Original Article
Effect and mechanism of SHED on ulcer wound healing in Sprague-Dawley rat models with diabetic ulcer

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Abstract: To evaluate the effect of stem cells from human exfoliated deciduous teeth (SHED) upon the ulcer wound healing and evaluate the mechanism underlying the role of SHED in Sprague-Dawley rat models with diabetic foot ulcer. The rats with diabetic ulcer were established and treated with SHED, mesenchymal stem cell (MSC) and PBS, respectively. The expression of vascular endothelial growth factor (VEGF), endothelial nitric oxide synthase (eNOS), matrix metalloproteinase-2 (MMP-2) and MMP9 at both protein and RNA levels was quantitatively measured. The serum levels of VEGF, IL-1β, TNF-1α and IL-10 were detected by ELISA. The remaining tissues were fixed in 4% chloral hydrate for hematoxylin and eosin (H.E) staining and immunohistochemical staining. MSC and SHED administration could reduce ulceration area and accelerate wound healing at 7 and 14 d after treatment as compared with the control group (all $P<0.05$), which were validated by H.E and immunohistochemical staining. Western blot results revealed that the expression levels of VEGF, eNOS, MMP2 and MMP9 proteins in the MSC and SHED groups were considerably up-regulated compared with those in the control group at different time points (all $P<0.05$). The same trend was also observed in the mRNA expression of these cytokines detected by RT-PCR. At 3-d after treatment, no statistical significance was noted in the IL-10 level among three groups, but the IL-10 concentration in the SHED and MSC groups was significantly down-regulated at 7- and 14-d post-treatment (all $P<0.05$). SHED administration, similar to MSCs, could accelerate wound healing, promote angiogenesis and suppress inflammatory responses in rat models with diabetic ulceration.

Keywords: SHED, diabetic ulcer, mesenchymal stem cell, cytokine

Introduction

Diabetes mellitus (DM) is one of the most prevalent chronic diseases worldwide [1]. Patients diagnosed with DM may present with ulceration and multiple types of skin lesions. Diabetic foot ulceration is one of the major complications resulting from DM, which has been considered as the main cause of diabetic foot syndrome. As an inherent physical mechanism, wound healing is a complex process that involves with simultaneous actuation of soluble mediators, blood cells, extracellular matrix (ECM) and parenchymal cells.

Instead, DM is capable of impeding the procedures of the wound healing process through prolonging the maturation of granulation tissues and reducing wound tensile strength [2]. Moreover, the incidence of DM is likely to lead to a series of pathophysiological changes involved with ECM, endothelial nitric oxide synthase (eNOS) and matrix metalloproteinase (MMP). Specifically, diabetic patients present with accumulation of eNOS inhibitor but decrease in eNOS production due to renal dysfunction induced by high glucose levels. MMP activity has been proven to considerably increase due to high levels of pro-inflammatory cytokines [3]. In particular, MMP-2 and MMP-9 have been found to be over-expressed in chronic diabetic ulceration [4]. However, the underlying mechanism responsible for MMP activity alteration remains elusive.

Multiple investigations have demonstrated that mesenchymal stromal cells (MSCs) play a vital role in accelerating wound healing [1-4]. However, a majority of MSCs are derived from bone marrow aspiration or from umbilical cord,

which is an extremely invasive procedure for the donors and the source of umbilical cord is highly limited. Recently, stem cells from human exfoliated deciduous teeth (SHED) have captivated widespread attention as a novel stem cell source, which may alleviate surgical invasiveness and trauma of bone marrow aspiration and resolve the challenge of rare sources. Previous study [5] has demonstrated that SHED is a population of stem cells which can extensively proliferate and possess multi-potential differentiation, thereby probably serving as a feasible cell source for repairing excisional defects, inducing bone regeneration and angiogenesis and accelerating wound healing. Hara et al. [6] have explored the characteristics of SHED compared with MSCs and proposed that SHED might be a promising approach for wound repair and tissue regeneration. In 2015, Yuka et al. [7] have demonstrated that SHED exerts a therapeutic effect upon ischemia-reperfusion injury-induced acute kidney injury in vitro. Multiple preclinical investigations [8-10] have evaluated the therapeutic effects of SHED in various animal models, such as bone defect, cord injury and skin ulcer, etc.

In the present study, the authors attempted to investigate the therapeutic effect and underlying mechanism of SHED transplantation upon wound healing in rat models with diabetic ulceration compared with administration of MSCs from bone marrow mesenchyme (MSCs-BMM) and phosphate buffered saline (PBS) control, aiming to offer more evidence for this novel cell source for repairing damage and regenerating tissues.

Materials and methods

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Stomatological Hospital of Peking University. The protocol was approved by the Committee on the Ethics of Animal Experiments of Stomatological Hospital of Peking University. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize animals' suffering.

