Bone marrow mesenchymal stem cells accelerate the hyperglycemic refractory wound healing by inhibiting an excessive inflammatory response

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Abstract. The aim of the present study was to evaluate the healing effect of bone marrow-derived mesenchymal stem cells administered to hyperglycemia model mice with skin wounds, and to explore the underlying mechanism contributing to their effects in promoting refractory wound healing. A full-thickness skin wound mouse model was established, and refers to a wound of the skin and subcutaneous tissue. The mice were randomly divided into three groups: Blank control group, hyperglycemic group and a hyperglycemic group treated with stem cells. Wound healing was monitored and the wound-healing rate was determined at 3, 6, 9, and 12 days following trauma. The structure of the organization of new skin tissue was observed by hematoxylin and eosin staining, and expression levels of the inflammatory cytokines interleukin (IL)-6 and tumor necrosis factor (TNF)-α were determined from 1 to 6 days following trauma. The wound healing of the hyperglycemic group was slower than that of the blank group, and the hyperglycemic mice treated with stem cells presented faster healing than the hyperglycemia group. The horny layer and granular layer of the skin were thinner and incomplete in the new skin tissue of the hyperglycemic group, whereas the new skin wound tissue basal layer was flat and demonstrated better fusion with the wound edge in the other two groups. The expression of inflammatory cytokines (IL-6 and TNF-α) was significantly increased in all three groups, with continuously higher expression in the hyperglycemic group and decreased expression in the other two groups over time. Hyperglycemia refractory wounds are likely related to the excessive expression of inflammatory cytokines surrounding the wound area. Stem cells may be able to alleviate the excessive inflammatory reaction in the wound tissue of hyperglycemic mice, so as to promote wound healing.

Introduction

A chronic wound results in an ulcer of the skin area, and is clinically characterized as requiring a long period of healing, skin tissue infection and defects or necrosis. Consequently, the wound is not able to repair in a timely manner through normal processes, eventually leading to skin tissue dysfunction and anatomical defects (1,2). Diabetic foot is a serious complication of diabetes, which is caused by peripheral nerve dysfunction and leads to a decline in the defensive function of the lower limbs. Furthermore, peripheral nerve dysfunction may occur, and is accompanied by peripheral artery disease, resulting in poor blood circulation to the extremities, finally causing ulcer and gangrene (3). Many factors contribute to the development of diabetic foot, which negatively affects wound healing. When normal skin tissue is injured, a local inflammatory response is required to initiate the wound healing process. A moderate local inflammatory response helps to improve immunity and promote wound healing (4,5). However, patients with diabetic foot usually present an excessive local inflammatory response in the wound tissue (6). The injury related to an excessive local inflammatory reaction contributes to inflammation imbalance, and is a major contributor to the development of chronic skin ulcer (7). Bone marrow-derived mesenchymal stem cells (BMSCs) have the ability for self-renewal and omni-directional transformation into specific cell populations, and are highly effective for repairing skin wounds (8,9). Previous studies have indicated that stem cells possess immunoregulation properties (10,11). Therefore, the authors speculated that stem cells may be able to regulate the excessive inflammatory response in the diabetic refractory wound healing process, in order to promote wound healing. In the current study, a hyperglycemic full-thickness skin wound mouse model was established, and the mice were intravenously injected with BMSCs to assess their effects on the hyperglycemia refractory wound

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healing process. Furthermore, the expression of the inflammatory cytokines interleukin (IL)-6 and tumor necrosis factor (TNF)-α was evaluated to explore the mechanism underlying the MSC-induced promotion of refractory wound healing.

