identification of the isolated fibroblasts. To stain the nucleus, the slide was immersed in hematoxylin for 1 min and then rinsed with water. Dehydration and clarification were performed by placing the slides in 50, 70, and 96% alcohol. Cells were counted using a Fisher Scientific hemocytometer and an 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 min. Finally, the percentage of living and dead cells was examined using a Beckman-Coulter Navios flow cytometer.[15,16]

Culture of fibroblasts in collage hydrogel

First, the hydrogel was prepared on the desired plate. After reaching the desired density, the 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 fibroblasts were used for wound dressing.[17,18]

Hydrogel cytotoxicity assessment

To measure cell viability, 1 x 10^5 fibroblasts were cultured in RPMI medium in a 96-well plate. After 24 h of incubation, the cells with hydrogel were treated. After 48 h of exposure of the cells to the hydrogel, 20 µL of 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT) solution was added (0.05 mg/well) to the plates in the dark. After 5 h of incubation of the plates, 100 uL of dimethyl sulfoxide solution was added to each well, and after 10 min of stirring, the resulting color intensity was measured by the Elisa reader at 570 nm. After culturing with glutaraldehyde, drying in room air, sectioning, coating with gold, the cultured cells were subjected to SEM imaging.[16-23]

Animal experiments

Male Wistar rats (42) weighing 200-220 g were purchased from Pasteur Institute (Tehran, Iran). The animals were kept in metal cages with access to water and food. Rats were exposed to 12 h of darkness and 12 h of light at 23 °C for 1 week under special conditions in terms of light and temperature. The rats were divided into 4 groups (6 in each group): (a) control group (regular 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 company) intraperitoneally into rats. Symptoms of diabetes, such as binge drinking and urinary incontinence, appeared in rats three days after the injection. Glucose levels in blood samples taken from the tail were measured using a glucometer 4 days after injection. Rats with blood glucose levels 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 to the wound using Vaseline gauze and transparent adhesive tape. Images of the wound area were captured on the days 7, 14, and 21 after the wound using a digital camera (S9 +, Samsung, South Korea). ImageJ was measured on a mm scale.

Histological examination

Animals were exterminated by the spinal method on the 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 hematoxylin and eosin (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 IX 70).

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 the Scanner 75, tpm taberna pro medium 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 the days 7, 14, and 21 after treatment in the wound area.

Statistical analysis

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

RESULTS

Microstructure and morphology of collagen

The color of the collagen hydrogel was clear and the results showed that UV light was sufficient for 1 h to sterilize the collagen and hydrogel samples (Figure 1A). The microstructure of the collagen samples exhibited a relatively rough surface with a spongy composition and appeared smooth and uniform (Figure 1B).

Isolated cell viability and cell count

The results indicated that a high percentage of fibroblasts were alive on the day of isolation, whereas a small number of them died, and this method was suitable for isolation (Figure 2).

Macroscopic observation and wound area measurement

The results revealed that the wound section in the group receiving fibroblasts and collagen cells compared with the control group and groups receiving each factor alone showed the highest improvement on the days 0, 7, 14, and 21 (Figure 3). Furthermore, 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.

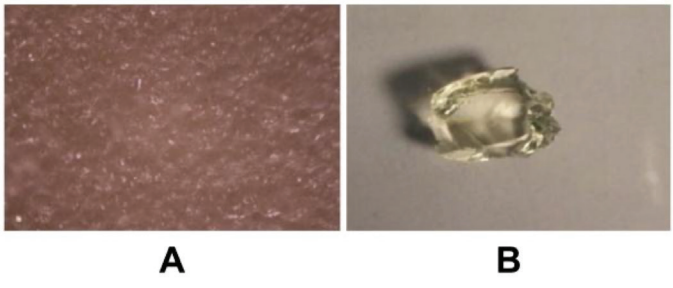

Figure 1. Digital images of (A) hydrogel samples and (B) microstructure of collagen (scale in nm)

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, 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. The epithelial tissue was observed on the 7th day after treatment and increased for 14 days. On the 7th day, angiogenesis in the collagen hydrogel group was significantly less than that in the three experimental groups (Figure 4).

