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Original research

Allogenous skin fibroblast transplantation enhances excisional wound healing following alloxan diabetes in sheep, a randomized controlled trial

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HIGHLIGHTS

- The role of fibroblast in wound contraction is already proven.
- It has been shown that use of autologous keratinocytes and fibroblasts in pigs enhance re-epithelialization.
- This study showed allogenous skin fibroblast transplantation can accelerate wound healing in alloxan diabetic sheep.

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ABSTRACT

Background: Healing of skin wound is a multi-factorial and complex process. Treatment of diabetic wounds is still a major clinical challenge. Recently, stem cell transplantation to chronic wounds is favored. The objective of this study was to evaluate effects of pre-labeled allogenous skin fibroblasts on healing of ovine diabetic wound model.

Methods: Eight 4-month-old Iranian Makoui wethers were used in this study. Alloxan monohydrate was used for induction of diabetes. In each wether two excisional wound were created on dorsum of the animal. Wounds of one side were randomly chosen as treatment group ($n = 8$), and wounds of the other side were considered as control group ($n = 8$). Pre-labeled skin fibroblasts with bromodeoxyuridine were used in wounds of one side as treatment. Photographs were taken in distinct times for planimetric evaluation. Wound samples were taken for BrdU detection and histopathologic evaluations on day 21 post-wounding.

Results: The planimetric study showed closure of fibroblast treated wounds is significantly faster than control group ($P < 0.05$). Immunohistochemical staining with anti-bromodeoxyuridine antibody indicated presence of transplanted cells in the wounds. Histopathologic evaluations of H&E stained sections disclosed significantly increasing of re-epithelialization, number of fibroblasts, and number of blood vessels in treatment group in comparison to control group ($P < 0.05$).

Conclution: The results of this study indicated that allogenous skin fibroblast transplantation can positively affect wound healing in diabetic sheep.

1. Introduction

Healing of skin wound is a multi-factorial and complex process [1]. Among these, treatment of chronic wounds, especially in diabetic patients, is a major clinical challenge [2]. The full mechanisms of impaired wound healing in diabetes mellitus have not yet been demonstrated [3]. Nonetheless, increased inflammatory cells, disturbed extracellular matrix synthesis and remodeling, and re-epithelialization problems are evident in these wounds [2]. Additionally, angiopathy and retardation of granulation tissue formation may also be present [4]. It is suggested that cellular and molecular signals that normally encourage wound healing are not

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exist in diabetic wounds, and this is the major factor of delayed healing [5].

Currently, application of epidermal growth factor (EGF) by various methods is the most common therapy for such wounds [6]. It is shown that recombinant human platelet derived growth factor hastens diabetic wound closure [7]. Wang et al. [5] reported that fibroblast growth factor (FGF) along chitosan cross-linked collagen sponge in diabetic rats accelerate healing of such wounds. Use of vascular endothelial growth factor (VEGF) also accelerates diabetic wound healing [8]. Recently, stem cell transplantation to chronic wounds is favored [9]. Several researchers have evaluated effect of adipose-derived mesenchymal stem cells in diabetic models [3,9,10]. Since the most important cells of the skin are fibroblasts and keratinocytes [11], using of these cells also have been considered [12]. The role of fibroblast in wound contraction is already proven [13]. It has been shown that use of autologous keratinocytes and fibroblasts in pigs enhance re-epithelialization [12].

However, it is still unclear that whether transplanted cells to the wound remain in the wound area and participate in proliferative events or simply stimulate host cells migration. The objective of this study was to evaluate if allogenous skin fibroblast survive in ruminant wounds, and how they affect wound healing process in diabetic ruminant model as well.

2. Materials and methods

2.1. Experimental design

Eight 4-month-old Iranian Makoui wethers weighting 25.25 ± 1.25 kg (ranging from 22 to 27 kg) at arrival were used in this study. After diabetes induction, two excisional wounds were created on the dorsum of each animal. Wounds of one side were randomly chosen as treatment group ($n = 8$), and wounds of the other side were considered as control group ($n = 8$). The overall health of the wethers was monitored before and throughout the study. The animals were kept in a barn of Veterinary Teaching Hospital of Urmia University, Iran and were acclimatized to the experimental conditions for 14 days. The animals had free access to hay and tap water throughout the study. Wool of the animals was clipped a week before surgery. Ear tags were used after local infiltrative anesthesia by 1 mL of lidocaine HCl 1% solution (Shahid Ghazi Pharmaceutical Co, Tabriz, Iran). All experimental procedures were approved by the Advisory Committee of the Urmia University Research Council. None of the animals were killed or died during or after the study.

