Original Article

The Efficacy of Interfollicular Epidermal Stem Cells versus Bone Marrow-Derived Mesenchymal Stem Cells in Cutaneous Wound Healing in Diabetic Rats

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Abstract

Objective: Many sources of stem cells, such as bone marrow stem cells, embriyonic stem cells, adipose-derived stem cells, epidermal stem cells were used extensively for diabetic wound healing. This study investigated whether interfollicular epidermal stem cell versus bone marrow-derived mesenchymal stem cell effectiveness in the enhancement of diabetic wound healing. **Methods:** The streptozotocin-induced Spraque-Dowley rats with 5 mm punch biopsy were used. Rats were divided into three groups: group I diabetic controls receiving no stem cells; group II, rats receiving bone marrow-derived mesenchymal stem cells; group III, rats receiving interfollicular epidermal stem cells. Wound healing was assessed clinically that regarding healing time and wound size. **Results:** Clinical results showed that wound size was significantly reduced in mesenchymal and interfollicular epidermal stem cell-treated groups as compared with controls. Complete wound-healing times were 19.4±2.85 days in bone marrow-derived mesenchymal stem cells group versus 20.3±3.45 days in interfollicular epidermal stem cells group and 24.7±4.17 in the control group. In the measurement of the wound area, there were no significant differences between the bone marrow-derived mesenchymal stem cells group (P=0.115) and interfollicular epidermal stem cells group (P=0.085). **Conclusions:** Interfollicular epidermal stem cells were found as effective as bone marrow-derived mesenchymal cells in the treatment of the diabetic wound.

Keywords: Bone marrow, diabetes, stem cells, wound

Introduction

Diabetes is a common major health problem in the world. Poor wound healing process in foot ulcers extremely serious condition in diabetic patients.[1-3] Wound healing requires a well-orchestrated integration of the complex biological and molecular events. Cell proliferation and migration, extracellular matrix deposition, and remodeling are the main steps in normal wound healing.[4-6] In diabetic models, altered expression of many molecular factors contributes to wound healing process. Reduced chemotactic ability influences inflammatory cells into the diabetic wound tissue.[1,2] New therapeutic modalities for chronic wounds are currently being used in clinical studies.[6,7] Recently, topical or systemic adult stem cell-based therapy has been widely used for diabetic wound repair and tissue regeneration.[8-10] Several preclinical and clinical studies showed that different types of stem cells, such as bone marrow-derived mesenchymal stem cells (BMSCs), endothelial progenitor cells, adipose-derived stromal cells, and epidermal stem cells (ESCs), have been used for wound healing.[10-13] BMSCs which are pluripotent stem cells are capable of differentiation into different types of cells, such as fibroblasts, adipocytes, vascular endothelial cells, and epithelial cells.[11-15] BMSCs promote that ability to migrate to the wound area, regeneration of damaged tissue, and stimulate proliferation and differentiation promote for wound healing.[12,13,15-18] On the other hand, ESCs are primitive, unique,

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multipotent stem cells that located in interfollicular epidermis and around the hair follicles. Interfollicular ESCs (IESCs) are crucial for wound coverage and restoring epidermal maintenance.[19-21] IESCs and their progenitors induce the proliferative phase in wound healing process and help to restore the barrier function of the skin.

In this present study, considering these previous clinical studies, we try to compare the beneficial effects of bone marrow mesenchymal stem cell and IESC populations in the healing of the wounds in diabetic rats.

Materials and Methods

Forty streptozotocin-induced diabetes Sprague-Dawley (SD) rats were selected; these rats were divided into three groups (n = 10). Group I – diabetic controls received 0.5 ml phosphate-buffered saline (PBS) with no stem cells; Group II – rats receiving BMSCs; and Group III – rats receiving IESCs. Group IV was used tissue sample source for the bone marrow mesenchymal stem cells and IESCs.

Animals

Adult male, SD rats, the average weight of 250–300 g, were used for the experimental process. The rats consumed standard rat chow and water *ad libitum*. The protocol for this study was approved by the Gulhane Investigation and Development Center (AR-2005/49).

Diabetic rat models

Diabetes was induced, after 24-h starvation, by intravenously injecting streptozocin (60 mg/kg in 0.9% NaCl, adjusted to a pH 4.0 with 0.2 Mol/l sodium citrate). The rats were anesthetized with methoxyflurane before injection. Diabetes was verified 7 days later by evaluating blood glucose levels with the use of glucose oxidase reagent strips Glikolyzer (GA-100- Kyoto Daiichi Kagaku Co Ltd. Kyoto Japan). Rats with a blood glucose level 300 mg/dl were considered to be diabetic and also after 1 week of having this level of blood glucose, 5 mm punch biopsy obtained for wounds. Glucose levels were measured every 3 days regularly. Age-matched normal SD rats served as controls.