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 with the control group ($p < 0.001$). In addition, the amount of skin thickness in the group receiving fibroblast stem cells + collagen hydrogel significantly differed 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).

The percentage of skin density in the group receiving fibroblast stem cells and fibroblast stem cells + collagen hydrogel significantly increased compared with that in 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 with that in the groups receiving fibroblasts and collagen hydrogel ($p < 0.001$, Figure 6).

Gross skin elasticity (R2) significantly increased in the group receiving fibroblasts + collagen hydrogel compared with the control group and the group receiving collagen hydrogel ($p < 0.01$). In addition, the net skin elasticity (R5) increased

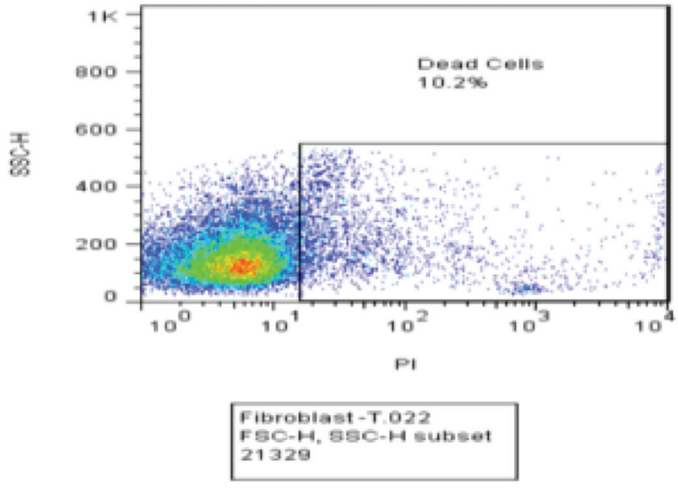

Figure 2. Viability of fibroblast cells isolated from the foreskin on the day of isolation

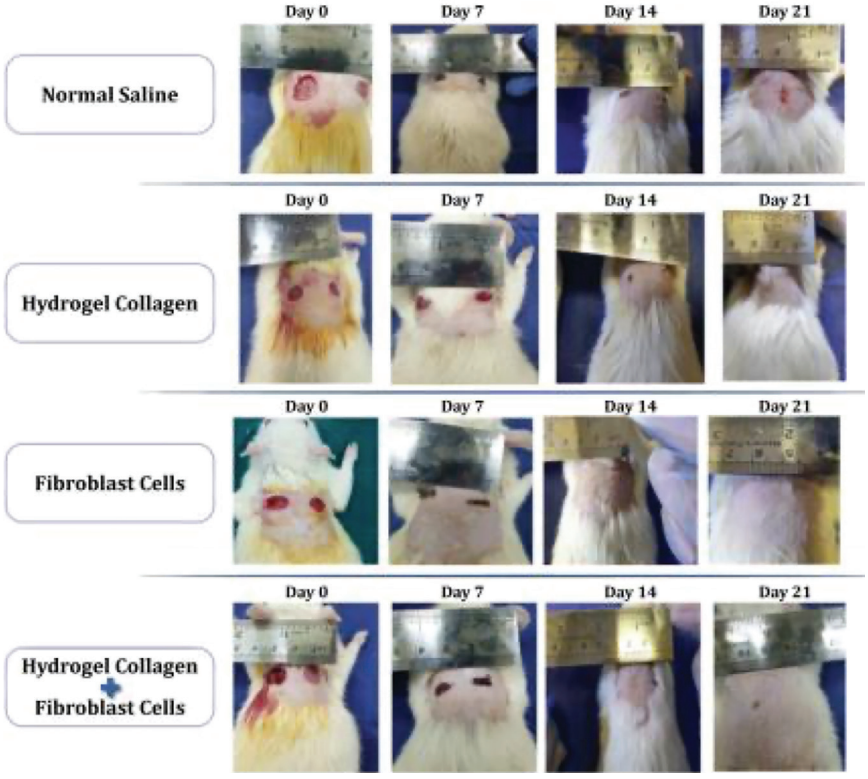

Figure 3. Wound closure on the days 0, 7, 14, and 21 in control and experimental rats

