The effect of heme oxygenase-1 complexed with collagen on MSC performance in the treatment of diabetic ischemic ulcer

Chunli Hou, Lei Shen, Qiang Huang, Jianhong Mi, Yangxiao Wu, Mingcan Yang, Wen Zeng, Li Li, Wen Chen, Chuhong Zhu

*Corresponding author.
E-mail address: zhuch99@yahoo.com (C. Zhu).

**Department of Anatomy, Key Lab of Biomechanics, Third Military Medical University, Chongqing 400038, China**
**Department of Anatomy, Qiqihar Medical College, Heilongjiang 161006, China**
**Department of Orthopaedics, Southwest Hospital, Third Military Medical University, Chongqing 400038, China**
**Department of Orthopaedics, Traditional Chinese Medicine Hospital, Shaping Ba District, Chongqing 400038, China**

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**Abstract**

Diabetic ischemic ulcer is an intractable diabetic complication. Bone marrow mesenchymal stem cells (BMSCs) have great potential in variety of tissue repair. In fact, poor cell viability and tolerance limit their ability for tissue repair. In addition, it is difficult for stem cells to home and locate to the lesion. In this study, we explore whether over-expression of heme oxygenase-1 (HO-1) in BMSCs complexed with collagen play an important role in treatment of diabetic ischemic ulcers. *In vitro*, over-expression of HO-1 promoted the proliferation and paracrine activity of BMSCs and the conditioned medium of BMSCs accelerated HUVECs migration and proliferation. These processes were closely related to Akt signaling pathway and were not dependent on Erk signaling pathway. *In vivo*, in order to make BMSCs directly act on the wound, we choose a solid collagen as a carrier, BMSCs were planted into it, ischemic wounds of diabetic mice were covered with the complex of BMSCs and collagen. The results indicate that the complex of HO-1-overexpressing BMSCs and collagen biomaterials can significantly promote angiogenesis and wound healing. These preclinical findings open new perspectives for the treatment of diabetic foot ulcers.

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**1. Introduction**

Diabetic ischemic ulcer is an intractable diabetic complication. There has been no good treatment method to date, and amputation is often the end result, creating a significant economic pressure and burden for both patients and society. In recent years, stem cell transplantation has been considered as a new therapeutic strategy for diabetic foot ulcers.

Bone marrow mesenchymal stem cells (BMSCs) have been used in a variety of tissue engineering strategies because of their multi-differentiation potential, immunomodulatory and tissue repair properties [1]. Wu and his colleagues [2] reported that the implantation of BMSCs can significantly promote wound healing by increasing the formation of epithelial cytoplast and local angiogenesis in wounds. In addition, artificial skin including BMSCs or injecting BMSCs around the wound can also accelerate wound healing in diabetic rats [3,4]. Although BMSCs have great therapeutic potential for damaged tissue, poor cell viability and tolerance limit their ability for tissue repair. Studies have confirmed that diabetes can damage BMSCs, decrease their proliferation and secretion as well as their anti-apoptosis and differentiation potential [5–8]. Exogenous BMSCs can survive for only a short time in subjects and ulcers relapse quickly, how to enhance stem cell viability in diabetic ischemic ulcers micro-environment is a key problem.

Oxidative stress induced by high glucose is believed as the central pathogenesis of the chronic complications of diabetes, and it is also an obstacle for stem cell therapy. The blockade of oxidative stress is very beneficial for the prevention and treatment of chronic diabetic complications. It is well known that heme oxygenase-1 (HO-1) is an important antagonist of oxidative stress. HO-1 plays an important role in regulating many pathophysiological processes, especially in protecting tissues and organs, and it has been a research focus in many fields, including research on organ transplantation, ischemia-reperfusion injury, and cardiovascular diseases [9–11]. One study found that the expression of HO-1 was significantly decreased in diabetics in comparison with non-diabetics. Tang et al. [12] reported that HO-1 can prevent graft cell death in the ischemic heart. The up-regulation of HO-1 can
reduce oxidation products and protect endothelial cells against damage and loss, thus reducing the vascular complications of diabetes.