Isolation, culture, and labeling of the stem cells

Bone marrow mesenchymal stem cells

BMSCs were obtained from the long bones (tibia and fibula) of 12 weeks of SD rats by surgical operation. Sterilization for long bones was done with alcohol for 4 h. After the distraction head of the bone, mesenchymal stem cells were collected by aspiration. Aspiration material resuspended in complete culture medium supplemented with 1% penicillin-streptomycin (Sigma, USA, P4333). Cells were incubated at 37°C, in 5% humidified CO_2 for 14 days. Media were changed every 5 days. When cell population reaches to the 80%–90% confluence, cultures were washed with PBS (P5493, Sigma, USA) and cells were trypsinized with 0.25% trypsin (Sigma, USA, T1246) in 1 ml ethylenediaminetetraacetate (EDTA) (Sigma, USA, E6758) for 5 min at 37°C. After centrifugation (at 2,400 RPM for 20 min at room temperature), cell pellets were resuspended and incubated in culture flasks. After then, 100 ml of BMSCs suspension were used for the flow cytometer. According to criteria proposed by the Mesenchymal and Tissue Cell Committee, BMSC must express CD29, and lack expression of CD45. The BMSC positive for CD29 (Sigma, USA, SAB) and negative for CD45 (Sigma, USA, Ox-1) were used in this study. Flow cytometer showed the presence of marker CD29 on more than 80% and absence of CD45 (<1% of cells).

Interfollicular epidermal stem cells

The 5 mm punch skin biopsy was taken from the dorsal part of the rats. A sample of the skin was maintained for 6 h in DMEM, containing penicillin and streptomycin (50 μg/ml each), gentamicin (10 μg/ml), and an antimycotic agent (amphotericin B, 10 μg/ml). Then, the sample of the skin was kept in a plastic dish with 0.5% dispase II (Boehringer; Mannheim, Germany); for 18 h in a refrigerator (12°C). Each enzyme-treated piece was dissected horizontally it into two halves. The dermal surface was removed. Cells were incubated in a solution of 0.05% trypsin and 1% EDTA (both from Sigma) for 15 min at room temperature and the enzyme activity was then blocked with 2 ml of medium containing 10% FCS. After centrifugation, the supernatant was removed, and keratinocytes were gently resuspended in serum-free growth medium and cultured on six-well culture plates (Falcon), at a final seeding, the cell density of approximately 20×10^6 viable cells per well. After 12-h incubation at 37°C, unattached cells were removed by aspiration, and attached keratinocytes were then maintained in culture. Primary keratinocytes that formed colonies were left to grow until they reached about 70% subconfluence in the culture plate. Cultures were kept at 37°C in a 5% CO_2-in-air atmosphere in a humidified incubator.

Separation of the interfollicular epidermal stem cells

ESCs, especially located at basal layer which are called IESCs express different stem cell marker such as α_6 integrin (CD 49f) and β_1 integrin (CD 29). ESCs were tried to found using by fluorescence-activated cell sorting (FACS) analyzing according to their cell surface markers.[22,23] We analyzed only freshly isolated cells. ESCs were examined using two-color fluorescence dot plots for α_6 integrin (CD 49f-Sigma USA SAB) and β_1 integrin (CD 29-Sigma USA SAB). Dual staining was performed using fluorescein isothiocyanate (FITC)-conjugated (CD 49f) monoclonal antibody and FITC-conjugated (CD 29) monoclonal antibody from Serotec (Raleigh, N. C). FITC/Mouse Ig G1: RPE Ab from Serotec was used as a negative control. Labeling reactions were performed in the dark for 45 min at 4°C. The cells were then resuspended in PBS containing 2% FCS at 3×10^6/ml. All samples were analyzed immediately by a FACSCalibur flow cytometer (Becton-Dickinson USA). The experiments were repeated at least twice using the same conditions and settings. Cells positive for CD 49f and CD 29 accepted as IESCs and they were used in this study.

Wound healing model

The animals were anesthetized using chloroform inhalation. After cleaning the hair, skin was disinfected by ethyl alcohol.

Full-thickness skin specimens, including subcutaneous tissue were excised using the four different 5 mm punch (total area: 28 mm^2) biopsy device (Stiefel) from the dorsal side of rats.