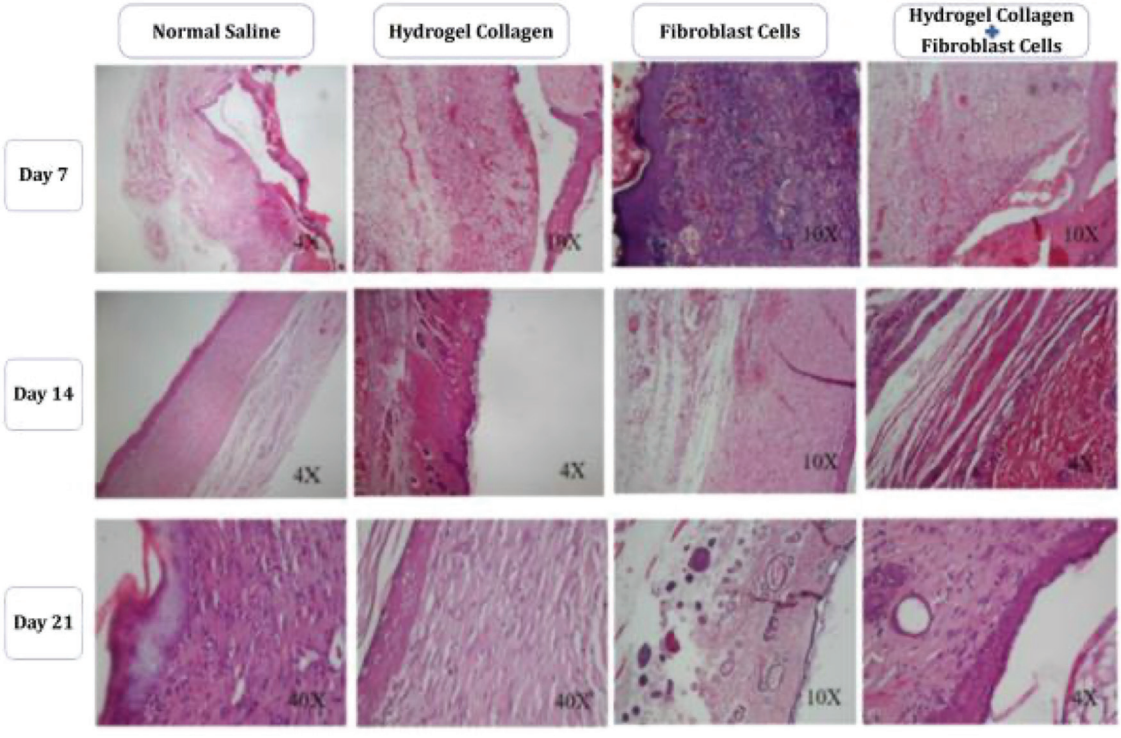

Figure 4. Histological sections of harvested wound area specimens on the days 7, 14, and 21 in the control and experimental groups

significantly in the group receiving fibroblast stem cells and fibroblast stem cells + collagen hydrogel compared with 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 with the control group ($p < 0.001$) (Figures 7-9).

The results obtained from ultrasound imaging showed that the wound thickness increased on the day 21 in all groups compared

with the days 7 and 14, which increased primarily in the group receiving fibroblasts + collagen hydrogel, and then the collagen group had the highest amount (Figure 10).

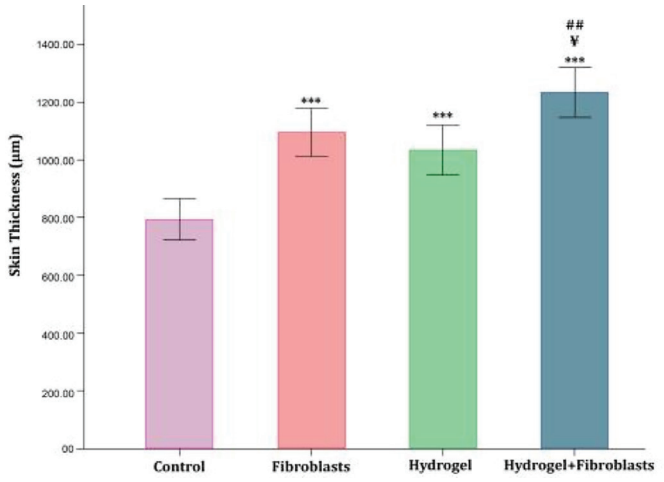

Figure 5. Data obtained from measuring the effects of fibroblasts and collagen hydrogels in the studied groups on skin thickness during the healing of diabetic wounds in an animal model

