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Adipose-derived stem cells improve neovascularization in ischemic flaps in diabetic mellitus through HIF-1α/VEGF pathway

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Abstract. – OBJECTIVE: To investigate the improvement effect of adipose-derived stem cells on neovascularization in an ischemic flap in diabetes mellitus (DM), and to explore the mechanism of hypoxia-inducible factor 1α (HIF-1α)/vascular endothelial growth factor (VEGF) pathway.

MATERIALS AND METHODS: A total of 60 male Sprague-Dawley (SD) rats were divided into control group, model group, and adipose-derived stem cells (ADSCs) group. The survival rate of the flap and the number of new blood vessels were measured. The content of VEGF was determined by enzyme-linked immunosorbent assay (ELISA) kit. Then, the expressions of HIF-1α and VEGF in each group were measured by immunohistochemistry. Reverse transcriptase polymerase chain reaction (RT-PCR) method and Western blotting assay were used to detect the mRNA and protein expression of HIF-1α and VEGF in each group.

RESULTS: Compared with control group, the flap survival rate of model group was decreased significantly, and the number of new blood vessels was also decreased significantly. Compared with model group, the flap survival rate of ADSCs group was increased significantly, and the number of new blood vessels was also increased significantly. The results of ELISA showed that compared with control group, the level of VEGF in model group was lower than that in model group, and the level of VEGF in the ADSC group was significantly higher than that in the model group. IHC results showed that both HIF-1α and VEGF proteins were decreased significantly in model group, whereas the expression of HIF-1α and VEGF in the ADSCs group was increased significantly. The results of RT-PCR and the Western blotting showed the mRNA and protein expressions in model group were all decreased, while those in ADSCs group were significantly increased ($p < 0.05$).

CONCLUSIONS: ADSCs can improve the neovascularization of diabetic ischemic skin by regulating the HIF-1α/VEGF pathway.

Key Words:
Adipose derived stem cells, HIF-1α/VEGF pathway, Diabetic ischemic flap, Angiogenesis.

Introduction

Diabetic mellitus (DM) is a kind of very common chronic metabolic dysfunction. DM microangiopathy is a common and severe complication of DM, severely affecting the life quality of the patients[1]. Flap transplantation is one of the most effective methods for treatment of tissue injury in plastic surgery[2]. However, necrosis of transplanted flaps caused by distant ischemia has been occasionally identified[3]. Therefore, it is urgent to search for an effective method to solve the ischemic necrosis during flap transplantation. ADSCs, as a kind of mature stem cells, are derived from the adipose tissues with the potential of multi-directional differentiation. Various studies have been identified that adipose-derived stem cells (ADSCs) have a definite inducing effect on differentiation of vascular endothelial cell[4,5]. However, the effect of ADSCs on the angiogenesis of ischemic flap in DM has been scarcely reported[5]. In this study, we investigated the role of ADSCs in hypoxia inducible factor 1α (HIF-1α)/vascular endothelial growth factor (VEGF) pathway, and explored how ADSCs improved the angiogenesis of ischemic flap in DM.

Materials and Methods

Experiment Animals and Grouping

A total of 60 male Sprague-Dawley (SD) with a weight of (200 ± 20) g were purchased from Bei-

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jing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). After one week of acclimatization with free access to food and water, these rats were randomly divided into three groups: blank control group (n=20), DM model group (n=20) and ADSCs group (n=20). For rats in blank control group, they were fed normally, and those in the DM model group and the ADSCs group were used for establishing the DM models and preparing the flap on the back. In addition, rats in the ADSCs group also received the local intervention with ADSCs. This study was approved by the Animal Ethics Committee of The Second Hospital Affiliated to Soochow University Animal Center.

Major Reagents

VEGF enzyme-linked immunosorbent assay (ELISA) kit (Shanghai Yu Bo Biological Technology Co., Ltd., Shanghai, China); bicinchoninic acid (BCA) protein quantification kit (Beyotime, Shanghai, China); TRIzol kit for extraction of total RNA (Tiangen, Beijing, China); reverse transcription kit for Real-time polymerase chain reaction (RT-PCR; Tiangen, Beijing, China); immunohistochemistry (IHC) kit (Boster, Wuhan, China); anti-glyceraldehyde-phosphate dehydrogenase (GAPDH), HIF-1α and VEGF monoclonal antibodies and secondary antibodies (Abcam, Cambridge, UK).