At present, cell homing and positioning to the lesion is still a difficult problem in stem cell therapy. In order to make BMSCs directly act on the wound, we choose a solid collagen as a carrier. BMSCs were planted into the solid collagen biomaterials, the wounds were covered with the complex of BMSCs and collagen. We speculate that the new complex plays an important role in the treatment of diabetic ischemic ulcers. In further, we hope to explore a non-invasive, convenient and more effective method to treat ischemic diabetic ulcers of the lower limb.

2. Materials and methods

2.1. Cell culture and cell group

We purchased human umbilical vein endothelial cells (HUVECs) and GFP-transfected human bone marrow mesenchymal stem cells (BMSCs) from Cyagen Biosciences (Guangzhou, China). The cells were routinely cultured according to the instructions of the supplier. Cells were used between passage 4 and passage 6 in the following experiments. Cobalt protoporphyrin (Copp) is known as an inducer of HO-1, it can specifically promote HO-1 expression in a variety of cells. On the contrary, Sn-protoporphyrin (Snpp) is an inhibitor of HO-1, it can inhibit HO-1 expression in many kinds of cells. In this experiment, we up-regulated or inhibited HO-1 expression by stimulating BMSCs with Copp (Santa, 20 μm) or Snpp (Sigma, 10 μm). The cells were divided into five groups; one group (control group) was cultured in a high-glucose medium (30 mM glucose), and the other four groups were cultured in a high-glucose medium with different stimulating factors, including Copp or Snpp, or Copp and Akt inhibitor (Copp + Akt), or Copp and Erk inhibitor (Copp + Erk). These media were used for the treatments described below.

2.2. Expression of HO-1 in BMSCs

In the experiment, we extracted cyttoplasm protein by protein extraction kit (Beyotime Institute of Biotechnology, China) and determined the role of Copp or Snpp on HO-1 expression in BMSCs through the ELISA assay (R&D Systems) according to the manufacturer’s instructions.

2.3. The effect of HO-1 on the proliferation and paracrine of BMSCs

2.3.1. MTT assay

BMSCs were harvested with 0.25% trypsin-EDTA (Hyclone Laboratories) when the closure rate was about 80% and were transferred to a 96-well plate at 10^5 cells/well. The BMSCs were cultured in different medium (Copp, or Snpp, or Copp + Akt, or Copp + Erk) for 3 days. MTT [3-[4,5-dimethylthiazol-2-yl] 2, 5-diphenyltetrazolium bromide] was dissolved in PBS at 5 mg/ml and filter sterilized. An aliquot (20 μl) of the MTT solution was added to each well incubation of the BMSCs continued at 37 °C, 5% CO2 for 4 h. The medium was removed, and 150 μl of DMSO was added to each well. After 15 min of orbital shaking, the plate was placed in an incubator for 5 min to eliminate air bubbles. The absorbance at a wavelength of 492 nm was measured using a plate reader.

2.3.2. ELISA assay

The conditioned medium was collected after BMSCs were cultured for 3 days. Vascular endothelial growth factor (VEGF) in the conditioned culture supernatants were determined using ELISA assay (R&D Systems) according to the manufacturer’s instructions.

2.4. The function of the conditioned medium of BMSCs to proliferation and migration of HUVECs

2.4.1. MTT assay

BMSCs were harvested with 0.25% trypsin-EDTA when the confluence rate was approximately 80% and were transferred to a 96-well plate at 10^5 cells/well. The medium consisted of RPM 1640 with the different treatments (conditioned medium of BMSCs/1640 – 1/5); the ensuing steps were the same as described above (2.3.1 MTT assay).

2.4.2. Cell scratch experiment

HUVECs were plated in 6-well plates at 5 × 10^6 cells/well. When cells covered the bottom of the well, a scratch was made with a sterilized P1000 pipette tip (a width of 0.4–0.5 mm), and the HUVECs continued to be cultured in the different media (conditioned medium of BMSCs/1640 – 1/5) after cleaning the striped cells with PBS. After 24 h, the HUVECs were fixed in 4% paraformaldehyde and were then stained with 0.5% Crystal violet (Sigma). The scratches were analyzed with an OLYMPUS BX50 microscope, and the scratch area was measured using IPP software (Media Cybernetics).