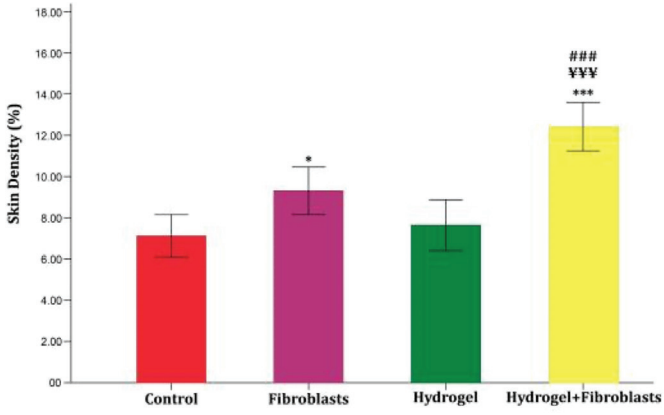

Figure 6. Data obtained from measuring the effects of fibroblasts and collagen hydrogels in the studied groups on the percentage of skin density in the healing of diabetic wounds in an animal model

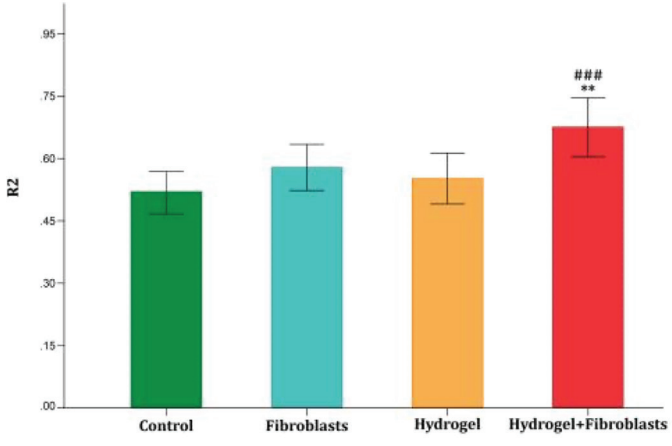

Figure 7. Data from the measurement of the effects of fibroblasts and collagen hydrogels in the studied groups on the amount of gross skin tension (R2) during the healing of diabetic wounds in an animal model

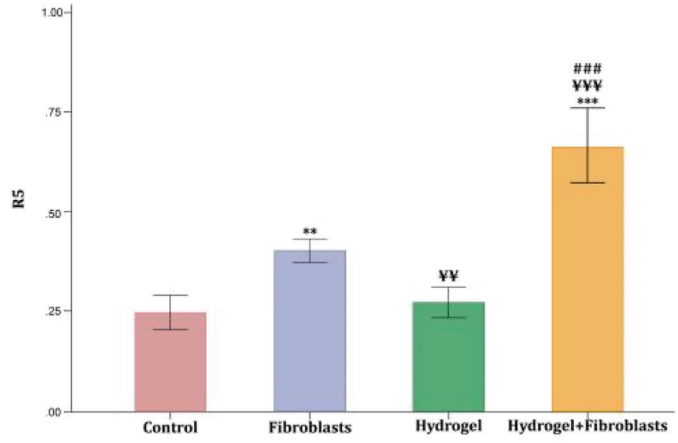

Figure 8. Data obtained from measuring the effects of fibroblasts and collagen hydrogels in wounds in an animal model the studied groups on the amount of skin elasticity (R5: net elasticity) in the healing of diabetic wounds