Materials and methods

Isolation, culture and identification of mice BMSCs. Mouse BMSCs were cultured using the tissue-explants adherent method (12). BALB/c mice at 8 weeks of age were anesthetized with 10% chloral hydrate (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), and cuts were made in the femur and tibia. The bone marrow was flushed out from the femur and tibia punctures with Dulbecco's Modified Eagle's medium (DMEM; Promega Corporation, Madison, WI, USA), and centrifuged at 37°C to separate the cells (2,250 x *g*, 5 min). The cells were re-suspended in DMEM containing 10% FBS (Sigma-Aldrich; Merck KGaA), and maintained in a saturated humidified incubator at 37°C, in a 5% CO_2 atmosphere, to observe their growth. When the cells reached 80% confluence, they were digested, centrifuged at 37°C and 2,250 x *g*, and subcultured in DMEM without serum. To identify BMSCs, flow cytometry was used to detect MSC-specific markers: CD29, CD44, Sca-1, CD45 and CD34. When the BMSCs at the third passage grew to 80% confluence, the cells were harvested with trypsin digest solution. Following washing the cells with 4°C PBS three times, the cell density was adjusted to $1.0x10^5$/ml with 4°C PBS containing 0.2% BSA (Promega Corporation) and fixed in 4% formalin (Sigma-Aldrich; Merck KGaA). Then the cells were centrifuged at 350 x *g* for 5 min. Prior to antibody treatment, the cells were blocked with goat serum at room temperature for 10 min (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Following this, the supernatant was discarded, and the remaining cells were stained with primary monoclonal antibodies against mice CD29 (catalog no. 46-0291-82), CD44 (catalog no. 46-3476-87), Sca-1 (catalog no. 47-0544-36), CD45 (catalog no. 46-2217-44; 1:25; Invitrogen; Thermo Fisher Scientific, Inc.) and secondary CD34 (catalog no. 46-5562-33; 1:50; Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 2 h, and then conjugated with fluorescein isothiocyanate, respectively. The cells were visualized with propodium (PI) staining. Finally, the expressions of these molecules on cells were detected by flow cytometry (FACScan; BD Biosciences, Franklin Lakes, NJ, USA).

Preparation of hyperglycemia mice. A total of 24 BALB/c mice, with a male/female ratio of 1:2, weighing 20 to 25 g, (provided by the Xinxiang Medical University Laboratory Animal Center, Xinxiang, China) were accustomed to a 24 h light/dark cycle and had free access to food and water prior to fasting for 24 h, and then received a peritoneal injection of 70 mg/kg body weight streptozotocin (Sigma-Aldrich; Merck KGaA). The weight and blood glucose level changes were monitored every day, and mice with a blood glucose level of >16.7 mmol/l were selected as the hyperglycemia mice group. The mice were sacrificed following anesthesia with diethyl ether. The study was approved by the ethics committee of Xinxiang Medical University (Xinxiang, China).

Establishment of the full-thickness skin wound mouse model. The dorsi of the mice were shaved, and the mice were anesthetized via intramuscular injection of 10% chloral hydrate, followed by disinfection with 70% alcohol. Circular full-thickness sections of the skin, 1 cm in diameter, were excised from both sides of the back, and the wounds were stanched with sterile gauze.

Animal experiments and grouping. The mice of the full-thickness skin wound model were randomly divided into three groups, with eight mice per group: A blank control group (no treatment); hyperglycemic mice with a full-thickness skin wound and no further treatment; and hyperglycemic mice with a full-thickness skin wound treated with BMSCs at a dose of $6x10^7$ cells/kg body weight.

Wound-healing rate. Photographs of the wounds of the three groups of mice were captured with a digital camera at 3, 6, 9 and 12 days following treatment, and analyzed using Image-Pro Plus software (version, 6.0; Media Cybernetics, Inc., Rockville, MD, USA). The edge of the wound was marked and the wound area was measured. Then, the rate of wound healing was calculated three times, as follows: wound healing rate = [(initial wound area - wound area) / initial wound area] x 100%.

Conventional hematoxylin and eosin staining. A total of 15 days following treatment, new skin tissue samples were collected from the wounds of mice, fixed in 4% formaldehyde, paraffin-embedded, sliced and stained with hematoxylin and eosin for observation of the skin organization structure under inverted microscope.

Enzyme-linked immunosorbent assay (ELISA) for IL-6 and TNF-α expression. Skin tissue samples around the wound were collected from each group before and at 1-6 days once daily following treatment, weighed and crushed with liquid nitrogen. Radioimmunoprecipitation assay buffer (Sigma-Aldrich; Merck KGaA) was added to the crushed samples to obtain the lysate, which was centrifuged (3,913 x *g*, 10 min) to collect the supernatant for determination of IL-6 and TNF-α protein concentrations with ELISA kits according to the manufacturer's protocol (catalog no. KHO0411; Elabscience Biotechnology Co., Ltd., Wuhan, China).