2.2. Diabetic conscious sheep model

For diabetes induction, 10% solution of alloxan monohydrate (Sigma Aldrich Co, Dorset, UK) was infused at a dose of 60 mg/kg into external jugular vein, after 24 h of fasting, similar to procedure performed in dogs [14]. Alloxan was injected immediately after dissolving in normal saline solution because of its very short half-life in saline and blood. Diabetes establishment was confirmed by measuring blood glucose and serum insulin levels a week later.

2.3. Blood glucose and serum insulin assays

Blood samples were taken in the fasted state from external jugular vein in one-week intervals. Blood glucose levels were determined using glucometer device (On-Call Plus; ACON Biotech Co Ltd, Hangzho, Zhejiang, China). Serum insulin levels were measured using ovine insulin ELISA kit (ALPCO Diagnostics, Salem, NH, USA).

2.4. Fibroblast isolation, culture and labeling

Dermal fibroblasts were isolated by previously described method [15]. Briefly, skin biopsy was obtained from ear edge of a 4-month-old sheep under local infiltration anesthesia after shaving and strict aseptic technique. The sample was immersed into phosphate buffered saline (PBS) supplemented with 2% antibiotics and brought to the Biotechnology Research Center of the Urmia University within an hour. Using sterile surgical blade and forceps, the epidermis and hypodermis were removed and the sample was cut into 2–4 mm [2] pieces. The skin pieces were washed two times with PBS in a Petri dish and were adhered onto culture flasks containing Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Grand Island, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone Labs, South Logan, USA) and 1% antibiotics. Cells were cultured at 37 °C in 5% CO_2 and humidified environment. The dishes were left undisrupted for 48 h to avoid tissue dislodging. Thereafter, dishes were observed under inverted microscope daily. The growth medium was changed every third day. The first overgrowth of dermal fibroblasts was observed after 3–4 days. When cultivated cells around the explants reached to 60–80% confluent, they were dissociated from dish using trypsin solution and after centrifuging in 1200 rpm for 10 min, were used for next passages.

For tracking the cells in the skin wounds, the cells were pre-labeled with bromodeoxyuridine (BrdU; Sigma Aldrich Co, Germany) before injection to the wounds. To do this, 10 μL of a 1 mM BrdU working solution was added directly to each mL of tissue culture medium after fourth sub-culture. The treated cells were then incubated over night. Finally, cells were dissociated from dishes as previously mentioned. Following centrifugation, the supernatant was removed and the residuum of 1 mL containing cells in PBS [13,16] was used in the wounds within 30 min. Cell count estimated by means of a hemocytometer.

2.5. Surgical wound model

After alloxan injection, a rest period of seven days for rabbits, and 12 days for rats and mice is usually allowed to stabilize animal's condition [17]. Two weeks after diabetes induction, the sheep's dorsum were shaved and under local anesthesia and aseptic surgical preparation, two squares measuring 2 × 2 cm (one square on dorsum of each side, approximately 15 cm apart) were outlined using a marker. Subsequently, the full-thickness demarcated areas of skin were removed by a scalpel. The wounds were left undressed after hemostasis. One mL of allogenous cell suspension containing about 10^6 fibroblasts in PBS was injected into margins of the treatment wounds. Control wounds received the same volume of PBS.

2.6. Planimetric analysis

Photographs were taken immediately after wounding and after 3, 7, 10, 14 and 21 days by means of a digital camera while a ruler was placed near the wounds. The wound areas were analyzed by Measuring Tool of Adobe Acrobat 9 Pro Extended software (Adobe Systems Inc, San Jose, California, USA) and wound closure percentage was calculated using the following formula: [18]

$$\text{Percentage of wound closure} = (A_o - A_t)/A_o \times 100,$$

where A_o is the original wound area and A_t is the wound area at the time of imaging..

Table 1
Wound closure percentage (mean ± SD) over various times compared with first day of wounding.

	Day 3	Day 7	Day 10	Day 14	Day 21
Treatment	46.75 ± 7.08^{a*}	45.50 ± 11.70^{a*}	60.25 ± 3.30^{a*}	78.75 ± 5.90^{b*}	90.75 ± 4.50^{b*}
Control	36.00 ± 3.74^{a}	37.25 ± 3.94^{a}	44.00 ± 2.82^{a}	61.25 ± 14.93^{b}	77.75 ± 7.76^{c}

^{abc} Means in the each row followed by the same letter are not significantly different ($P \geq 0.05$). Asterisk (*) indicates significant difference ($P < 0.05$) between treatment and control wounds in each time point.