Animal model establishment

Sixty Sprague-Dawley rats, weighing 180 to 200 g, were used to establish diabetic models by fed with high-fatty acid diet consisting of 60% calories, 35% protein and 5% carbohydrate for 3 weeks and then administered with *Streptozotocin* at a dosage of 10 mg/kg once daily for 7 d and a 4-mm excisional wound was created on the dorsal side of the right hind foot. In the SHED group (n=30), 50 μl of PKH26-labeled SHED cells at a density of 1×10^6 were transplanted into the right hind foot and an equivalent quantity of PBS was injected into the contralateral side. In the MSC group (n=30), the right hind foot was administered with 50 μl of MSCs according to the methods described above. In the control group (n=5), 50 μl of PBS solution was administered into bilateral feet. The experimental procedures were conducted in accordance with the Guidelines for Animal Experimentation of our hospital.

hMSCs and SHED culture

MSC-BMMSC (Jing-Meng Stem Cell Technology Co.Ltd, Beijing, China) were inoculated in Dulbecco's modified eagle's medium consisting of a low level of glucose supplemented with 10% fetal bovine serum, 100 μg streptomycin (Sigma, USA), 100 U penicillin G and 0.25 μg amphotericin B (Sigma, USA) at 37°C in an environment containing 95% humidified air and 5% CO_2. According to the methods proposed by Gronthos [11], the deciduous teeth were repeatedly washed by PBS, isolated, crushed and mingled with 3 mg/ml type I collagenase and 4 mg/ml dispase at a ratio of 1:1, water bathed at 37°C for 1 h, digested and subsequently centrifuged at 1000 rpm/min for 5 min. The supernatant was discarded and the pallet of SHED was re-suspended in an appropriate volume of culture solution and 70-μm filtered. The cell suspension was inoculated into 25 cm² flask, supplemented with an appropriate amount of penicillin-streptomycin solution, -MEM solution containing 20% FBS, cultured at 37°C in 5% CO_2 for 3 d. Half of the culture solution was exchanged every 3 or 4 d. When monolayer cell confluence was observed, the cells were subject to passage at a ratio of 1:3.

Cell transplantation

Sixty SD diabetic model rats with hind foot ulcer were randomly divided into the MSC and SHED groups (n=30), anesthetized with intraperitoneal injection of 4% chloral hydrate. Prior to cell transplantation, SHED was labeled with fluro-
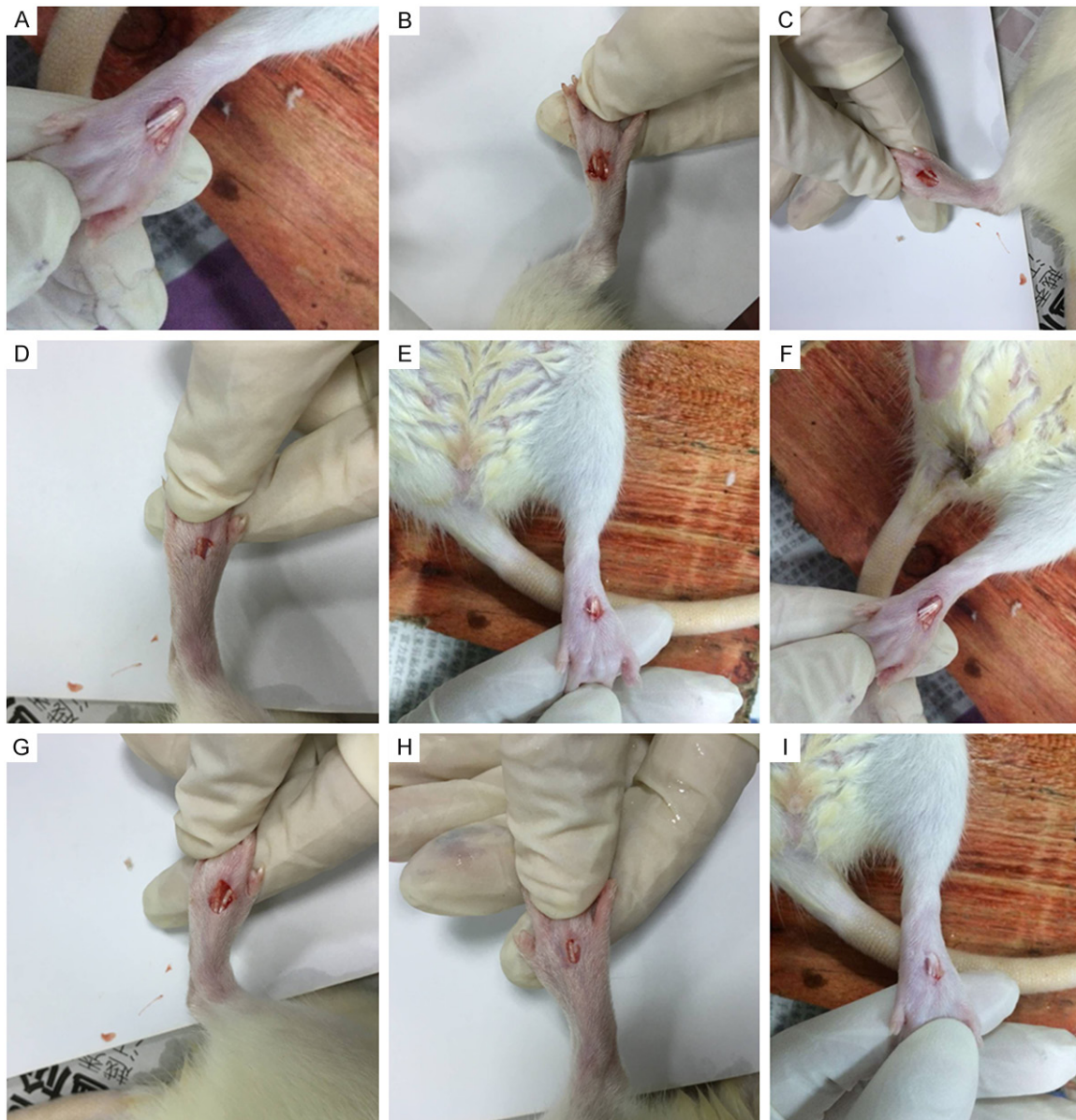
Figure 1. Macroscopic observation of the healing area at 3-, 7- and 14-d after treatment. A-C illustrating the healing process of the rat diabetic ulcer at 3-, 7- and 14-d after treatment in the control group; D-F revealing the gross appearance of the rat diabetic ulcer at 3-, 7- and 14-d after SHED treatment; G-I illustrating the healing process of the diabetic ulcer in the rat limb at 3-, 7- and 14-d after MSC administration.