Statistical analysis. All statistical analyses were performed using SPSS software version 19 (IBM SPSS, Armonk, NY, USA). Data are expressed as the mean ± standard error of the mean. The differences between groups were tested using Student's t-test or one-way analysis of variance, followed by Fisher's least significant difference test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Culture and identification of stem cells. Newly generated cells could be seen after 5-7 days. The cells grew, adhering to the wall, and were shuttle-shaped or polygonal; their distribution was mostly non-uniform (Fig. 1). Examination
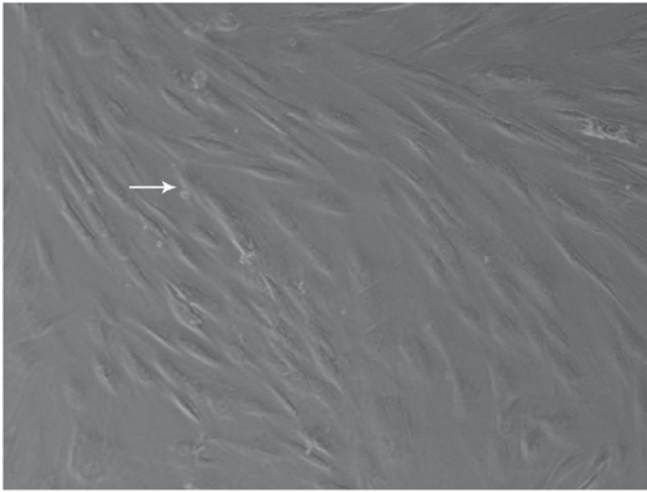
Figure 1. Morphological characteristics of mice bone marrow mesenchymal stem cells (magnification, x100). New cells were observed following 5-7 days. The cells grew, adhering to the wall, and were shuttle-shaped or polygonal in a non-uniform pattern. The arrow denotes the BMSC cells.

by flow cytometry demonstrated that the phenotype of the cells conformed with the typical phenotypic characteristics of BMSCs: CD29(+), CD44(+), Sca-1(+), CD45(-) and CD34(-) (Fig. 2). Specifically, CD29, CD44 and Sca-1 expression constituted >95% of antigen expression, and expression of CD45 and CD34 expression accounted for <2%.

Healing of the full-thickness skin wound. Fig. 3 presented the wound healing rates of the three groups. Obvious scar tissue appeared around the wounds of the blank control group at 3 days following treatment, whereas the hyperglycemia group and hyperglycemia + stem cells group presented no scar tissue. At 6 days following treatment, obvious scar tissue was still visible in the blank control group, however, there were clear signs of healing in the blank group and hyperglycemia + stem cells group. At 9 days, the wound healing percentages of the blank control group, and the hyperglycemia + stem cells groups, demonstrated significant differences, when compared with the hyperglycemia group. At 12 days, the wounds of the blank control group were almost completely healed. In addition, the hyperglycemic mice treated with stem cells demonstrated good wound healing; yet the hyperglycemia group still had a large area that had not healed.

Histological examination of full-thickness skin wounds. A total of 15 days following treatment, hematoxylin and eosin staining was applied to the new skin tissue of the wounds of the three groups, which were observed with an inverted microscope. In the blank control group, the basal layer of new skin tissue was uniform, the horny and granular layers were thick, demonstrating good fusion with the wound edge (Fig. 4A). In the hyperglycemia group, the new skin wound tissue basal layer was flat, and the prickle cell layer, granular layer, and hierarchical structure of the horny layer were visible, but the horny layer and granular layer were thinner and incomplete, presenting poor fusion with the wound edge (Fig. 4B). In the hyperglycemic mice treated with stem cells, the new skin

tissue basal layer was uniform, the horny layer and granular layer were thinner, and fusion with the wound edge was better (Fig. 4C).

IL-6 and TNF-α expression. Fig. 5A demonstrated the concentration of IL-6 in the skin tissue homogenate of mice. Prior to trauma, the IL-6 concentration in the skin tissue of normal mice and hyperglycemic mice were very low (<5 ng/g), which dramatically increased in all three groups following trauma. Over time, the concentration of IL-6 in the blank control group gradually reduced to a visibly consistent level at 3 days following trauma. In the hyperglycemic group, the concentration of IL-6 decreased slightly from day 1 to day 6 within the scope of 45-28 ng/g. In the hyperglycemic mice treated with stem cells, the concentration of IL-6 decreased which was slightly higher than that of the blank group (P=0.521).

