Original Article
The influence of autoblood treated with an improved preservation solution on the wound healing of diabetes mellitus mouse models and the functional mechanism of the HIF-1α signaling pathway in wound healing

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Abstract: Objective: This paper aims to analyze the promotion effect of autoblood treated with an improved preservation solution (IPS) on the wound healing (WH) of diabetes mellitus (DM) mouse models. Methods: 60 healthy male SD mice were selected as research materials and divided into a standard group (SG), which included 20 mice, an improved group (IG), which included 20 mice, and a blood preparation group (BPG), which included 20 mice, after building the DM mouse models so as to compare the WH in the three groups. The SG was transfused with autoblood treated with a standard preservation solution (SPS), the IG was transfused with autoblood treated with IPS, and the BPG was respectively kept in vitro of the IPS and SPS for one week. Results: (1) The wound healing rate (WHR) of the IG was higher than it was in the SG at 1 d, 4 d, 7 d, and 14 d after the autologous blood transfusion (ABT) (P<0.05). (2) Using real-time fluorescent quantitative PCR (RT-qPCR), we found that the mRNA expression levels of HIF-1α, vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and Hsp90 in the IG were higher than they were in the SG (P<0.05). (3) Using immunohistochemical staining (IHC staining), we found that the positive expression rates (PER) of HIF-1α, VEGF, and EGF in the IG were higher than they were in the SG (P<0.05). (4) After silencing the HIF-1α in the fibroblasts using siRNA, the measurement results based on the Western blot method showed that the protein expression levels of HIF-1α, VEGF, and MMP-2 in the IG were higher than they were in the SG (P<0.05). (5) According to the CCK8 quantification, the fibroblast viability (FV) of the IG was higher than it was in the SG at 24 h, 48 h, and 72 h after the ABT (P<0.05). (6) According to the quantification using flow cytometry (FCM), the cell quantity (CQ) of the IG was much higher than it was in the SG in the G0/G1 stages and much lower than it was in the SG in the G2/M stage (P<0.05), and there was no significant difference in the CQ between the IG and the SG in the S stage (P>0.05). Conclusion: Autoblood treated with IPS accelerated the WH of the DM mouse models, and the activated HIF-1α signaling pathway regulated the FV to enhance the cell viability, strengthen cell proliferation and migration, and thus accelerate the WH.

Keywords: Improved preservation solution, autoblood, diabetes mellitus mouse models, wound healing, HIF-1α signaling pathway, functional mechanism

Introduction
Affected by the changes in lifestyle and dietary behavior, the incidence of DM is increasing gradually. In the past, DM was mainly treated using drugs to control the blood glucose. But with the continuous development of medical techniques and treatment concepts at present, some DM patients are now treated with operations. And the enhanced operative rate of DM accordingly increases the need for blood transfusions [1, 2].

Previously, homologous blood transfusion (HBT) was always the main method of clinical blood transfusion. But its deficiencies are gradually becoming highlighted with its wide application. Now, with increased research, the application value of ABT is being increasingly recognized [3, 4]. More than 60% of patients actively select
ABT for their selective operation in developed countries, over half of patients refuse to receive HBT, and the autoblood storage rate exceeds 80% in America [5]. ABT is a new method of blood supply and it will substitute HBT gradually with the development of medicine and finally become an important method of blood conservation [6]. Compared with HBT, ABT is safer. But there is a shortage of blood with regard to ABT. In order to solve this problem, a lot of relevant studies have been carried out clinically to reform the methods of ABT [7]. Currently, the available methods include salvaged autotransfusion, acute normovolemic hemodilution (ANH), and preoperative autologous blood donation (PABD) [8].

In order to make the best use of autoblood, it is very important to preserve and process the autoblood properly. In vitro operation and improper preservation must be avoided so that the original dysfunction of the red blood cells (RBC) in DM patients will not become more severe. For this purpose, mice were selected for the animal experiment in this study so as to analyze the influence of autoblood treated with IPS on the WH of DM mouse models and thus provide guidance for DM patients treated with ABT.

Materials and methods

Materials

60 healthy male SD mice, 8 months old and weighing 26 g-30 g, were selected and raised in different cages in a clean, strictly-controlled environment. Under a light-dark cycle of 12 h, the humidity was about 50% and the temperature was 19-21°C. The mice, fed with standard solid feedstuff, could drink water freely, which was stopped 6 h before the experiment. This study was approved by the Affiliated Hospital of Southwest Medical University Ethics Committee for Experimental Animals, and the operation procedures were conducted in strict accordance with Nursing and Guideline for Experimental Animals.