Table 1. Comparison of wound area among three groups at different time points (unit: mm^2)

Group	3 d	7 d	14 d
Control group	11.91±0.20	9.06±0.31	8.42±0.15
SHED group	11.70±0.16	6.97±0.34*	4.58±0.23*
MSC group	11.55±0.25	5.70±0.35*	3.92±0.19*

Note: *represents compared with the control group at the same time point.

rescent linker PKH26 (Sigma, USA) at a density of 2×10^4 mol/L for 1×10^6 SHED for 3 min and then terminated by adding 2 ml FBS strictly according to the manufacturer's protocol. The obtained SHED was washed twice by using α-MEM solution. The next day, 50 μl PBS containing SHED at a density of 1×10^6 was injected into the ulceration site of the right hind foot and an equivalent volume of PBS was administered into the contralateral side. In the MSC group, the equivalent quantity of MSCs and PBS was injected into the ulcer site and the control side. In the control group, the same volume of PBS was administered into bilateral hint feet. The
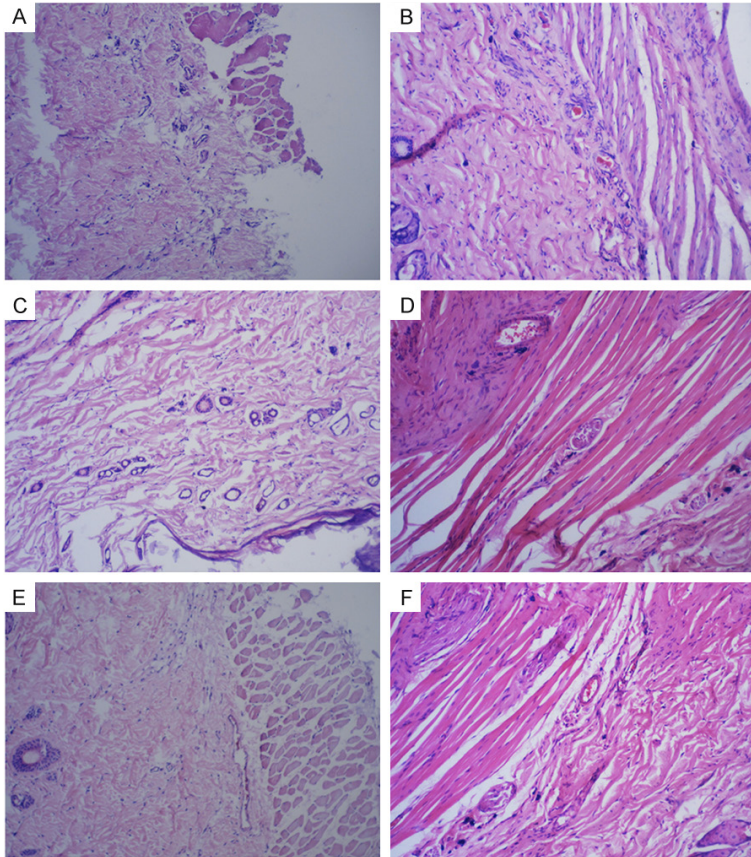
Figure 2. HE staining of diabetic ulcer tissues in the control, SHED and MSC groups. A and B revealing a higher quantity of inflammatory cells and thinner granulation tissues in the control group compared with those in the MSC and SHED groups; C, D (SHED group) and E, F (MSC group) illustrating the process of epithelialization was significantly accelerated in comparison to that in the control group.

animals were sacrificed at 3-, 7- and 14-d after cell transplantation.

H.E and immunohistochemical staining

The tissue was collected, fixe in 4% paraformaldehyde, embedded in paraffin and sliced into 4 μm sections. After 5-min hematoxylin staining, the sections were washed twice in distilled water, treated with hydrogen ethanol for 30 min, dehydrated and transparent handling in gradient ethanol and dimethylbenzene for 15 min and the sections were finally sealed with natural balsam for microscopic observation. For immunostaining, the ulcerative tissue was incubated with type I collagen at a diluted ratio of 1:100, type III collagen diluted 1:50, CD31 antibody at a dilution of 1:50 (BD Bioscience) at 4°C vernight and incubated by vascular endothelial growth factor (VEGF) for 1 h. Following H.E staining, the slices were rinsed for 10 min

and dehydrated in ethanol and dimethylbenzene and sealed for subsequent microscopic observation [12].