Experiment Methods

Preparation of ADSCs

ADSCs were prepared from the cells isolated from the adipose tissues collected from SD rats and amplified *in vitro*.

Model Establishment

In model group and ADSCs group, DM rat models were prepared through intraperitoneal injection of streptozotocin in small dose in combination with the high-glucose and -lipid feedstuff followed by establishment of flaps on the back. Additionally, local transplantation was performed for the ischemic flaps in DM rats in the ADSCs group.

Detecting the Level of VEGF Through ELISA Method

In each group, 6 rats were taken for collecting the blood through the abdominal blood. Then, in accordance with the instruction of kit, we assayed the level of VEGF in local tissues and peripheral blood with microplate reader and ELISA kit.

IHC Staining

Paraffin-embedded sections in each group were placed in the xylene (10 min/time, twice), and then treated with the ethanol in gradient concentrations for 5 min. After antigen retrieval, sections were rinsed with phosphate buffered saline (PBS; 3 min/time, three times). Streptavidin-peroxidase staining method was applied followed by rinsing with PBS (3 min/time, 3 times). Then, the PBS was removed, and the normal goat serum working solution was used for incubation of sections at room temperature followed by treatment with antibodies of HIF1α and VEGF in a wet box at 4°C refrigerator overnight. After sections were washed with PBS (5 min/time, 3 times), secondary antibodies were added on the sections for incubation at 37°C for 15 min followed by rinsing with PBS (5 min/time, 3 times) and incubating with working solution. Then, sections were incubated at 37°C for 15 min, and washed with PBS (5 min/time, 3 times). With the freshly prepared diaminobenzidine (DAB) solution, color development of sections was observed and controlled under the microscope followed by re-dyeing with the hematoxylin, 1% hydrochloric acid alcohol differentiation for 20 s, bluing and dehydration in gradient ethanol. Sections were mounted with the neutral balsam, and placed under the microscope (200×; Nikon Eclipse TE2000-U, Nikon, Japan) for observation and analysis.

Real-Time Polymerase Chain Reaction (RT-PCR) Analysis

Tissue samples in all groups were rapidly transferred into the Eppendorf (EP: Hamburg, Germany) tubes supplemented with 1.5 mL RNAiso plus, and placed at room temperature for 5 min for sufficient lysis of samples. After centrifugation at 12000 g for 5 min at 4°C, supernatant was taken, where 0.2 mL chloroform was added and well mixed followed by placing at room temperature for 5 min. Next, tubes were again centrifuged at 12000 g for 15 min at 4°C, and in the supernatant, we added the isopropanol in the same volume. After the solution was well mixed, it was placed at room temperature for 10 min followed by centrifugation at 12000 g for 5 min at 4°C with the supernatant being discarded carefully; this step was repeated for one more time. After the RNA sediment was fully rinsed, the liquid was completely removed, and RNase-free water was added. Then, the solution of total RNA (1 μg/μL) was prepared with RNase-free water, and used to prepare the reaction solution of reverse transcription in accordance with

the instruction of RT reagent kit with gDNA Eraser. In the solution, RNA samples were added for obtaining cDNA that was later preserved at -20°C. Thereafter, in accordance with the instruction of SYBR®Premix Ex Taq™ II (Tli RNaseH Plus), we assayed the level of mRNA. The corresponding RNA primers are listed in Table I.