Fig. 5B presents the concentrations of TNF-α in the skin tissue homogenates of the mice. Prior to trauma, the concentrations of TNF-α in the skin tissue of normal mice and hyperglycemic mice were 10-20 ng/g, and demonstrated a dramatic increase in all three groups following trauma. As time increased, the concentration of TNF-α in the blank group gradually reduced to approximating the normal level at 3 days following trauma. In the hyperglycemic group, the concentration of TNF-α remained higher than 30 ng/g throughout the study period. In the hyperglycemic mice treated with stem cells, the concentration of TNF-α significantly decreased, which was slightly higher than that of the blank group.

Discussion

Chronic wounds result in ulceration to the skin area and tend to take a long time to heal, frequently becoming infected, leading to defects or necrosis. Furthermore, the wound is often not repaired through the normal processes, leading to skin tissue dysfunction and anatomic defects (11,12). In addition, the chronic wound area is characterized by long-term high expression levels of inflammatory cytokines, indicating an inflammation imbalance, which is an important contributor to the development of chronic skin ulcers (7). Owing to their characteristic of multipotent differentiation, stem cells have been previously demonstrated to accelerate wound healing in a number of studies (13-15). Therefore, in the present study, the authors evaluated the potential of intravenous BMSCs on refractory wound healing in a hyperglycemia full-thickness skin wound mouse model. In addition, the expression levels of inflammatory cytokines were measured to explore the mechanism by which BMSCs may promote refractory wound healing.

The results indicated that the wound healing of the hyperglycemia group was slower than that of the blank group, and the hyperglycemic mice treated with stem cells presented faster healing than the hyperglycemia group. The horny layer and granular layer were thinner and incomplete in the new skin tissue of the hyperglycemic group, whereas the new skin wound tissue basal layer was flat and demonstrated better fusion with the wound edge in the other two groups. These results suggested that BMSCs could accelerate the wound healing process in hyperglycemic mice. To further explore the mechanism by which stem cells promote the process of refractory
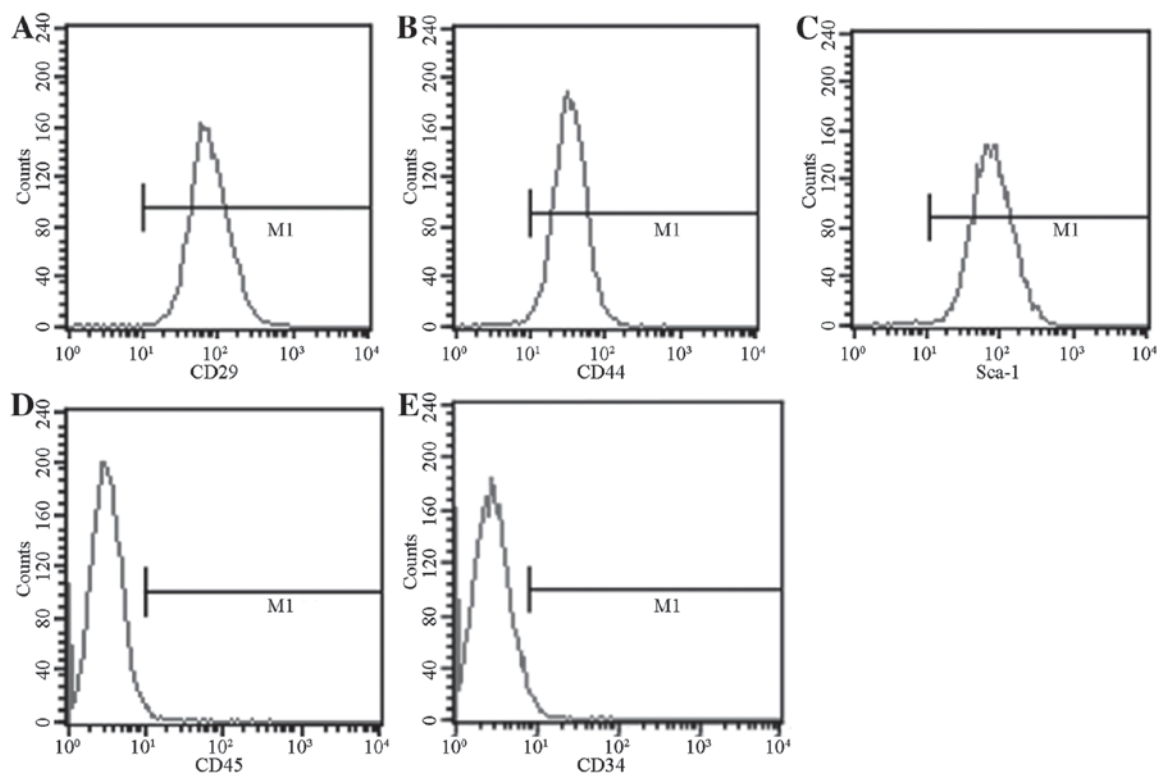
Figure 2. Surface markers of mice BMSCs analyzed using flow cytometry. The surface markers analyzed were: CD29, CD44, Sca-1, CD45 and CD34. The phenotype of the cells conformed with the phenotypic characteristics of BMSCs: (A) CD29(+), (B) CD44(+), (C) Sca-1(+), (D) CD45(-) and (E) CD34(-). M1 denotes the negative control. BMSCs, bone marrow mesenchymal stem cells; CD, cluster of differentiation.
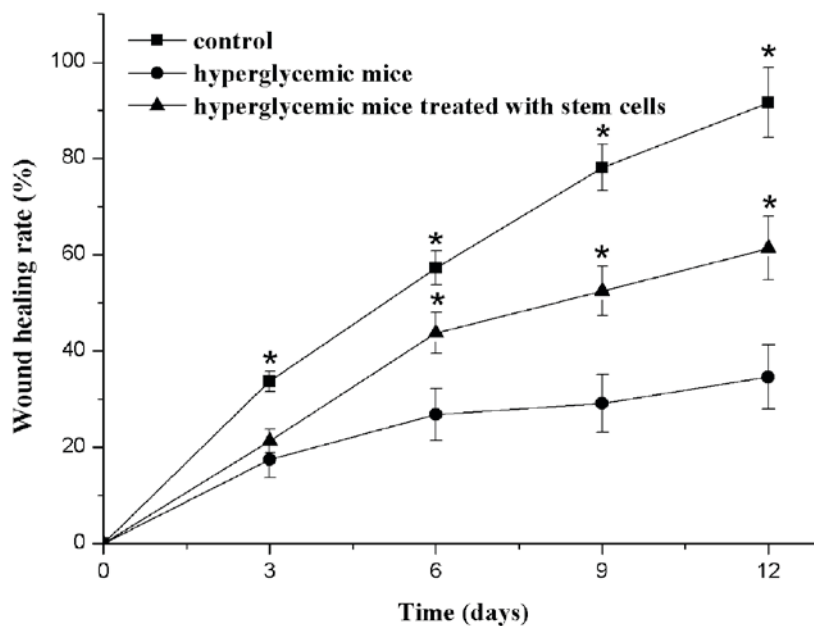