2.4.3. Transwell chamber experiment

HUVECs were harvested with 0.25% trypsin-EDTA when the confluence rate achieved 75%, and 10^4 cells were loaded into the upper chamber of a 24-well transwell migration insert (pore size of 5 mm). The lower chamber contained basal medium with the different conditioned media of the BMSCs (conditioned medium of BMSCs/1640 – 1/5). After 6 h, the cells on the upper side of the membrane were wiped away, and cells on the lower side of the membrane were fixed with 4% parafomaldehyde and stained with 0.5% crystal violet. The cell morphology was immediately determined by light microscopy, and the number of cells migrating into the lower chamber was determined.

2.5. Growth of BMSCs in biomaterials

The hybrid electrospinning nano-fiber mesh was made by polyactic acid 80%, silk protein 10% and collagen 10% using a previously described procedure [13]. BMSCs were seeded in the biomaterials at 8 × 10^6 cells/material; cells were divided into three groups. The first group was added high concentration glucose (30 μM) as control group; the second group was added high concentration glucose (30 μM) and Copp (20 μM), and the third group was added high concentration glucose (30 μM) and Snpp (10 μM). Three days after, BMSCs were fixed with 4% paraformaldehyde and determined by Leica TCS SP5 laser scanning confocal microscope.

2.6. Animal procedures

Six- to eight-week-old male C57BL/6j mice were streptozotocin-induced with diabetes (Sigma, i.p., 40 mg/kg). Four weeks after the diabetes induction, the mice were depilated on their hindlimb area and underwent bilateral hindlimb ischemia by ligature and electro-coagulation under anesthesia. Full-thickness wounds were simultaneously created in the dorsal thigh skin of both legs using a sterile 5-mm-wide biopsy punch. To evaluated the healing potential of HO-1-overexpressing BMSCs in the model of diabetic ischemic ulcer. The wounds were covered with collagen alone or collagen containing 2 × 10^5 BMSCs or 2 × 10^5 HO-1-overexpressing BMSCs. For a detailed experimental procedure, please see references [14–17]. All the procedures complied with the standards stated in the Guide for the Care and Use of Laboratory Animals (Third Military Medical University) and were approved by the ethics committee of Third Military Medical University.

2.7. The rate of wound closure and histology

Photographs were taken on day 0, day 7 and day 14, the wound area was analyzed using IPP software 6.0 (Media Cybernetics). On day 14 postoperative, the animals were sacrificed using an overdose of anesthetic (0.3% pentobarbital, i.p.), and the wounds and surrounding skin were removed. The tissues were fixed in 4% paraformaldehyde, and the survival and distribution of the BMSCs were analyzed by fluorescence microscopy (BMSCs with green fluorescence). The vascular endothelial cells were labeled by von Willebrand Factor (vWF, 1:200, overnight; BOSTER Corp., Wuhan, China). Capillary profiles were recognized by fluorescence microscope, the capillary density was determined in a blind manner [14,18].

2.8. Statistical analysis

The experimental data were analyzed by SPSS 18.0, t-test and variance analyses were performed and the results were expressed as the mean ± SEM. Each experiment was repeated at least three times. All p values were two-tailed, and p value <0.05 was considered statistically significant.

3. Results

3.1. Expression of in HO-1 BMSCs

In an Enzyme-Linked Immunoassay (Fig.1), high concentration glucose promoted HO-1 expression of BMSCs in the early stage, it indicates that HO-1 plays an important role in cellular oxidative stress reaction. Copp significantly improved HO-1 expression in BMSCs, and the difference was significant compared with high glucose group (p < 0.01). On the contrary, Snpp significantly inhibited HO-1 expression in BMSCs. So in the following experiments, we achieved the up-regulation or inhibition of HO-1 expression in BMSCs through Copp or Snpp respectively.
Values are mean ± SEM.