Western blot

Following 50.0 μg/mL GA intervention for 24 h, total protein was extracted for determination of concentration of protein by BCA assay. Western blot analysis was employed to measure the expression levels of VEGF, eNOS, MMP2 and MMP9 proteins. Tanon-4100 chemiluminescence analyzer (Tanon-4100, Biotanon Shanghai, China) was used for detection and photograph. The integral optical density value of the target protein was calculated and statistically compared with that of GAPDH (UV-2450, Shimadzu). The relative expression of the target protein was calculated.

RT-PCR

Total RNA extraction was performed using Trizol reagent (Takara, Japan). The total volume of reaction system was 20 μl, supplemented with 5 μl 5×RT buffer, 5 μl dNTP, 0.5 μl RNA template, 5 μl random primer (oligdt), 1 μl reverse transcriptase M-MLV, 0.5 μl RNAase inhibitor RNAsin, 10.5 μl water free from Rnase, denaturalization at 42°C for 60 minutes and annealing at 95°C for 5 min. Collagen I α: forward primer GATTC-ACCTACAGCACGCTTGT, reverse primer GGATG-GAGGGAGTTTACACGAAG; MMP2 forward primer CCTACACCAAGAACTTCCGACTATC, reverse primer CACTGTCCGCCAAATAAACCGA; MMP9 forward primer CGACTCCAGTAGACAATCCTTGC, reverse primer AACTTCCATACCGACCGTCCT; VEGF forward primer GCAGCATAGCAGATGTGAAT, reverse primer GTCTGCGGATCTTGGAC; GAPDH forward primer GTTACCAGGGCTGCCTTCTC, reverse primer GATGGTGATGGGTTTCCCGT.

ELISA

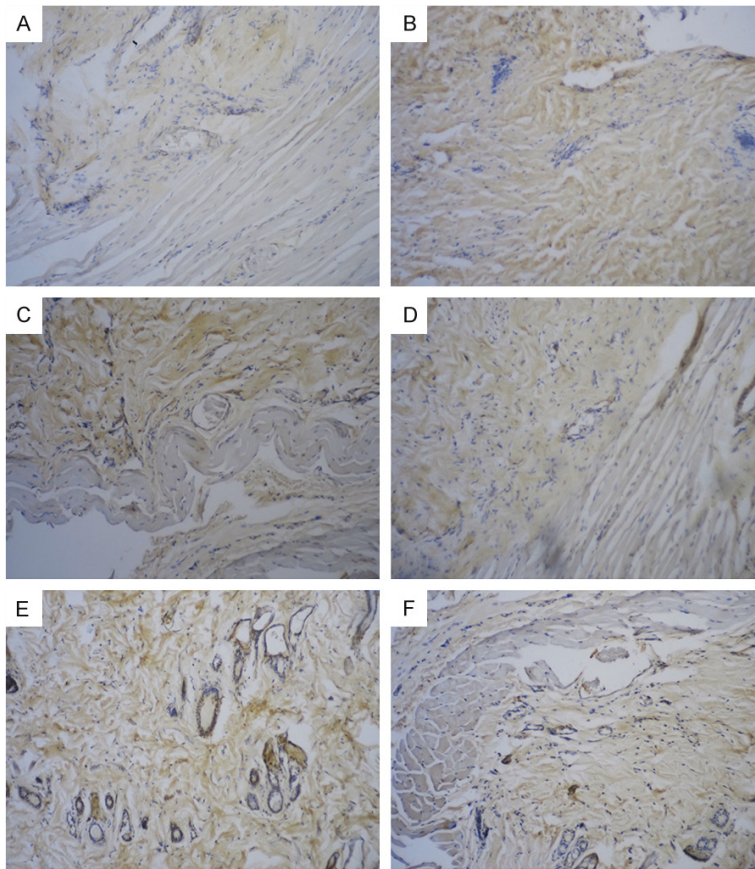The serum levels of VEGF, IL-1β, TNF-1α and IL-10 were detected by ELISA. After correspond-

Figure 3. Immunohistological staining of the diabetic ulcer tissues in the control (A: right hind limb; B: left hind limb), SHED (C: right hind limb; D: left hind limb) and MSC groups (E: right hind limb; F: left hind limb). (C-F) revealing slighter inflammatory cell infiltration and more granulation tissues and newly-developed blood vessels and accelerated epithelialization in the SHED (C and D) and MSC groups (E and F) in comparison to those in the control group (A and B).

ing administration, the VEGF level in the peripheral blood was measured daily. A portion of 1.5 ml peripheral blood sample was collected via venous route, centrifuged at 5000 r/min for 4 min and then 300 µl sampling was prepared for ELISA. Meantime, the changes in the levels of IL-1β, TNF-1α and IL-10 were simultaneously quantitatively measured strictly according to the manufacturer's instructions.