Western Blotting Analysis

Tissues in all groups were rinsed with the icy normal saline, and prepared for assaying the protein concentrations with the BCA kit followed by preservation at -80°C. In accordance with extraction kit of total protein, immunoprecipitation (IP) cell lysis buffer (containing phenylmethanesulfonyl fluoride and protease inhibitor), the tissues were sufficiently grinded on ice. Thereafter, tissues were centrifuged for 10 min at 4°C and 12000 g for homogenization, and the supernatant was centrifuged again for 20 min at 4°C and 12000 g. The supernatant was used for quantification of protein in accordance with the protein kit instruction. Protein sample containing the same volume of protein was loaded in the sampling holes for electrophoresis under 220 V until the bromophenol blue reached the bottom of gel. According to the molecular weight of target proteins, gel was cut and placed in the transfer buffer. Polyvinylidene difluoride (PVDF) membrane was also cut based on the size of gel. Then, the PVDF membrane was soaked in methanol for 10 s, and transferred into the transfer buffer together with the filter paper. In sequence of positive electrode, filter paper, PVDF membrane, filter paper and negative electrode, these materials were sequentially placed in the membrane-transferring machine for membrane transfer under a constant voltage of 110 V to transfer the protein on gel to the membrane. Thereafter, PVDF membrane, together with the transferred protein, was blocked with 5% skimmed milk on a shaker for 3 h, and incubated with the corresponding primary antibody (1:1000) overnight at 4°C. Next, the membrane was sufficiently rinsed with Tween 20 and Tris-buffered saline (TTBS; 10 min/time, 3 times), and incubated with secondary antibody (1:2000) for 1 h at room temperature followed by rinsing with TTBS (10 min/time, 3 times). Thereafter, enhanced chemiluminescence (ECL) reagent was added for color development and photographing.

Table I. Primer sequences of corresponding genes in RT-PCR analysis.

Gene	Primer sequence
HIF-1α	5'-3' AAGTCTAGGGATGCAGCAC
	3'-5' CAAGATCACCAGCATCTAG
VEGF	5'-3' ATGGCAGAAGGAGGAGGG
	3'-5' CGAAACGCTGAGGGAGGCT
β-actin	5'-3' AGTGTGACGTGGACATCCGCAAAG
	3'-5' ATCCACATCTGCTGGAAGGTGGAC

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA) was applied in statistical analysis, and experiment data were presented as mean ± standard deviation (SD). As for data analysis, One-way ANOVA test was used for comparison between groups followed by Least Significant Difference (LSD). $p < 0.05$ suggested that the difference had statistical significance.

Results

Survival Rate of Flaps and Number of New Vessels

As shown in Figure 1, compared with blank control group, the survival rate of flaps and quantity of new vessels in model group were significantly decreased. Compared with model group, we found that those two indexes in the ADSCs group were also increased.

Detecting the Level of VEGF Through ELISA

As shown in Figure 2, we found that compared with blank control group, the level of VEGF in model group was significantly decreased. Meanwhile, compared with model group, the level of VEGF in the ADSCs group was significantly increased.

IHC Results of HIF-1α and VEGF Proteins

As shown in Figure 3, we performed IHC experiment for HIF-1α and VEGF proteins in each group, and found that compared with blank control group, the expressions of HIF-1α and VEGF in model group were significantly decreased. However, when compared with model group, the expressions in the ADSCs group were increased.

MRNA Expressions of HIF-1α and VEGF

Compared with blank control group, we found that the mRNA expressions of HIF-1α and VEGF in model group were significantly