Methods

Model preparation: All the mice were adaptively raised for 7 d and then were intraperitoneally injected with 50 mg/kg streptozotocin (STZ mixed with sodium citrate-hydrochloric acid buffer solution in the concentration of 0.1 mmol/l and a pH value of 4.5) for 5 d. Their blood was collected from the caudal vein to measure their blood glucose. The blood was collected from the caudal vein to measure their blood glucose at 7 d, 14 d, 28 d, and 56 d after the injection of STZ. The models were prepared successfully if the blood glucose was ≥16.7 mmol/L.

Mice grouping: 60 mice were randomly divided into the BPG (the blood was collected from the heart after the mice were anesthetized, and then the mice were killed), the SG (the autoblood treated with SPS for 1 week was transfused), and the IG (the autoblood treated with IPS for 1 week was transfused). Each group included 20 mice.

Collection and preservation of the autoblood: In the BPG, 0.6 ml blood was collected from the heart after the mice were anesthetized, and then the mice were killed. The blood was kept in pre-sterilized centrifuge tubes containing anticoagulant and then was stored respectively in SPS and IPS. After mixing, it was stored in the refrigerator at 5°C. The mice were raised in different cages after the blood collection. They could drink and eat freely, specifically, SPS, at pH 5.8, containing 12 mmol/L citric acid, 2.2 mmol/L adenine, 20 mmol/L NaHCO3, 70.1 mmol/L NaCl and 110 mmol/L sugar, and IPS, at pH 6.5, containing 55 mmol/L mannitol, 12 mmol/L NaHCO3, 55.5 mmol/L sugar, 26 mmol/L NaCl and 2 mmol/L adenine.

Establishment of wound models and ABT: After the skin of the back was sterilized, the mice were intraperitoneally injected with 35 mg/kg 1% nembutal for anesthesia. After the anesthesia took effect, the skin was shaved and sterilized. A 2-cm-long open wound was made on both sides of the spine. Then the mice were raised in different cages without medication after the preparation of the wound models. The autoblood stored in different preservation solutions was transfused back at 1 d, 2 d, and 3 d after the preparation, with the total blood transfusion volume of 0.5 ml within 3 days for each mouse. The wound part was recorded and the WH area was calculated 0 d, 1 d, 4 d, 7 d, and 14 d after the preparation of the wound models, with the calculation method = (initial
wound area × residual wound area)/initial wound area × 100%. The mice were killed on the 14th day to collect the wound surface and its surrounding full-thickness tissues for later analysis.

The follow-up experiment and analysis included IHC [9], separation, cultivation, and identification of the fibroblasts [10], the Western-blot method [11] and RT-qPCR measurement [12], which was performed according to the normal operation methods in the corresponding references.

Observation targets

WHR: The WHR was calculated and compared between the SG and IG respectively at 1 d, 4 d, 7 d, and 14 d after the preparation of the wound models and ABT.

mRNA level: The SG and IG were compared in terms of their mRNA levels of HIF-1α, VEGF, EGF and Hsp90, which were measured using RT-qPCR. The total RNA of the mouse skin was determined using the Trizol method. The RNA concentration was determined using a nucleic acid protein analyzer. According to the instructions of the cDNA synthesis kit, 5 μg of total RNA was reversed-transcribed to produce the cDNA. The cDNA was obtained and placed in a freezer at -20°C. The primer sequences of HIF-1, VEGF, EGF, and Hsp90 were synthesized by the Takara Biotechnology (Dalian) Co., Ltd. The specific synthesis process was as follows: 1) With cDNA300 ng, 1 × PCR buffer solution, d NTPs 200 μmol/L, 80 pmol/L of each forward and reverse primer, 0.5 U TAQ polymerase, 25 μL of PCR reaction was performed; 2) The appropriate amount of ddH₂O was added to achieve a total volume of 50 μL, and it was mixed gently and then centrifuged; 3) Set up the PCR program: pre-degeneration at 94°C for 5 min, degeneration at 94°C for 30 s, annealing at 54.5°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 10 min, and then preserved at 4°C; 4) Agarose gel electrophoresis was performed, and the results were observed under ultraviolet light; 5) The gel image analysis system was used to perform the density scanning of the electrophoresis strip. Primer sequence: HIF-1α: F: 5’-CGTTCCTTCATCA GTTGTC-3’, R: 5’-TCA-