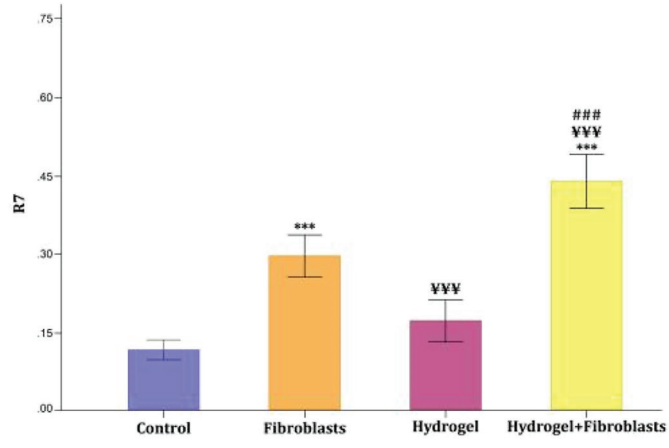

Figure 9. Data obtained from measuring the effects of fibroblasts and collagen hydrogels in the studied groups on the amount of skin elasticity (R7: recovery after deformation) during the healing of diabetic wounds in an animal model

DISCUSSION

Although many studies have shown that collagen hydrogels and fibroblasts are effective in wound healing,[1,4] 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 fibroblasts can heal the wound at the right time and by what mechanisms it does this repair. According to the results, collagen hydrogel did not exhibit significant cytotoxic effects on neonatal foreskin-derived fibroblasts and is a biosafe substance in wound healing. The results revealed that the wound cross-section had the highest healing rate in the group receiving fibroblasts and collagen simultaneously. In addition, over time, the thickness of the wound shell in this group was greater than that in other groups, and the wound

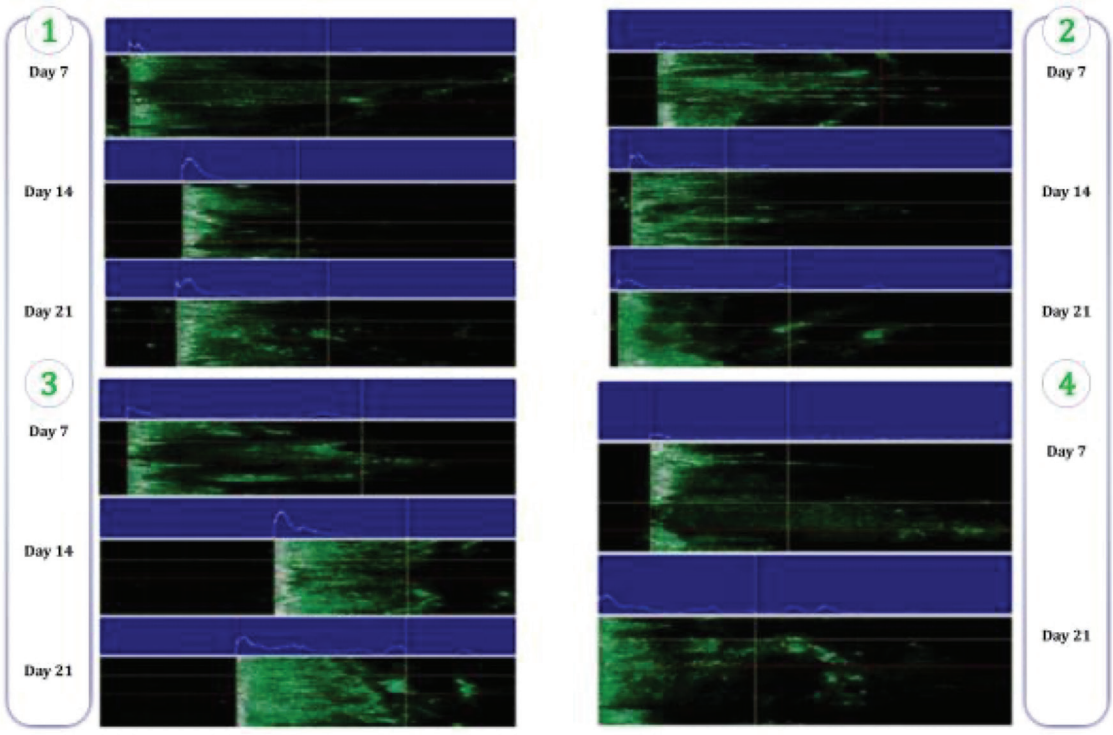

Figure 10. Ultrasound imaging for measurement of the density and thickness of the wound area skin using a 75 MHz ultrasound probe on the days 7, 14, and 21 after the treatment