2.7. Histopathologic analysis

Since the proliferative phase of wound healing lasts about 21 days [19], tissue biopsies for histopathologic evaluations were taken under local infiltration anesthesia after 21 days of wound creation. The specimens were fixed in 3.7% formaldehyde solution, and embedded in paraffin. Five μm thickness sections were cut and stained with hematoxylin and eosin (H&E). The slides were assessed under light microscope for epidermal thickness, number of fibroblasts, and number of newly formed blood vessels. The observer was blinded to the experimental design.

The thicknesses of the epidermal areas formed were measured in ten different places along the wound surface by using a graticule eyepiece lens and the average of these areas were taken as the thickness of the epidermis. The number of newly formed blood vessels and fibroblasts were counted by special planimetric lens in 0.25 mm^2 microscopic field. Ten different areas in the sections were counted and mean values were taken into account.

2.8. Detection of allogenous cells in the wound biopsy

To identify the transplanted cells in the wound, 5 μm thick sections of paraffin embedded tissues were prepared as previously mentioned. After routine deparaffinization, immunohistochemical (IHC) staining was done using BrdU detection kit (BrdU IHC Kit; Kamiya Biomedical Co, Seattle, WA, USA). The slides were evaluated by light microscope.

2.9. Statistical analysis

All experimental results were presented as means ± standard deviation (SD). Differences were considered significant at $P < 0.05$. Planimetric data were analyzes with repeated measures analysis of variance (ANOVA) using SAS Proc Mixed software (Version 9.2, SAS Institute Inc, Cary, NC, USA). The models included the fixed effects of treatment, day and their interactions. The models were compared using likelihood ratio test statistic. The correlation structure between the repeated measures over time were examined by including SP (POW) [spatial power law], SP (GAU) [Gaussian], and SP (SPH) [Spherical] to the model and the appropriate covariant structure was selected based on the lowest value of Akaike information criterion (AIC) statistic. After fitting the model, the assumptions of normal distribution, equal variance and unusual observations were checked using the residuals. If a significant fixed effect was detected, differences between least squares means were compared using the Bonferroni test. Insulin and glucose concentrations and histopathologic parameters were analyzed by Student's *t*-test.

3. Results

Serum insulin concentration of the wethers decreased from 88.53 ± 8.17 pmol/L to 14.92 ± 7.89 pmol/L after alloxan injection and confirmed diabetes development. Blood glucose level also increased from 3.57 ± 0.54 mmol/L to 11.06 ± 3.16 mmol/L after diabetes induction. Both of these differences were statistically significant ($P < 0.05$). Average body weight of the animals reached from 25.25 ± 1.25 kg at arrival to 21.75 ± 2.62 kg at the end of the study. Additionally, polyuria was apparent several days after alloxan administration.

As primary outcome (endpoint), wound closure percentage of treatment and control groups over time points were measured and could be found in Table 1. Treatment wounds were significantly different from control wounds on days 10 and 14 after wounding. Furthermore, time had significant effect on wound closure of all wounds, as shown in table ($P < 0.05$).

Histopathologic parameters including epidermal thickness, fibroblast cells count and number of new blood vessels are expressed in Figs. 1–3, respectively. All of these parameters in treatment wounds were significantly higher than control wounds ($P < 0.05$). Not only epidermis was thicker in treatment wounds (Fig. 4A and C), but also fibroblast proliferation and collagen fibers in this group were more than control wounds (Fig. 4B and D). IHC staining of paraffin embedded sections with anti-BrdU antibody

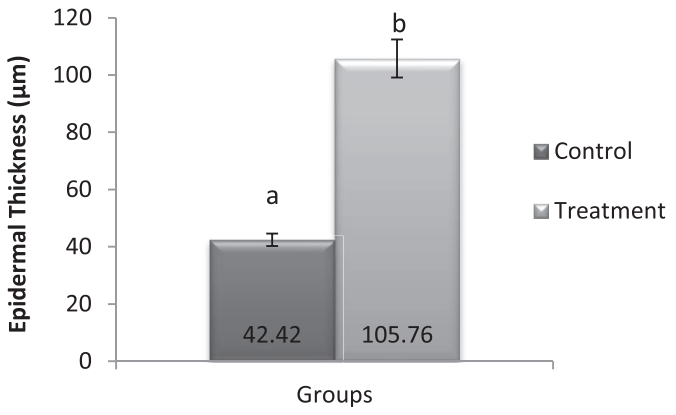

Fig. 1. Epidermal thickness (μm) of treatment and control wounds (mean ± SD). ^{ab} different letters indicates significant difference ($P < 0.05$) between the groups.