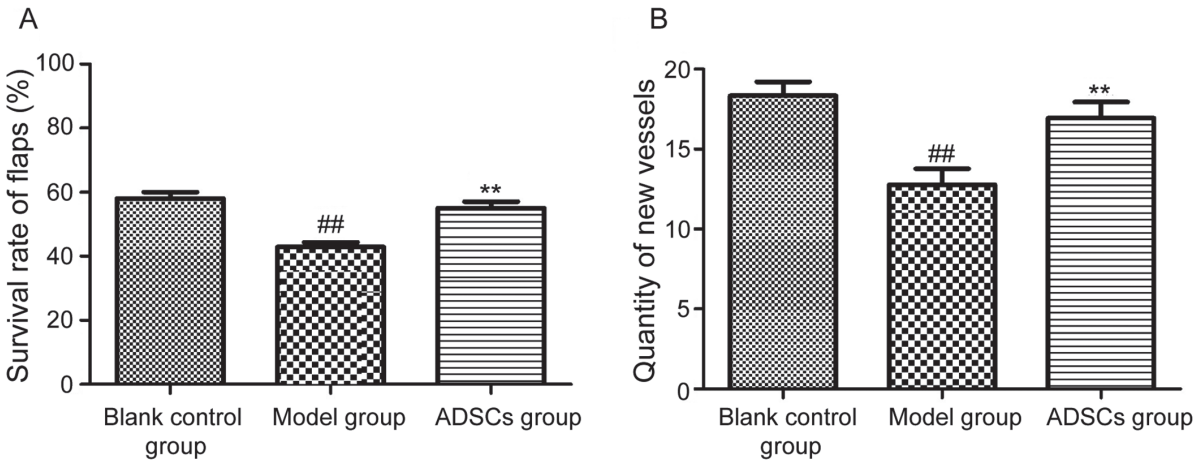

Figure 1. Results of the survival rate of flaps and quantity of new vessels in all groups. ***A,*** Survival rate of flaps: compared with blank control group, the survival rate of flaps in model group is significantly decreased, $^{\#\#}p < 0.01$; compared with model group, the survival rate of flaps in the ADSCs group is also remarkably increased, $^{**}p < 0.01$. ***B,*** Quantity of new vessels: compared with blank control group, the quantity of new vessels in model group is significantly decreased, $^{\#\#}p < 0.01$; compared with model group, the quantity of new vessels in the ADSCs group is also remarkably increased, $^{**}p < 0.01$.

decreased. In comparison with model group, the mRNA expression levels of HIF-1α and VEGF in the ADSCs group were significantly elevated (Figure 4).

Western Blotting Results of HIF-1α and VEGF Proteins

Western blotting results showed the protein expressions of HIF-1α and VEGF. As shown in Figure 5, we found that, in comparison with blank control group, the protein expressions of HIF-1α and VEGF in model group were significantly decreased. At the same time, comparison with model group showed that the protein expressions of HIF-1α and VEGF in the ADSCs group were significantly elevated.

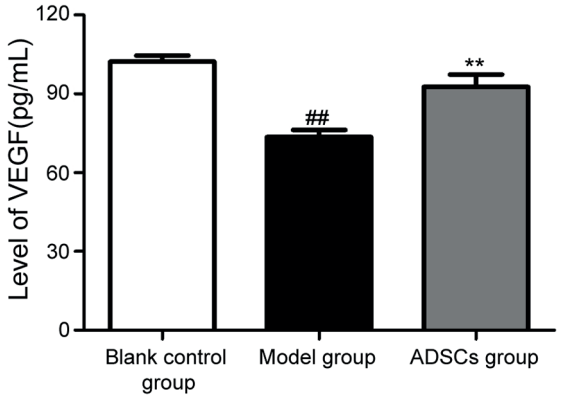

Figure 2. Level of VEGF. Compared with blank control group, the level of VEGF in model group is significantly decreased, $^{\#\#}p < 0.01$; while compared with model group, the level of VEGF in the ADSCs group is significantly increased, $^{**}p < 0.01$.

Discussion

In recent years, with the gradual increase in the living condition, tremendous changes have been seen in life style and diet of people, which have rapidly increased the incidence rate of DM[6]. DM, as a kind of chronic metabolic disorder severely threatening the health of human beings[7], can lead to various serious complications, and also severely affect the life quality of human beings. In clinical practice, complications of skin or soft tissue caused by DM are frequent, and it is urgent to find out an effective method for treatment of DM[8-10].

However, pathogenesis of DM remains unclear, and the ischemic flaps caused by DM are also a problem that should be solved urgently. In recent years, flaps have become one of the repairing methods in surgery, especially the plastic surgery[11-13]. ADSCs are the somatic stem cells isolated from the adipose tissues, and have the potential of multi-directional differentiation and many functions[14-17]. It has been confirmed that HIF-1α and VEGF, secreted by ADSCs, can promote the vascular regeneration, and improve