PER of the indexes: The SG and IG were compared in the PER of HIF-1α, VEGF and EGF, and the results were observed using IHC staining. The detailed process is as follows: The wound surface and adjacent tissues were fixed, dehydrated, and embedded in paraffin, and then they were sliced into 5 μm thickness sections, and then dried, dewaxed, and rehydrated. The sections were incubated in 1% of H₂O₂ for 10 min at room temperature, washed with PBS, and then heated in the antigen recovery buffer at 97°C and let stand for 15 min for cooling. After that, the sections were incubated with rabbit VEGF (ab39256), EGF (ABGF 6995), HIF-1α (ab88932), and HSP90 antibodies for 1 h at room temperature, and then they were washed three times with PBS. After removing the PBS solution, 1 drop of polymer reinforcement was added and incubated at room temperature for 20 min, and then the sections were washed three times with PBS. After removing the PBS solution, horseradish peroxidase-coupled goat anti-rabbit Ig G antibody was added and incubated at 37°C for 40 min and then washed with PBS three times. The PBS was removed, and a drop of freshly prepared diaminobenzidine solution was added to each section, and then the sections were observed under a microscope for 5 min. The sections were stained with diaminobenzidine solution, redyed with hematoxylin, differentiated with 0.1% HCl, and rinsed with tap water and bluing. Then the sections were dehydrated and dried with gradient alcohol, made transparent with xylene, sealed with neutral balsam, and dried and observed.

Protein expression: The SG and IG were compared in terms of their protein expression levels of HIF-1α, VEGF, MMP-2, and HSP90, which were measured through the Western-blot method. Skin tissues and cells were washed twice with PBS, and a RIPA lysis buffer was added into the homogenizer to achieve homogenate. Process: After 30 min of lysing, the lysate was collected using a centrifuge tube, mixed on a
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shaker for 10 min, centrifuged at 12000 r/min at 4°C for 30 min. After collecting the upper serum, the protein concentration in the supernatant was determined using the BCA method, and the volume of the sample was adjusted. 50 μg of total protein was dissolved in a 2 × SDS buffer and boiled at 100°C for 5 min. 15-20 µL of the sample was loaded to SDS-PAGE gel electrophoresis (10 cm × 10 cm) at a 10% concentration. The protein was transferred to a polyvinylidene fluoride (PVDF) membrane using the wet transfer method, blocked with 5% skim milk at room temperature for 1 h, and incubated with the primary antibodies β-actin (1:1000), HIF-1α (1:100), VEGF (1:200), and EGF (1:200). The membrane was washed three times with TBST, and HRP-labeled secondary antibodies and goat anti-mouse Ig G polyclonal antibodies (1:2000) were used to incubate it at room temperature for 1 h. After development, it was analyzed using an American DOCTM XR system. The ratio of the gray value of the target protein band to the gray value of the β-actin band reflected the expression level of the target protein.

FV: FV was detected using the CCK8 method and compared between the SG and IG respectively at 0 h, 24 h, 48 h, and 72 h. After transfection for 6 h, the cells were isolated with 6-10 mL 0.25% trypsin and inoculated on a 96-well plate (3000/well). 10 μL CCK8 reagent was added to the medium at 10°C and reacted in an incubator of 5% CO₂ and at 37°C for 1 h. The value of D450 nm was determined.

Cell cycle of fibroblasts: The CQ was measured using FCM and compared between the SG and IG respectively in the G0/G1 stage, the S stage, and the G2/M stage. The cells in each group were treated with PBS solution (containing 2.5 g/L trypsin and 0.4 g/L EDTA) and were then collected and placed in a 5 mL centrifuge tube. After washing twice with PBS, the cells were centrifuged at 900 r/min for 5 min. The supernate was removed, and 1 mL of PBS was used to suspend the cell particles. The Cell suspension was fixed with 2 mL of anhydrous ethanol, sealed, and stored at 4°C. The cells were adjusted to 1.0 × 10⁵/well and divided into two FACS tubes. The apoptosis was stained with an Annexin V-FITC apoptosis assay kit, and the proportion of cells in each phase was determined using flow cytometry.

Statistical methods

SPSS 22.0 was used for the statistical analysis. The measurement data were represented as the mean ± standard deviation. T tests were used for the comparisons between two samples, and a one-way analysis of variance (ANOVA) was used for the comparisons between multiple samples. P<0.05 meant that the difference had statistical significance.

Results

WHR

The WHR was (5.36±0.89)% in the SG and (16.86±0.96)% in the IG 1 d after the ABT; (30.49±7.78)% in the SG and (44.85±8.03)% in the IG 4 d after the ABT; (52.38±8.71)% in the SG and (86.43±9.48)% in the IG 7 d after the ABT; and (64.52±9.23)% in the SG and (92.23±5.41)% in the IG 14 d after the ABT, which indicated that the difference in the WHR between two groups had statistical significance (P<0.05) (Figure 1).