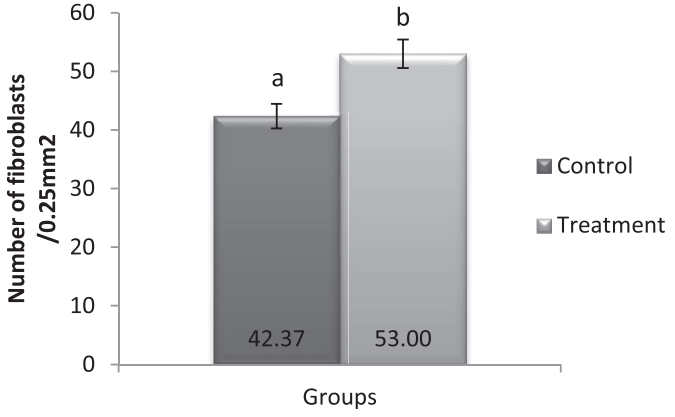

Fig. 2. Number of fibroblasts/0.25 mm^2 (mean ± SD). ^{ab} different letters indicates significant difference ($P < 0.05$) between the groups.

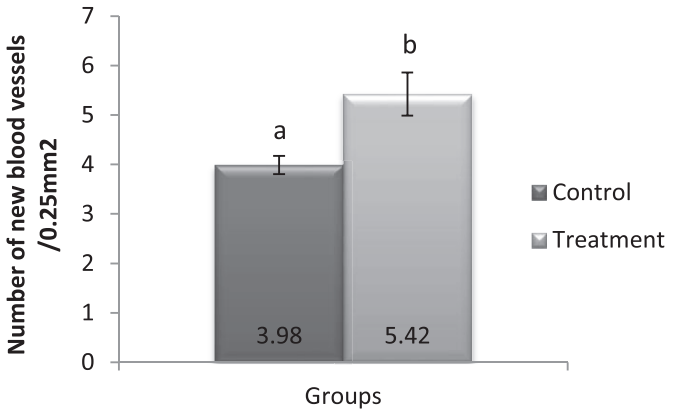

Fig. 3. Number of new blood vessels/0.25 mm^2 (mean ± SD). ^{ab} different letters indicates significant difference ($P < 0.05$) between the groups.

showed some of the transplanted allogenous cells have survived in wound area after 21 days of transplantation (Fig. 5). Histopathologic results are the secondary outcomes of this study.

4. Discussion

Crucial role of fibroblast in wound healing [15], led us to investigate its effect in compromised diabetic wound healings in ovine model. Although diabetes develops in sheep [20], to the best of our knowledge, diabetic wound healing in ovine model has not been documented. The results of this study disclosed direct effect of allogenous transplanted fibroblasts in promoting healing of skin wounds of diabetic sheep.

Although diabetes is primarily more important in humans than animals, much of what is known about diabetic wound healing has been derived from experimental wounds in animals [21]. Sheep is a model for wound repair and technologies that improve this process [22]. Alloxan monohydrate was used to diabetes induction in the present study. Although alloxan is the second most commonly used chemical for diabetes mellitus induction, alloxan diabetes is the best known drug-induced diabetic model. Inducing of both type I and type II diabetes mellitus is possible with proper dosage selection of alloxan. However, for unspecified reasons, streptozotocin is the most commonly used drug [17]. Alloxan applies specific effects on pancreas β-cells leading to complete inhibition of the insulin biosynthesis [23]. It forms superoxide radicals that establish a redox cycle, and undergo dismutation to hydrogen peroxide. Pancreas β-cells rapidly destroy by action of reactive oxygen species with a simultaneous considerable increase in cytosolic calcium concentration [17]. Since alloxan induced diabetes may be less stable and reversible due to the spontaneous regeneration of β-cells [24], we do not allow more than two week period between diabetes induction and wound creating. Accordingly, our results may represent healing properties of acute wounds instead of the chronic ones.

