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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..