Transportation of the stem cells

After isolation, stem cells were transported to the wound. Cytoline-2 microcarrier is macroporous and composed of the polyethylene and silica, was used for this procedure. Cytoline-2 microcarriers yield higher stem cell concentrations which can be sustained for a long time in the transplanted area. The stem cells at the 2–4 passages were used for loading onto the cytoline-2. Microcarriers were sterilized with 98% ethanol and spread over the 2 cm diameter culture flask. 2 mL cell suspension was plated into 2 cm diameter culture flask in two stem cell treatment group. They were incubated at 37°C with 5% CO_2 and shaken every 30 min over a period of 6 h to attach the cells onto the surface of the microcarrier instead of the base of the culture flask. After this step, 100.000 cells in one cytoline were transported into the wound area for each application. Approximately, 6–8 microcarries were seated in for each area (average 10^6 cells for each application). The exposed skin was covered by Tegaderm (3M Tegaderm™ Non-Adherent Contact Layer, USA). The wound area was washed and new stem cells transported to the wound area every 3 days until complete wound healing. In control group, 10 wounded rats were received 0.5 ml PBS (P5498, Sigma USA) injections without any stem cells each application.

The evaluation of wound healing

Wound size

Wound contraction was measured in 7th, 14th, 21th, and 28th postoperative days as a percentage reduction in area. Decreasing in the wound area was measured regularly by tracing the wound margin using on a tracing paper. The tracing is then placed onto graph paper, and the number of squares was counted. The total wound area was noticed each control and determined the differences from the initial measures of the wound area. The percentage of wound closure was calculated as (area of the original wound-area actual wound)/area of the original wound X100.

Time of complete healing

Time of the complete healing was recorded as the day on which wound healed completely. Healing was considered complete when the hairy skin covered the entire wound area and mean duration were noticed for complete healing was calculated for all groups.

Photographic documentation

The wounds were photographed at days 0, 7, 14, 28 with the rats in the prone position by using a digital camera (Canon EOS 100 D).

Statistical methods

Data were statistically analyzed to evaluate the difference between the groups. Data are presented as mean values ± SD. The results were considered statistically using the Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

Phenotypic characterization of stem cells

Flow cytometer analysis of the BMSCs from passage 2 showed that these stem cells expressed CD29 but were negative for CD 45. Flow cytometer showed the presence of marker CD29 expression more than 80% and absence of CD45 expression (<1% of cells). It was considered that the major population of detected cells were BMSCs (Not shown). On the other side, two-color flow cytometric analysis of CD29 and CD49b expression in ESCs was noticed. At passage 3, most of the IESCs showed high levels of CD 29 and CD 49b expressions (93% and 88% respectively) suggesting that the most of the cultured cells were IESCs [Figure 1].

Wound size

Digital photographs of the macroscopic gross differences of the wound area are shown in Figure 2. It was found that wound closure increased significantly in two stem cell groups compared to control toward to 14 days. On day 14, faster recovery was observed in stem cell treated groups compared with the control group. Healing of the wound area on day 21 is faster stem cell groups than the control group ($P = 0.18$ for BMSC and $P = 0.23$ for ESC). On day 28, healing of the diabetic wound in ESC and BMSC groups completely finished. Mean values of wound area (mm^2) in the diabetic wound of rats were shown in Table 1. There were no significant differences between the BMSCs ($P = 0.115$) and IESCs ($P = 0.085$) according to the closure of the wound area at day 21.

The percentage wound closure was shown in Figure 3. Wounds closure rate of healing found 80% in IESCs and 78% in BMSCs groups and 60% in the control group on day 21. Furthermore, there were significant differences between diabetic rats treated with PBS (Group I), and stem cell (BMSCs and IESCs)-treated groups ($P < 0.05$).

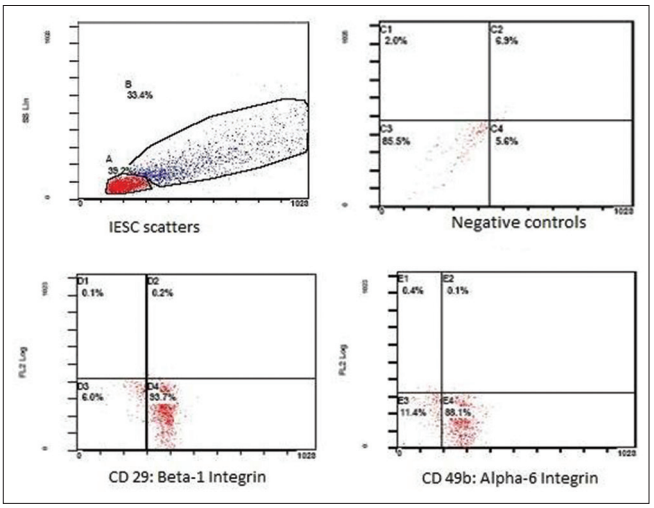

Figure 1: Flow cytometric analysis of cultured Interfollicular epidermal stem cells. Most of the stem cells highly expressed the surface markers, CD 29 and CD 49 (93% and 88% resp.)