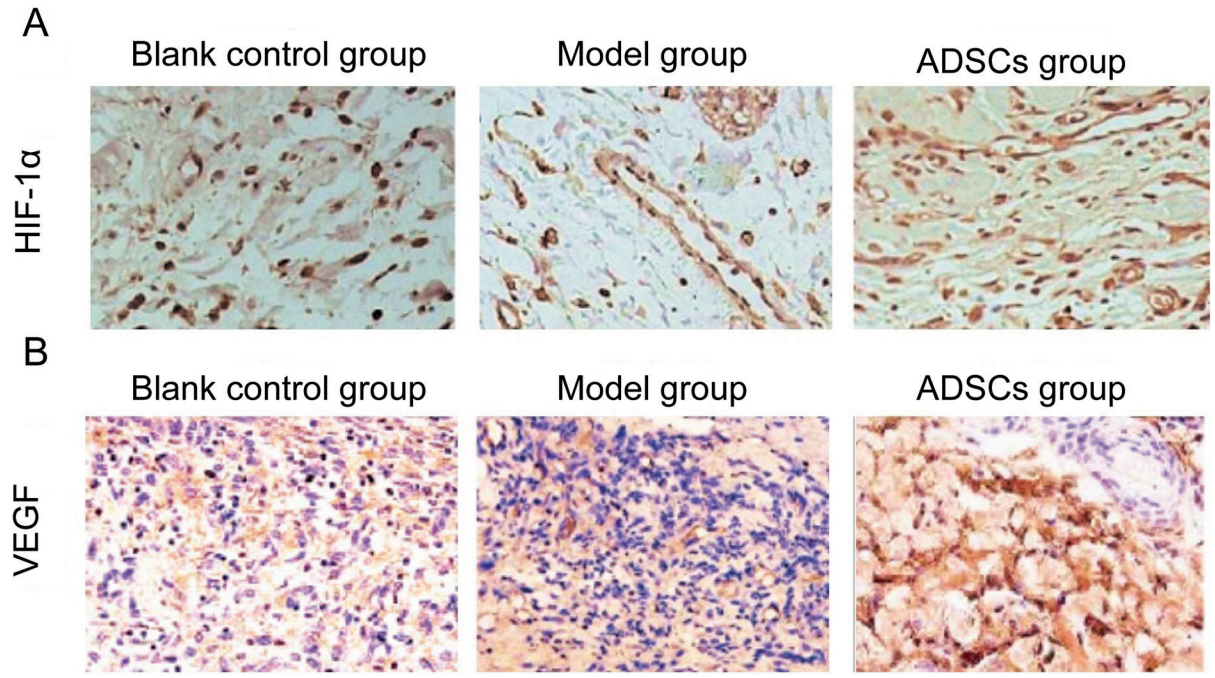

Figure 3. IHC results of HIF-1α and VEGF proteins (200×). Compared with blank control group, the expressions of HIF-1α and VEGF in model group are significantly decreased; however, when compared with model group, the expressions in the ADSCs group are increased.

the local blood supply[18-20]. In summary, HIF-1α/VEGF pathway may serve as a new direction in treatment of ischemic flaps in DM.

In this experimental study with the SD rats, we divided those rats into three groups: blank control group, model group and ADSCs group. We measured the survival rate of flaps and the quantity of new vessels, and assayed the content of VEGF through ELISA method. Furthermore, we performed the IHC assay to detect the expressions of HIF-1α and VEGF in each group. RT-PCR experiment and Western blotting were also carried out to detect the mRNA and protein expressions of HIF-1α and VEGF in each group. Our results showed that compared with control group, the flap survival rate of model group was decreased sig-

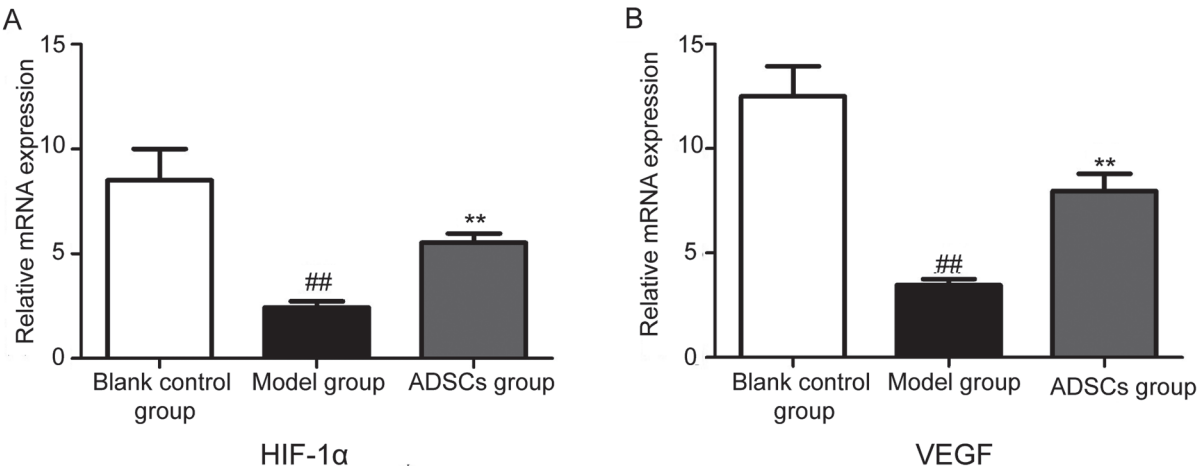

Figure 4. mRNA expressions of HIF-1α and VEGF. Compared with blank control group, mRNA expressions of HIF-1α and VEGF in model group are significantly decreased, $^{\#\#}p < 0.01$; in comparison with model group, the mRNA expressions of HIF-1α and VEGF in the ADSCs group are elevated, $^{**}p < 0.01$.