Results of all indexes

According to the RT-qPCR measurement, the mRNA expression levels of HIF-1α, VEGF, EGF, and Hsp90 in the IG were much higher than they were in the SG, which indicated that the difference had statistical significance (P<0.05) (Table 1 and Figure 2).
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Table 1. Comparisons of the mRNA levels of all indexes in the SG and IG using RT-q PCR measurement ($\bar{x} \pm s$)

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of mice</th>
<th>HIF-1α</th>
<th>VEGF</th>
<th>EGF</th>
<th>Hsp90</th>
</tr>
</thead>
<tbody>
<tr>
<td>SG</td>
<td>20</td>
<td>0.73±0.08</td>
<td>0.70±0.09</td>
<td>1.10±0.12</td>
<td>0.35±0.03</td>
</tr>
<tr>
<td>IG</td>
<td>20</td>
<td>1.76±0.12</td>
<td>4.91±0.33</td>
<td>1.46±0.15</td>
<td>0.79±0.08</td>
</tr>
<tr>
<td>t</td>
<td></td>
<td>31.939</td>
<td>55.043</td>
<td>8.381</td>
<td>23.031</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Figure 2. Comparison of the RT-qPCR results of all the indexes between the SG and IG. The levels of HIF-1α, VEGF, EGF, and Hsp90 in the IG were much higher than they were in the SG, which indicated that the difference was statistically significant ($P<0.05$) (Table 4 and Figure 4).

Protein expression

After the silencing of HIF-1α in the fibroblasts through siRNA, the protein expression levels of HIF-1α, VEGF, and MMP-2 were measured using the Western blot method, and the results showed that the protein expression levels of HIF-1α, VEGF, and MMP-2 in the IG were much higher than they were in the SG ($P<0.05$) (Table 5 and Figure 5).

Discussion

DM patients will spend a longer time on WH for all wounds, and the reason may be that the high blood glucose and poor blood circulation can easily lead to diabetic neuropathy, causing immune system deficiency and even wound infection [13]. WH includes a variety of dynamic processes, such as proliferation, epithelialization, inflammation, and remodeling. The inflammatory response is closely related to WH, and the uncontrolled inflammatory response will lead to poor healing, including chronic wounds, ulceration, and hypertrophic scarring, etc. [14, 15]. It has been verified through published studies that cell factors or angiogenic factors can effectively accelerate angiogenesis and WH, but the application value has not been reflected in the WH of DM patients [16]. Hence, the new and effective methods must be explored and used in the treatment of diabetic wounds.

The application advantages of ABT have been widely verified, including the prevention of pathophoresis, the saving of blood resources, the enhanced safety of blood transfusion, and the reduction of side reactions, etc. It is widely used in the treatment of surgical bleeding and anemia [17, 18]. DM patients are susceptible to injection due to their significant immunodeficiency.
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Table 2. Comparisons of the PER of all the indexes in the SG and IG after the IHC staining (\( \bar{x} \pm s, \% \))

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of mice</th>
<th>HIF-1( \alpha )</th>
<th>VEGF</th>
<th>EGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>SG</td>
<td>20</td>
<td>11.23( \pm )4.16</td>
<td>6.05( \pm )2.58</td>
<td>14.63( \pm )1.82</td>
</tr>
<tr>
<td>IG</td>
<td>20</td>
<td>64.58( \pm )7.99</td>
<td>50.13( \pm )8.69</td>
<td>38.84( \pm )4.21</td>
</tr>
<tr>
<td>( t )</td>
<td></td>
<td>26.486</td>
<td>21.767</td>
<td>23.606</td>
</tr>
<tr>
<td>( P )</td>
<td></td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Table 3. Comparison of the cell cycle of fibroblasts in the SG and IG (\( \bar{x} \pm s \))

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of mice</th>
<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>SG</td>
<td>20</td>
<td>67.95( \pm )4.58</td>
<td>15.93( \pm )3.12</td>
<td>16.15( \pm )3.95</td>
</tr>
<tr>
<td>IG</td>
<td>20</td>
<td>81.96( \pm )3.53</td>
<td>11.53( \pm )2.02</td>
<td>6.38( \pm )3.54</td>
</tr>
<tr>
<td>( t )</td>
<td></td>
<td>10.835</td>
<td>5.294</td>
<td>8.237</td>
</tr>
<tr>
<td>( P )</td>
<td></td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Table 4. Comparisons of the FV in the SG and IG at 24 h, 48 h, and 72 h (\( \bar{x} \pm s \))