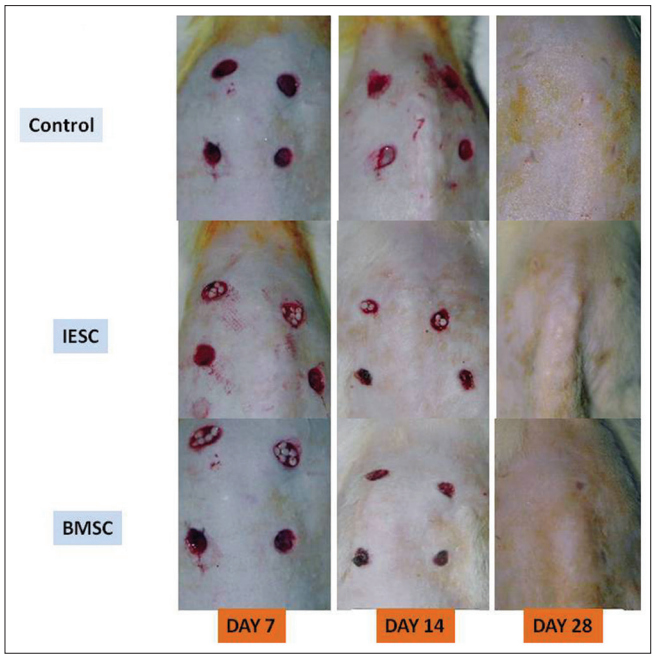

Figure 2: Representative images of diabetic wound healing treated with placebo, bone marrow-derived mesenchymal stem cell and interfollicular epidermal stem cell at different times at day 7, 14 and 28. The wound size was significantly reduced in bone marrow-derived mesenchymal stem cell and interfollicular epidermal stem cell group at day 14 as compared with the phosphate buffered saline control group. On day 28, all diabetic wounds in each group completely healed

Table 1: Mean Values of wound area (mm²)

Groups	Wound area in post wounding days			
	7th day	14th day	21th day	28th day
Diabetic control	27.1±3.5	16.4±4.7	11.5±1.9	2.5±0.9
Bone marrow SC	22.0±3.3	11.8±2.2	5.3±0.9	0.3±0.2
Interfollicular ESC	23.4±3.2	12.1±1.9	5.7±1.1	0.4±0.1

Complete healing time

Differences between the stem cells treated and non-treated group were noticed on the 7th day, 14th day, and 21th day. Complete wound-healing time was 19.4 ± 2.85 days in BMSCs, 20.3 ± 3.45 days in IESCs and 24.7 ± 4.17 days in the control groups. There were significant differences between diabetic rats treated with PBS and treated with stem cells groups ($P < 0.05$). Complete healing was completed on the 28th day in all groups [Figure 3].

Discussion

Foot ulcers in diabetes are a serious complication of diseases due to impaired wound healing process.[1,2] The wound healing process depends on multiple factors, including cell migration, extracellular matrix deposition, chemokine, and growth factor secretion.[5,6] There is an abnormal inflammatory response, impaired vascularization, defective collagen metabolism and dysfunction of the growth factors in diabetes.[4]

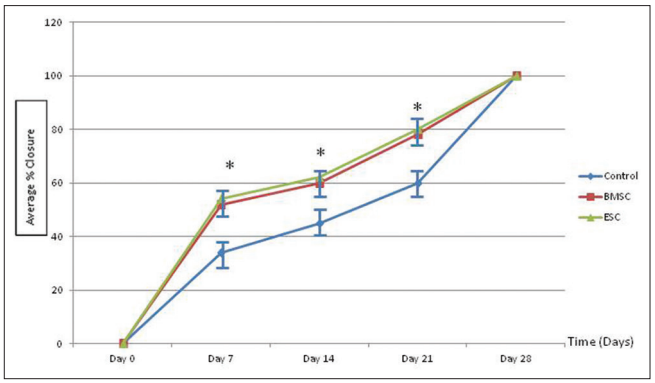

Figure 3: Percentage wound closure rates. Healing of the diabetic ulcer is expressed percentage closure (mean ± standard error). Wound sizes were measured at day 0, 7, 14, 21, 28. Bone marrow-derived mesenchymal stem cell and interfollicular epidermal stem cell-treated wounds showed greater percentage closure at day 7, 14, 21 compared with phosphate buffered saline controls. On day 28, all wounds in each group were completely healed. *Indicates a significant difference ($P < 0.05$)