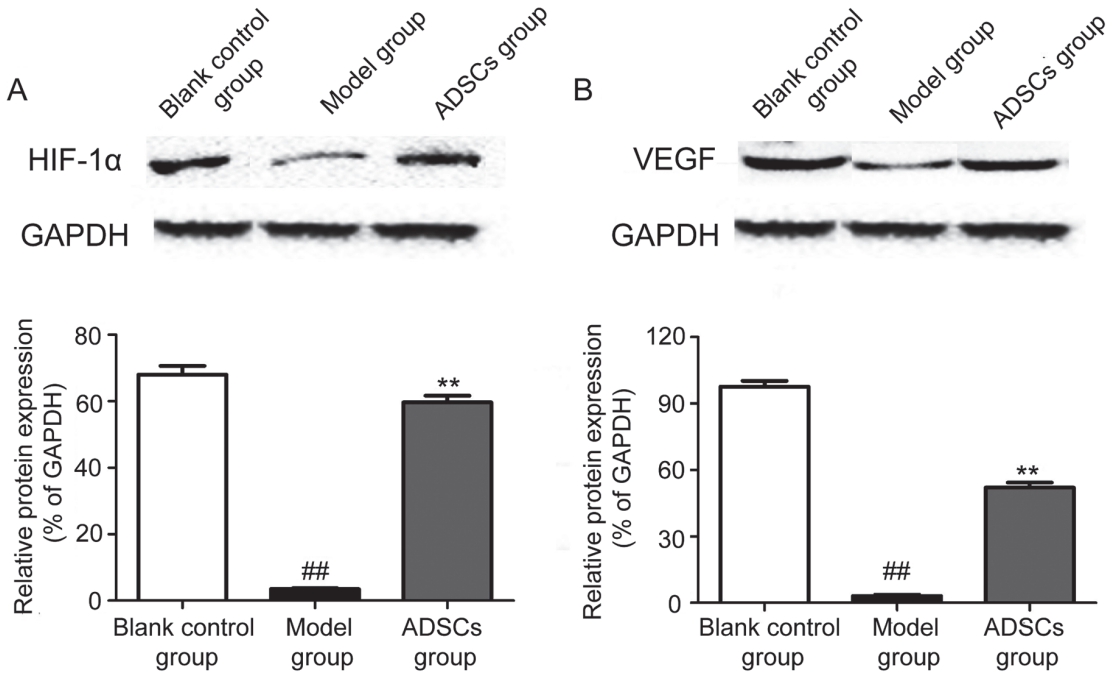

Figure 5. Protein expressions of HIF-1α and VEGF. Compared with blank control group, the protein expressions of HIF-1α and VEGF in model group are decreased, $^{\#\#}p < 0.01$; in comparison with model group, the protein expressions of HIF-1α and VEGF in the ADSCs group are significantly increased, $^{**}p < 0.01$.

nificantly, and the number of new blood vessels was also decreased significantly. Compared with model group, the flap survival rate of ADSCs group was increased significantly, and the number of new blood vessels was also increased significantly. The results of ELISA showed that compared with control group, the level of VEGF in model group was lower than that in model group, and the level of VEGF in the ADSC group was significantly higher than that in model group. IHC results showed that both HIF-1α and VEGF proteins were decreased significantly in model group, whereas the expression of HIF-1α and VEGF in the ADSCs group was increased significantly. The results of RT-PCR and the Western blotting assay showed the mRNA and protein expressions in model group were all decreased, while those in ADSCs group were significantly increased.

Conclusions

We suggest that ADSCs can improve the neovascularization of diabetic ischemic skin by regulating the HIF-1/VEGF pathway, which can provide new orientation for the treatment of ischemic flaps in DM.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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