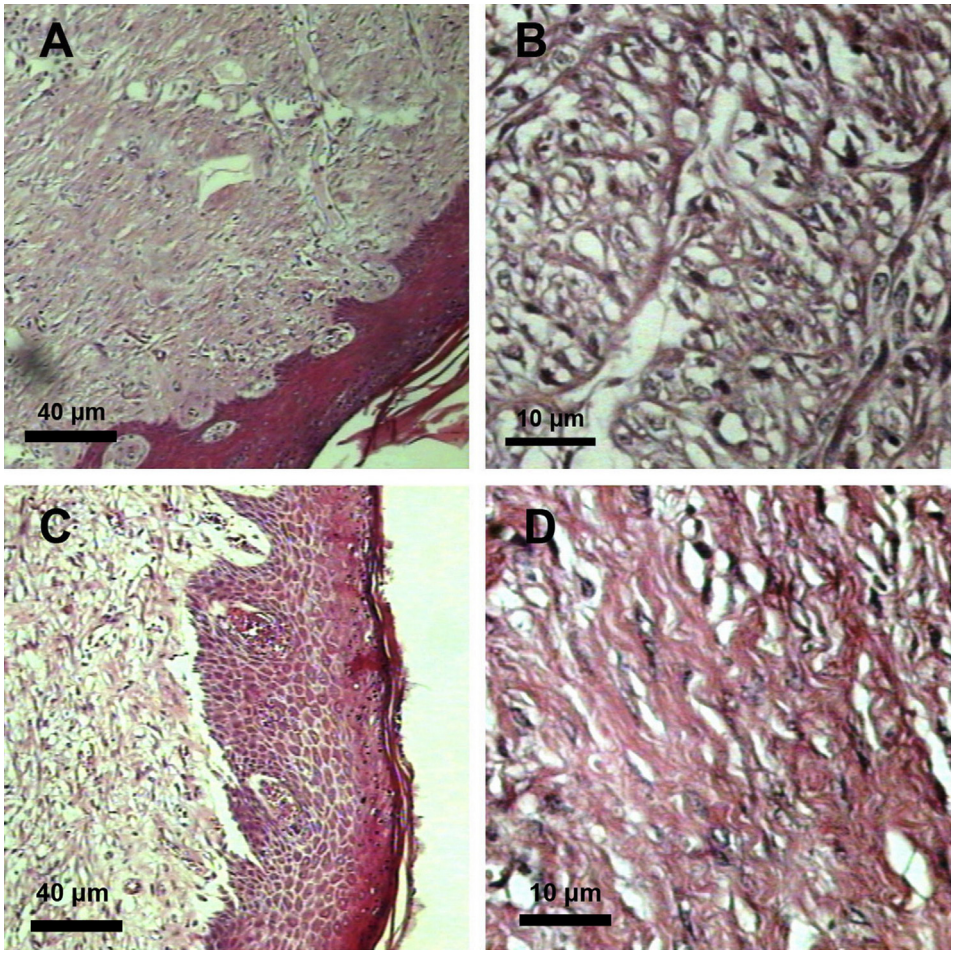

Fig. 4. Experimental wounds. A. Control group. Regenerated epiderm with loose texture underlying tissue. B. Control group. Repaired area showing loose texture. C. Treatment group. Growing epiderm with the underlying granulation tissue. D. Treatment group. Dense fibroblastic proliferation with collagen fibers. (H&E staining).

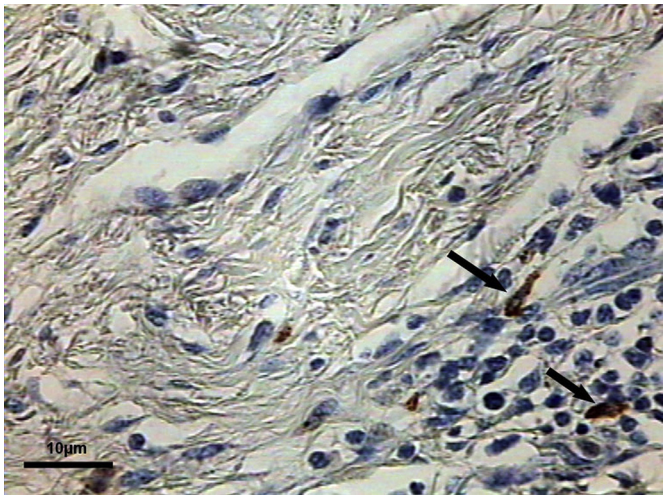

Fig. 5. Immunohishtochemical anti-BrdU staining of allogenous fibroblasts (arrows) discloses presence of them in wound area after 21 days of transplantation.