Statistical analysis

SPSS statistical software was used for data analysis (SPSS Inc., IL, USA). All data were expressed as mean ± standard deviation (SD). ANOVA was utilized to conduct multiple group comparison. A *P* value of less than 0.05 was considered as statistical significance.

Results

Gross observation

Forty rats were initially prepared for the establishment of diabetic models and 27 were eligible for subsequent model establishment of diabetic ulceration. The mean serum glucose level achieved up to (20.29±2.06) mmol/L, the average triglyceride concentration was calculated as (1.25±0.15) mmol/L and the mean level of total cholesterol was (2.30±0.15) mmol/L. As shown in **Figure 1**, the percentage of wound healing in the MSC group was the highest among all groups, followed by the SHED group and the healing status was the lowest in the control group at all times points. As illustrated in **Table 1**, no statistical significance was noted among three groups in the wound healing at 3 d after treatment (all *P*> 0.05). At 7 d, the wound area in both SHED and MSC groups was considerably reduced to (6.97±0.34) and (5.70±0.35) mm^2, significantly smaller compared with (9.06±0.31) mm^2 in the control group (both *P*<0.05). At 14 d, the wound area in the SHED and MSC groups was further decreased to (4.58±0.23) and (3.92±0.19) mm^2. The wound area in the MSC group was slightly smaller than that in the SHED group with no statistical significance at 3, 7 and 14 d, respectively (all P>0.05).

Histological observation

HE staining indicated that less inflammatory cells and thicker granulation tissues were observed in the MSC and SHED compared with the control group. In the SHED and MSC groups, the process of epithelialization was significantly accelerated in comparison to that in the control group (**Figure 2**). In the SHED and MSC groups, slighter inflammatory cell infiltration and more granulation tissues and newly-devel-
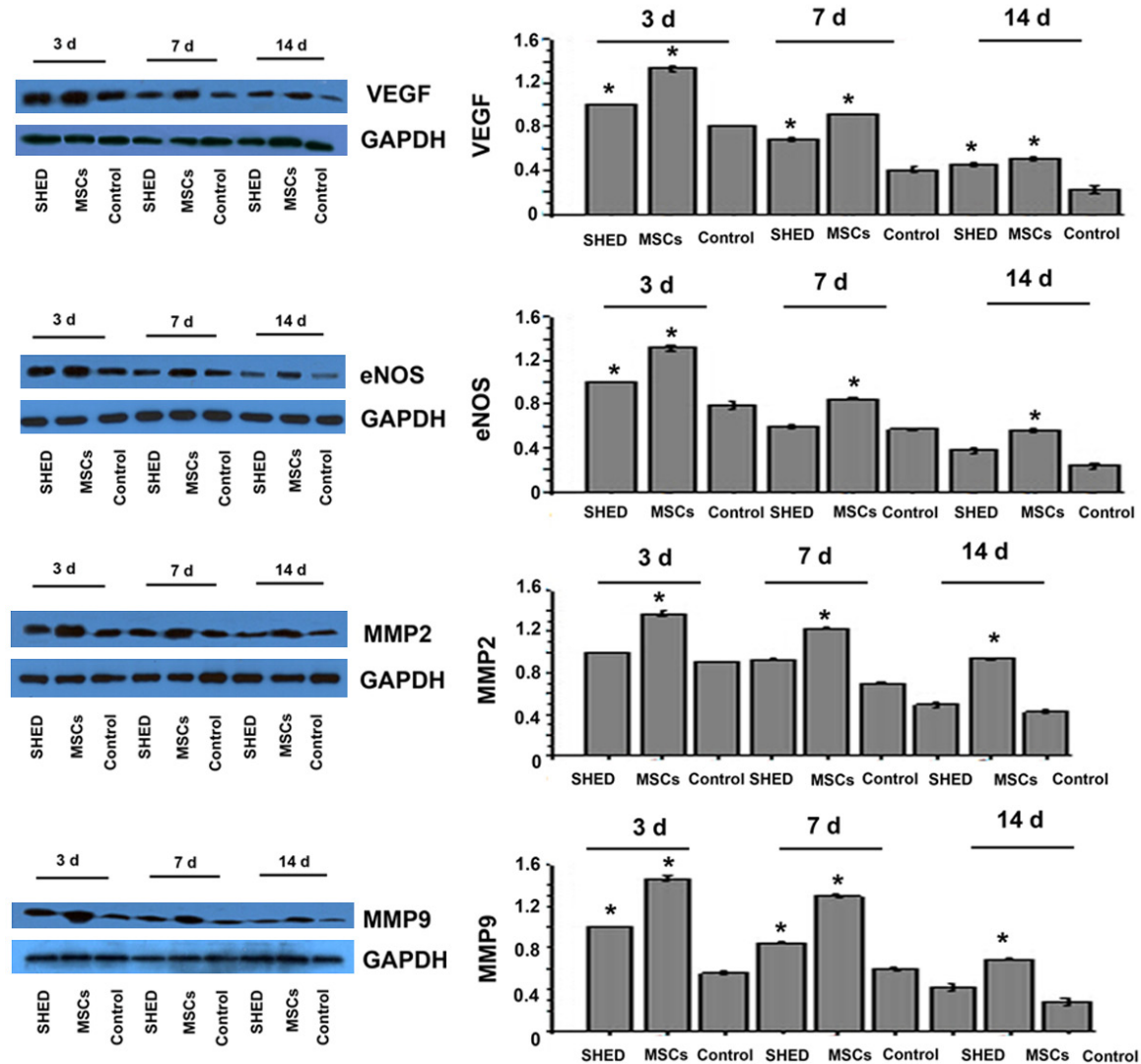
Figure 4. Western blot analysis of VEGF, eNOS, MMP2 and MMP9 among three groups at 3, 7 and 14 d after cell transplantation. The expression of VEGF in the SHED and MSC groups were significantly up-regulated compared with the control group at 3-, 7- and 14-d after cell transplantation. The expression of eNOS in the MSC group was significantly higher than the control group. Similar comparison results were seen in MMP2 expression among three groups. The expression of MMP9 in the MSC group was significantly up-regulated compared with the control group. The expression of MMP9 in the SHED group at 3 and 7 d were considerably higher compared with the control group.