Tissue engineering technologies have been widely used for the past 3 decades. It can provide many treatment options for wound healings instead of the traditional skin grafting.[8,10] Stem cells are regarded the master cells, capable of self-renewal properties, and promise enormous potential for tissue repair and regeneration.[9,24] The development of novel stem cell-based therapies shows promising results for the treatment of diabetic wound healing. The therapeutic potential of stem cell is due to promote to secrete proregenerative cytokines and other mediators.[8,9]

Many sources of stem cells, such as BMSCs, adipose-derived stem cells, and ESCs, were used extensively for wound healing.[8,10,11] BMSCs are fibroblast-like self-renewing stem cells in the bone marrow. These cells are nearly 10% of the hematopoietic stem cells in a number. BMSCs have been used widely successful in a streptozotocin-induced diabetic rat wound healing model.[20,25-27] The effects of the BMSC on diabetic wound healing were due to improving dermal matrix deposition, granulation tissue formation, and promote angiogenesis.[16,18,28,29] Addition to these effects, BMSCs may trans-differentiate into epidermal keratinocytes and differentiated skin.[27]

On the other side, ESCs are crucial for wound coverage and restoring epidermal maintenance.[26,30-32] ESCs are primitive, unique, multipotent stem cells. Different ESCs coming from the hair follicle, isthmus, infundibulum, and interfollicular epidermis contribute to wound healing.[7,11,24,33,34] Keratinocytes are derived from two different stem cell populations in the skin: IESC, located in the basal layer and hair follicular bulge stem cells (HFSC), located in the outer root sheath.[7,33,34] Unipotent IESC and their progenitors are essential for maintaining for interfollicular epidermis, therefore, basal layer is not only mitotically active layer and but also the interfollicular epidermis have its own stem cell population in the skin. IESCs possess high level of ß1 integrin and α6 integrin. Two

sources of the stem cells provide keratinocytes in the restore the wounded area.[34] In response to injury, cells from hair follicles and basal layer have been shown to migrate from stem cell area to the wound site.[32,34] It has been proposed that IESC-derived stem cells are the major long-term contributors to wound healing compare to HFSC.[21,30] Wound healing in normal skin is dependent on the replicative properties of IESC. IESC and their progenitors begin to proliferate and restore the barrier function of the epithelium.[34] On the contrary, Langton *et al*. suggested that keratinocytes from IESC contribute healing process without hair follicle stem cells.[35] There are some explanations about the effect of the IESC on wound healing. The differentiation of stem cells in a wound process is organized by the epigenetic mechanism. Epigenetic regulations of ESCs will be demonstrated enormous potential for diabetic wound healing.[36,37]

Previously, cultured autologous keratinocytes were used as single-cell sources for wound healing as first treatment modalities.[38,39] Repeated regular applications of the autologous keratinocytes and keratinocytes differentiated from embryonic and adipogenic stem cells were found effective treatment for wound healing in diabetes.[40,41]

The present study was the first experimental animal study using IESCs therapy for diabetic wound healing process compared to BMSCs population. IESCs and BMSCs groups were found equally effective in the treatment of streptozotocin-induced diabetic wound healing. The percentage wound closure increased significantly in two stem cell groups compared to control group at day 7, day 14, and day 21. On the other hand, the healing time of the wound area on day 21 is faster in two stem cells groups than the control group.

There are some concerns that we have to take into account before stem cell therapy for diabetic ulcers. It has also remained unclear which stem cell population, and delivery methods provide most effective methods. First, a selection of the most appropriate stem cell populations is a very important point for the treatment of the diabetic wounds. It can be speculated that IESCs are the most suitable stem cell source for wounds. Second, effective delivery methods are permitted to use to protect the stem cell source and provide functional enhancement. We used Cytoline-2 microcarriers for transplantation of the stem cell delivery. Cytoline-2 microcarriers yield higher stem cell concentrations which can be sustained for a long time in transplanted area. It may be useful to compare microcarriers to other delivery methods in the next experimental studies.

Conclusion

Our study demonstrated that the administration of IESCs was found equally effective as BMSC in the treatment of the diabetic wound healing. In the future, the availability of skin stem cells from the skin samples could provide new opportunities without the risk of immune rejection. The regenerative capacity of ESC from the skin will create new opportunities to develop stem cell-based therapies for wounds. It will be helpful to provide safer and more effective therapies for diabetic ulcers.

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Conflicts of interest

There are no conflicts of interest.

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