Insulin plays a central role in the control of glucose metabolism. This hormone enhances glucose consumption in muscle and adipose tissue cells and stimulates phosphorylation of glucose and synthesis of glycogen in the liver [25]. Although vast range of plasma insulin varying from 71.75 to 358.75 pmol/L has been reported in sheep [26], and the exact amount of hypoinsulinemia indicating diabetes has not been documented in this species as well, our data showed apparent decrease of serum insulin level a week after alloxan injection and we were convinced about diabetes development. Distinct concurrent hyperglycemia also confirmed this situation. Body weight of the animals decreased during the study. The rationale for this weight loss may be affection of carbohydrate, protein, and fat metabolism with insulin deficiency. Thus, muscle tissue undergoes catabolic metabolism for energy, and protein synthesis is inhibited, resulting in muscle wasting [27].

Use of allogenous cells has some advantages compared to autogenous cells: One can prepare these cells beforehand, whereas biopsy taking from animal suffering from a wound for cell culture needs much time; with this method, the problems of donor site in injured animal are not encountered; and finally, allogenous cells can be frozen and stored for a long time. Although, immunologic problems and probability of rejection are the most serious concerns of allogenous cells transplantation [28–30], the short-term survival of allogenous fibroblasts following intradermal injection is not a significant problem [31]. Allogenous dermal fibroblasts do not evoke an immune response, making it possible to utilize them in skin substitutes and possibly use them for therapy. The absence of immune response to allogenous dermal fibroblasts has been attributed to the absence of human leukocyte antigen (HLA)-DR expression by these cells [32]. Use of the cultured fibroblasts gained from the 5th to the 10th sub-cultures is the common approach for grafting and wound healing acceleration. By this method, surface antigenicity of the fibroblast is gradually eliminated as the number of sub-cultures increases. However, continuing the sub-culturing process from the 10th passage can result in chromosomal abnormalities [33]. In the present study, the 5th sub-culture was used to preventing from these abnormalities.

It is reported that cultured fibroblasts survive in living tissues [13]. Our data also disclosed improvement of diabetic wound healing by cultured allogenous fibroblasts. Tracing of transplanted pre-labeled cells by anti-BrdU staining showed that they are still present in the wound even after 21 days of transplantation and have not rejected. Thus, based on the results of our study, transplantation of the 5th sub-culture allogenous fibroblasts in diabetic sheep may be an appropriate method in wound therapy. Since host cell proliferation diminishes in later stages of diabetic wounds healing [21,34], and host fibroblasts of diabetic patients show some differences compared with normal fibroblasts, such as increased matrix metalloproteinase (MMP) synthesis that could degrade newly formed extracellular matrix [35], it is expected allogenous fibroblasts eliminate these adverse conditions. Despite transplanted fibroblasts were present in the wound, their population did not increase as time goes on. Other researchers have also mentioned that transplanted fibroblasts persist in a time dependant manner [36,37], and their number begin to decrease after two weeks [38]. Wound closure percentage of treatment group reached to 90.75 ± 4.5, while control group showed only 77.75 ± 7.7 percent of closure at the end of the study; and this difference was significant ($P < 0.05$). Histopathologic evaluations also revealed significantly increase of epidermal thickness, fibroblast population, and newly formed blood vessels ($P < 0.05$). Several researchers have investigated role of fibroblasts on wound healing. Despite Svensjo et al. [39] found little effect of these cells on full-thickness wound healing of normal pigs, fibroblast transplantation into diabetic pigs by Velander et al. [12] accelerated re-epithelialization. However, they did not find any effect on wound contraction. Our data could be justified by the fact that fibroblasts are active in proliferative phase of healing [40], and are responsible to angiogenesis [41], extracellular matrix and collagen synthesis, wound contraction, and excretion of growth factors and cytokines stimulating the wound healing [13].

Increasing in type-VII collagen by single intradermal injection of allogeneic fibroblasts in individuals with the recessive dystrophic epidermolysis bullosa is already proven [31,37]. Considering fibroblasts stimulate cell migration of the host by chemotaxis [19], we can presume transplanted cells have accelerate this process in the present study. Based on the results, it can be concluded that allogenous skin can accelerate wound healing in alloxan diabetic sheep.

Ethical approval

The animal care was in accordance with the institution guidelines.

Conflicts of interest

None.

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Author contribution

Siamak Kazemi-Darabadi and Farshid Sarrafzadeh-Rezaei: Study design, data collection and writing

Amir-Abbas Farshid and Bahram Dalir-Naghadeh: Data analysis

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