oped blood vessels and epithelialization were observed in comparison to that in the control group, as illustrated in **Figure 3**.

Western blot analysis

The expression levels of VEGF in the SHED and MSC groups were significantly up-regulated compared with those in the control group at 3-, 7- and 14-d after cell transplantation (all $P<0.05$). The expression levels of eNOS in the MSC group were significantly higher than those in the control group at different time points, whereas the expression levels of eNOS in the SHED were higher in comparison to those in the control group with no statistical significance ($P>0.05$). The similar findings were obtained in the levels of MMP2 when compared among three groups. The expression levels of MMP9 in the MSC groups were significantly up-regulated compared with those in the control group (all $P<0.05$). The expression of MMP9 in the SHED group at 3 and 7 d was considerably higher compared with those in the control group (both $P<0.05$), as revealed in **Figure 4**.
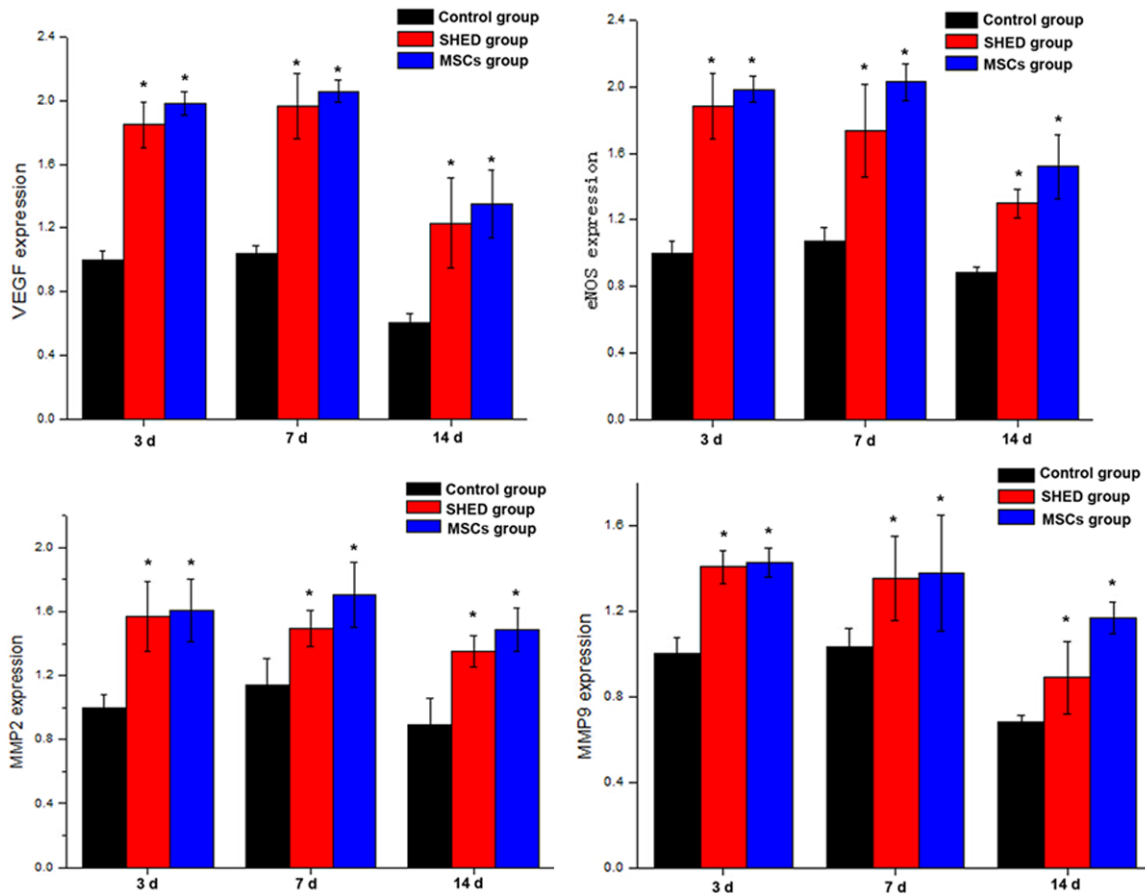
Figure 5. RT-PCR analysis of VEGF, eNOS, MMP2 and MMP9 among three groups at 3, 7 and 14 d after cell transplantation. At various time points, the expression levels of VEGF, eNOS, MMP2 and MMP9 mRNA in the SHED and MSC groups were significantly up-regulated compared with the control group. The expression of VEGF peaked at 7 d after cell transplantation in three groups. In the SHED and MSCs groups, the expression of MMP9 at 3 d following cell transplantation was the highest.