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of mice</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>SG</td>
<td>20</td>
<td>0.80( \pm )0.05</td>
<td>0.67( \pm )0.03</td>
<td>0.51( \pm )0.02</td>
</tr>
<tr>
<td>IG</td>
<td>20</td>
<td>0.93( \pm )0.05</td>
<td>0.84( \pm )0.03</td>
<td>0.73( \pm )0.03</td>
</tr>
<tr>
<td>( t )</td>
<td></td>
<td>8.222</td>
<td>17.920</td>
<td>27.288</td>
</tr>
<tr>
<td>( P )</td>
<td></td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
</tbody>
</table>

VEGF is one of the most important angiogenic factors after trauma. Its generation is closely associated with the downstream of hypoxia. HIF-\( \alpha \) is activated after hypoxia damage occurs, and the level of VEGF will be up-regulated through the transcriptional activation of the target genes. And in this way, the angiogenesis is accelerated [22].
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According to the results of this study, the expression of the downstream genes on the HIF-1α axis for wound surface were reduced in the SG, which implied that the DM mice had decreased wound angiogenesis and delayed WH. By contrast, the expressions of HIF-1α, VEGF, EGF, HSP90, and MMP-2 were increased in the IG, which implied that the application of IPS could enhance the HIF-1α signaling pathway in the wound. The hypoxic environment in the early stage of wound formation can stimulate HIF-1α and downstream signals and thus promote WH. Through this analysis, we found that HIF-1, as an important transcriptional regulator of the hypoxia response, is involved in the angiogenesis, survival, and metastasis of cells through the transcriptional activation of target genes. Under oxygen deficient conditions, HIF-1 can be combined with the hypoxia response element (HRE), which accumulates and leads to the expression of various angiogenic genes in the promoter region [23]. This study also showed that FV is significantly enhanced after the transfusion of autoblood treated with IPS through HIF-1α, which promotes the proliferation and migration of fibroblasts. It is considered that skin fibroblasts play a crucial role in wound contraction, tissue remodeling, and the deposition of the extracellular matrix. Fibroblasts have certain defects in cell proliferation, migration, and growth factor secretion in chronic wounds. As a key cellular process promoting wound healing, both oxygen-dependent hypoxia HIF-1α and hypoxia-dependent hypoxia HIF-1α can increase the proliferation of fibroblasts [24]. Chen et al. [25] indicated that the stability of HIF-1α is related to the maintenance of the p-Cofilin level, the rearrangement of actin filaments, the increase of the cell area, and the reduction of single-cell migration in the case of hypoxia, etc. This conclusion was also verified in this study, showing that the proliferation of fibroblasts is promoted by oxygen-dependent hypoxia HIF-1α and hypoxia-dependent hypoxia HIF-1α.

In conclusion, based on molecular biology, this study analyzed the influence of autoblood treated with IPS on the WH of DM mouse models and the specific functional mechanism of the HIF-1α signaling pathway in WH. In addition, it

Figure 4. Comparison of the cell cycles of the fibroblasts in the SG and IG. The cell cycles of the fibroblasts in the IG were much higher than they were in the SG at 24 h, 48 h, and 72 h (P<0.05). * means P<0.05 when SG and IG are compared at the same time.

Table 5. Comparisons of the protein expression levels in the SG and IG using the Western blot method (X ± s)

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of mice</th>
<th>HIF-1α</th>
<th>VEGF</th>
<th>MMP-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>SG</td>
<td>20</td>
<td>0.64±0.07</td>
<td>0.57±0.06</td>
<td>0.57±0.07</td>
</tr>
<tr>
<td>IG</td>
<td>20</td>
<td>1.29±0.12</td>
<td>0.88±0.10</td>
<td>0.94±0.11</td>
</tr>
<tr>
<td>t</td>
<td>20.924</td>
<td>11.888</td>
<td>12.691</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
</tr>
</tbody>
</table>

Figure 5. Comparison of the Western blot results of all indexes in the SG and IG. The levels of HIF-1α, VEGF, and MMP-2 in the IG were much higher than they were in the SG (P<0.05). # means P<0.05 when the SG and IG were compared in respect of the same index.
was also verified that autoblood treated with IPS has a good application value. However, there were certain deficiencies in this study. For example, there was no deep discussion about how autoblood treated with IPS promotes the WH of DM mice through the HIF-1α signaling pathway. And there was also no in-depth exploration of the mechanism of autoblood treated with IPS and HIF-1α in blood transfusions for diabetic wounds. Further analyses and experimentation should be conducted focusing on these deficiencies in the future so as to verify the effect of autoblood treated with IPS on diabetic WH in a more comprehensive way.

Disclosure of conflict of interest

None.

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