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 the day 21 in all 4 groups. This amount was significantly lower in the cellular hydrogel group than in the other three groups. Epithelial formation was observed on the 7th 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 that in the other three groups. On day 7, angiogenesis in the cellular hydrogel group was significantly lower than that in the other three groups. According to these results, Bai et al.[24] reported 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.[25] displayed 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.[1] 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.[6] 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. 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.[26] reported that fat-derived stem cells maximize signaling. Paracrine and angiogenesis *via* the PI3K/AKT pathway have synergistic effects on wound healing.[26] Nuschke[27] examined the effects of MSCs using MTT and immunohistochemistry. The results of this study showed that MSCs through tissue collagen deposition can reduce tissue inflammation and induce angiogenesis and wound healing. In another study, Kaisang et al.[28] examined the effects of collagen hydrogels cultured with fat-derived stem cells on wounds using histological methods in diabetic rats. Their findings showed that collagen hydrogels cultured with fat-derived stem cells improve and optimize stem cell function to increase diabetic wound healing.[27] In addition, they investigated the effects of hydrogels cultured with bone marrow MSCs (BMSCs) on the wounds of diabetic rats using histological and immunohistochemical methods. They reported that this treatment involved granulation, angiogenesis, and extracellular matrix secretion and showed that rapid epithelialization helps heal diabetic skin ulcers.[28] In a study, Branco da Cunha et al.[29] examined the effects of collagen and alginate hydrogels cultured on fibroblast cells by flow cytometry and reverse transcription-polymerase chain reaction. Immunohistochemical results confirmed that cultures of encapsulated skin fibroblasts enhance the morphology of various cells and that this biomaterial can regulate wound healing progress.[29] In another study, Yu et al.[9] 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 to heal wounds. Uysal et al.[30] studied the effects of MSCs, bone marrow-derived stem cells, and ASCs on histological and immunohistochemical methods on skin lesions in male Fisher mice, and their results

showed that the fastest wound healing rate was observed by MSCs while increasing the rate of epithelialization through angiogenesis. In a study, Rodriguez et al.[31] examined the effects of 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 displayed that the concomitant recipient group of fibroblasts and collagen cells with amazing ability increased the thickness and percentage of skin density in the wound area. In addition, 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, revealed significant incremental changes. According to these findings, Kittana et al.[32] showed that different concentrations of carbon nanotubes increase the thickness of the epidermis in the wound area. Luna et al.[33] examined the effects of bone marrow mesenchymal cells on skin wounds using histological and Western blotting methods in diabetic rats. The results indicated that bone marrow mesenchymal cells inhibit wound healing and are a treatment option for wounds in patients with diabetes.[33] Huang et al.[7] examined the effects of collagen hydrogel cultured with umbilical cord stem cells on diabetic wounds using histological methods in diabetic rats and found that collagen hydrogel is a desirable scaffold. Hydrogel-loaded stem cell factor as a dressing is a promising treatment for diabetic tissue regeneration.[34] In a study, Williams et al.[34] examined the effects of collagen hydrogels on wounds using histological and immunohistochemical methods in non-diabetic rats. The results showed that collagen hydrogel-treated wounds showed significant improvement compared with controls.[34] In contrast, Qiu et al.[35] studied the effects of fibroblasts on wound collagen production in diabetic rats and found that transplanted fibroblasts 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 their secretion. 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.[36]

CONCLUSION

The results of the present study indicated 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, stimulate 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 fibroblasts and diabetic ulcers, as well as to improve 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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Ethics

Ethics Committee Approval: All applicable international, national, or institutional guidelines for the care and use of animals were followed. 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).

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Authorship Contributions:

Concept: A.Z., M.M., Design: A.Z., Analysis or Interpretation: M.M., S.P., Writing: S.P.

Conflict of Interest: No conflict of interest was declared by the authors.

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