RT-PCR

Real-time PCR was performed to quantitatively measure the expression levels of VEGF, eNOS, MMP2 and MMP9 mRNA at 3, 7 and 14 d after cell transplantation. At different time points, the expression levels of VEGF, eNOS, MMP2 and MMP9 mRNA in the SHED and MSC groups were significantly up-regulated compared with those in the control group (all $P<0.05$). For all groups, the expression of VEGF peaked at 7 d after cell transplantation. In both the SHED and MSCs groups, the expression levels of MMP9 at 3 d following cell transplantation were the highest among different time points, as revealed in **Figure 5**.

ELISA

In the SHED and MSCs groups, the expression levels of VEGF were significantly up-regulated in comparison to those in the control group at 7 and 14 d after cell transplantation (all $P<0.05$), whereas the level of VEGF in the control group was slightly higher than that in other groups. In the SHED and MSCs groups, the levels of IL-10 at 7 and 14 d after cell transplantation were considerably down-regulated compared with that in the control group (all $P<0.05$). The expression levels of TNF-1α and IL-1β in the SHED and MSCs groups were significantly higher in comparison to those in the control group at 3, 7 and 14 d following cell transplantation (all $P<0.05$), as illustrated in **Figure 6**.

Discussion

The incidence of chronic skin wound and ulceration has been considered as one of the major complications for DM patients. At present, there has been no standard therapeutic strat-
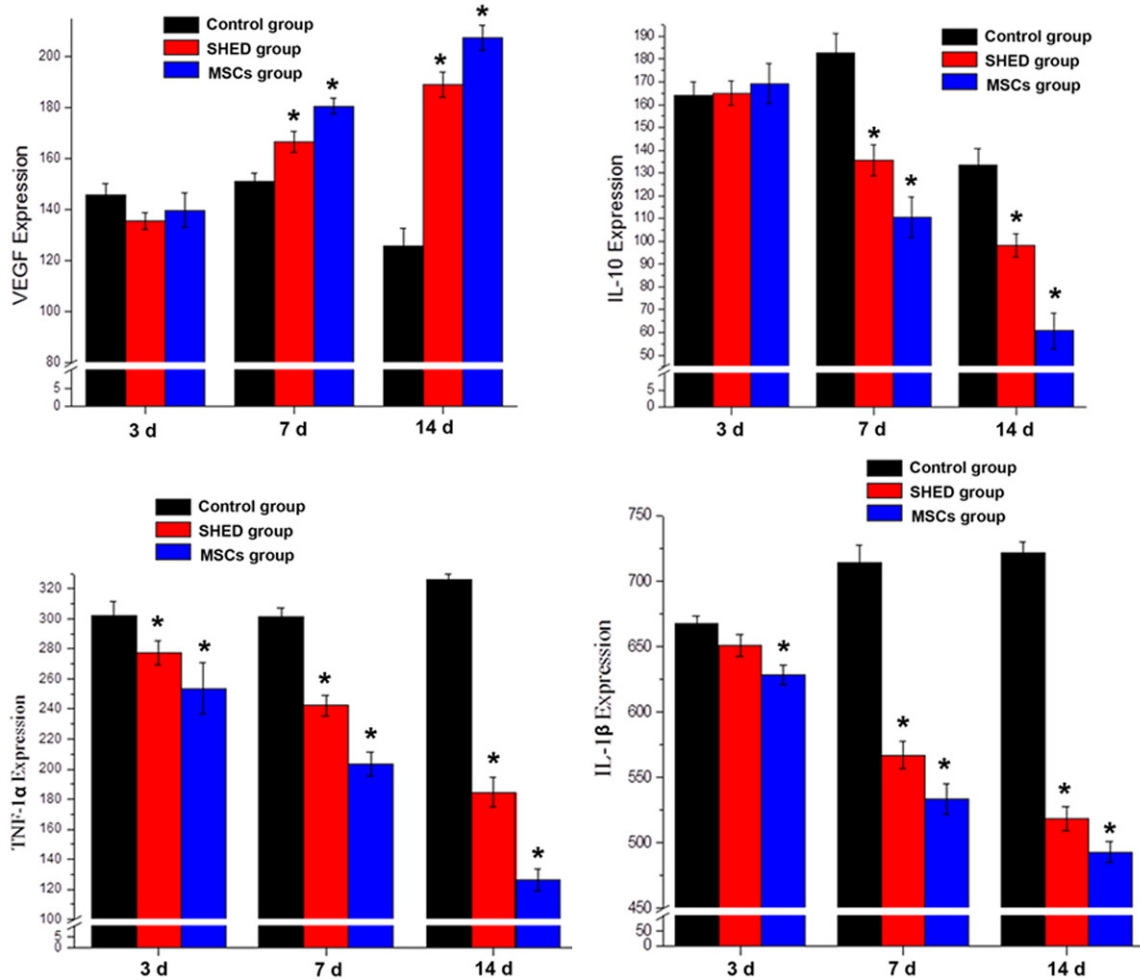
Figure 6. ELISA analysis of VEGF, IL-10, TNF-1α and IL-1β among three groups at 3, 7 and 14 d after cell transplantation. In the SHED and MSC groups, the expression of VEGF was significantly up-regulated in comparison to the control group at 7 and 14 d, whereas the level of VEGF in the control group was slightly higher than in SHED and MSC groups. The level of IL-10 at 7 and 14 d after cell transplantation was considerably down-regulated compared with the control group. The expression levels of TNF-1α and IL-1β in the SHED and MSCs groups were significantly up-regulated in comparison to the control group.