Figure 3. The wound healing rate of three groups over 12 days following treatment to induce hyperglycemia. Wound healing rate was calculated three times by the following equation: Wound healing rate=[(initial wound area-wound area)/initial wound area]x100%. Error bars represent the mean of three repeats ± standard deviation. *P<0.01 vs. control group.

wound healing, the authors detected the expression of inflammatory cytokines (IL-6 and TNF-α) during the wound healing

process. The expression of inflammatory cytokines (IL-6 and TNF-α) was increased in all three groups, with continuously
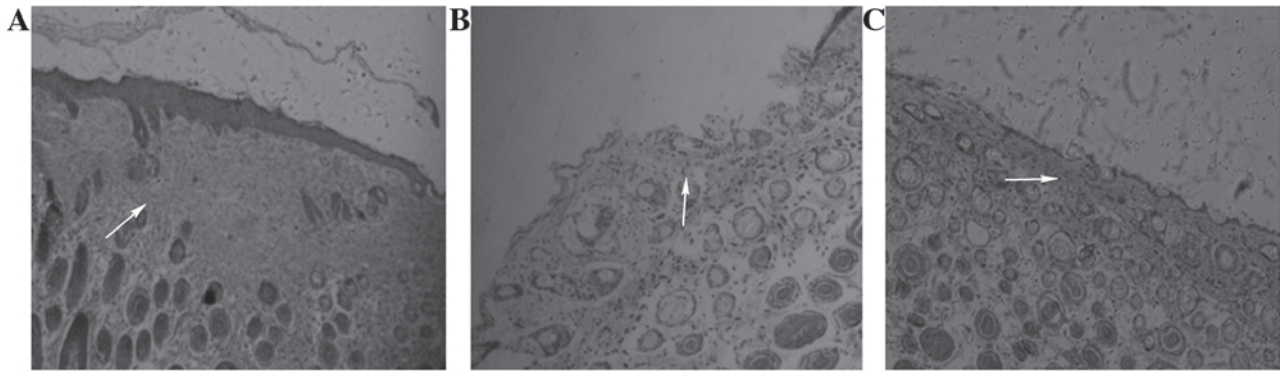
Figure 4. Histological analysis newborn skin tissue in the (A) blank control group, (B) the hyperglycemia group and (C) the hyperglycemia + stem cells group (magnification, x100). The arrow represents the new skin tissues.
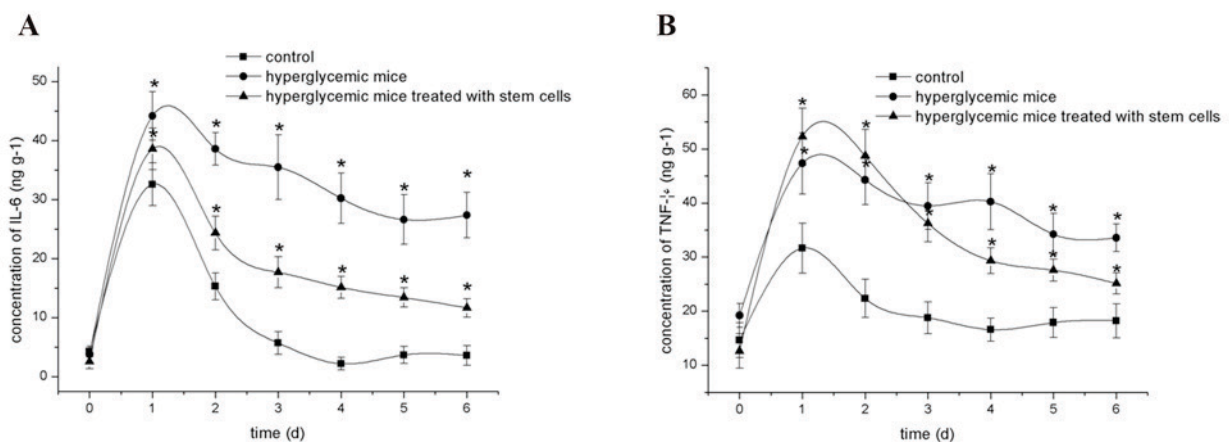

Figure 5. The content of inflammatory cytokines in mouse skin tissue of three groups following trauma. Concentration of (A) IL-6 and (B) TNF-α in the skin tissue homogenates of mice. Error bars represent the mean of three repeats ± standard deviation. *P<0.01 vs. control group. IL-6, interleukin-6; TNF-α, tumor necrosis factor-α.

higher expression in the hyperglycemic group and decreased expression in the other two groups over time. These results suggested that BMSCs could effectively alleviate the excessive inflammatory reaction in hyperglycemia, thus accelerating the wound healing process.

Inflammation is a normal physiological response to stimuli such as trauma, hemorrhage or pathogen infection in biological tissue (14). Following damage to normal skin tissue, a local inflammatory reaction is required to initiate wound healing: Neutrophils enter the wound area and secrete a large number of chemokines (15), inducing the mononuclear cells in the blood into the wound tissue to become macrophages. In addition, neutrophils secrete a large number of enzymes with antimicrobial effects, and then the neutrophils disappear through an apoptotic process (16,17). Macrophages can promote repair through inducing cell proliferation and migration in autocrine and paracrine manners (18) through the secretion of various chemokines and growth factors (19,20) and promotion of various cytokines into the wound area. A local inflammatory reaction that is not only excessive, but also long-lasting in a chronic wound, is generally referred to as a local excessive inflammatory reaction (21) and is one

factor contributing to the difficulty in wound healing (22). Stem cells can promote rehabilitation and reconstruction during injury repair in a variety of tissues and organs, largely due to their immune regulation effects (23,24). Many studies have demonstrated the involvement of BMSCs in the immune inflammatory response, which help to accelerate tissue regeneration. Specifically, BMSCs from the bone marrow were indicated to effectively reduce the inflammatory reaction (25,26). The current experiments confirmed that BMSCs can accelerate the healing process of a skin wound in hyperglycemia mice, and could effectively curb the inflammatory reaction during the process of wound healing. Therefore, the authors speculate that the difficulty of wound healing in hyperglycemia is related to an excessive inflammatory reaction, and that administration of BMSCs may effectively alleviate the excessive inflammatory reaction to accelerate the wound healing process.

Acknowledgements

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