egy for the healing of diabetic wound and ulceration complications because of the highly complicated process involved with wound healing. It has been widely accepted that the procedures of wound healing can be divided into four stages including the immediate response phase, inflammatory response phase, cell proliferation and migration response phase and resolution phase [13]. Therefore, a variety of factors, such as the inflammatory cells, serum cytokines and collagens, collectively participate in the process of wound healing [14]. It has been well documented that the accumulation and infiltration of inflammatory cells constantly occurs during the advanced phase of DM-induced chronic wound healing [15].

Consequently, in current investigation, SD rat models with diabetic ulcer were established successfully, and subsequently treated with transplantation of SHED and MSC as positive controls. Those animals were treated with an equivalent quantity of PBS used as negative controls.

Previous studies have demonstrated that MSCs can be applied to treat myocardial infarction [16], repair the injury of glomerular cells and restore kidney function [17]. Zhao et al. have reported that MSCs derived from umbilical cord matrix can be used to treat and repair foot ulceration in diabetic rat models by promoting epithelialization of ulcerous tissues and

enhancing the production of cytokeratin 19 from ECM formation [18]. In present investigation, MSCs were successfully transplanted into the right hind feet of the diabetic rats. Interestingly, the area of foot ulcerous wound in the MSCs group was the smallest in comparison with both the SHED transplantation and PBS injection groups at 7- and 14-d after cell transplantation, suggesting the MSC transplantation exerts a higher effect upon accelerating the process of wound healing compared with SHED administration. Moreover, the wound area in the animals transplanted with SHED was significantly smaller compared with that in the control rats, indicating that SHED transplantation could also accelerate the process of wound closure, whereas the healing rate was slightly slower in comparison to MSCs transplantation. These findings have been further validated by pathological observations. H.E staining indicated that less inflammatory cells and thicker granulation tissues were noted in the MSC and SHED groups compared with the control group, suggesting the process of epithelialization was significantly accelerated in comparison to that in the PBS controls.

Angiogenesis is deemed as a physiological process during which new blood vessels form and increase from pre-existing vascular tissues, which acts as a normal and pivotal event in cell growth, proliferation, formation of granulation tissues and wound healing [19]. VEGF has been proven to be one of the main factors contributing to the process of angiogenesis. Up-regulating the expression level of VEGF is a necessary physiological response to play its role in accelerating the angiogenesis. In current investigation, both western blot and RT-PCR demonstrated that the expression of VEGF in the SHED and MSCs groups was significantly up-regulated at both protein and mRNA levels compared with the PBS controls, indicating that MSC and SHED transplantation is capable of promoting the process of angiogenesis at an early stage probably at 3 d after corresponding treatment.

It has been noted that inflammatory cells can be observed under the skin affected with chronic wound during the advanced healing stage [20]. The expression levels of TNF-α and IL-10 have been regarded as pivotal factors of persistent inflammation in response to the incidence of chronic diabetic wound [21]. TNF-α belongs to the secreted protein family, which

has been proven to implement several cellular functions, such as cell growth, proliferation, differentiation, migration and apoptosis, etc. [22]. It has been widely recognized that down-regulating the expression levels of TNF-α is capable of alleviating the inflammatory response and accelerating wound closure [23]. IL-1β and IL-10 have been considered to play a necessary role in the occurrence and progression of inflammatory response, which can be significantly up-regulated along with the aggravation of cell inflammation [24]. In present study, the expression levels in the diabetic rats transplanted with MSCs and SHED were considerably down-regulated in comparison to those animals treated with PBS control at 3-, 7- and 14-d after cell transplantation. ELISA analysis has demonstrated that the expression levels of IL-1β and IL-10 are considerably down-regulated after MSCs and SHED transplantation in comparison to PBS administration, indicating that both SHED and MSCs transplantation can mitigate the severity of inflammation and simultaneously enhance the process of chronic wound healing induced by DM in rat models.

Conclusion

Taken together, similar to MSCs transplantation, SHED transplantation can also function to accelerate the healing process of wound ulceration in diabetic rats compared with those treated with PBS administration. However, the rate and efficacy of wound healing by SHED transplantation are slightly lower in comparison to those by MSCs administration with no statistical significance. This approach serves as a novel and promising method for promoting the healing of diabetic ulcer, which might resolve the surgical invasiveness and source rarity of MSCs transplantation.

Disclosure of conflict of interest

None.

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