3.2. The function of HO-1 to proliferation and paracrine activity of BMSCs

To explore the effect of HO-1 on BMSCs growth in high-glucose medium, we used MTT assay to examine BMSCs proliferation. When we added Copp to the high-glucose medium, the proliferation rate of BMSCs was significantly higher than the control group (*p < 0.01), the OD value (492 nm) of Copp group was 132% of that in control group, which suggests that HO-1 can promote BMSCs proliferation. On the contrary, the proliferation rate of BMSCs was significantly lower than the control group ($p < 0.01$) when the expression of HO-1 was inhibited by Snpp, the OD value was 63% of that in control group. When we added Copp and Akt inhibitor together to the high-glucose medium, the proliferation rate of BMSCs significantly decreased versus Copp group (#p < 0.01), the OD value was as the same as control group. However, there was little difference between Copp + Erk inhibitor group and Copp group. These results demonstrate that the over-expression of HO-1 can promote BMSCs proliferation. In contrast, when the expression of HO-1 is inhibited, the proliferation rate of BMSCs declines significantly. In addition, the process relies on the Akt signaling pathway and the Erk signaling pathway is not involved (Fig. 2a).

VEGF is a critical cytokine for angiogenesis. In this experiment, we used an ELISA to detect the VEGF level in the conditioned medium of BMSCs. We found that the amount of VEGF in Copp group was 152% compared with control group. On the contrary, which were significantly lower than control group when the expression of HO-1 was inhibited by Snpp ($p < 0.01$), the amount was 57% compared with control group. In addition, the amount of VEGF in Copp + Akt inhibitor group was 50% compared with Copp group, the difference was significant (#p < 0.01). But there was no significant difference between Copp + Erk inhibitor group and Copp group. These results demonstrate that over-expression of HO-1 can promote BMSCs to secrete VEGF. In contrast, when the expression of HO-1 is inhibited, the secretion of BMSCs declines significantly. In addition, the process relies on the Akt signaling pathway and the Erk signaling pathway is not involved (Fig. 2b).

3.3. The role of conditioned medium of BMSCs on proliferation and migration of HUVECs

The proliferation and migration of endothelial cells are very important for vascular repair and regeneration. Through the above experiments, we conclude that HO-1 can promote proliferation of BMSCs and secretion VEGF. So we speculate that the conditioned medium of BMSCs plays a role in the proliferation and migration of endothelial cells. In order to confirm our speculation, the proliferation of HUVECs was detected through MTT assay and scratch experiments (Figs. 3a–c). In addition, the migration of HUVECs was verified through a transwell chamber experiment (Fig. 3d and e). In MTT assay, the OD value of Copp group was 165% of the control value and was 175% of the value of Snpp group, it indicates that the medium of BMSCs + Copp group can improve HUVECs proliferation. In addition, the OD value of Copp + Akt inhibitor group was 64% of the value of Copp group, but there was no significant difference between Copp + Erk group and Copp group (Fig. 3a). The result of cell scratch experiment was consistent with MTT assay result (Fig. 3b and c). In transwell chamber experiment, the conditioned medium of Copp group promoted significantly HUVECs migration, and the migration rate was 140% compared with control group. The migration rate of Copp + Akt inhibitor group descended sharply compared with Copp group (#p < 0.01), but there was no significant difference between Copp + Erk inhibitor group and Copp group. These observations suggest that

![Fig. 1.](image1.png)

**Fig. 1.** Copp and Snpp regulated HO-1 expression in BMSCs. HO-1 expression was determined by ELISA when BMSCs were cultured for 72 h. *p < 0.01 (n = 8) versus the control group; #p < 0.01 (n = 8) versus Copp group; $p < 0.01 (n = 8) versus Copp + high glucose group; $p < 0.01 (n = 8) versus Snpp + high glucose group. Values are mean ± SEM.

![Fig. 2.](image2.png)

**Fig. 2.** HO-1 promoted BMSCs proliferation and VEGF secretion. (a) MTT assay showing the OD value (492 nm) of BMSCs cultured for 72 h in each group. *p < 0.01 (n = 18) versus the control group; #p < 0.01 (n = 18) versus the Copp + Akt inhibitor group. $p < 0.01 (n = 18) versus the control group. Values are mean ± SEM. (b) The VEGF concentration in conditioned medium was determined by ELISA. *p < 0.01 (n = 18) versus the control group; #p < 0.01 (n = 18) versus the Copp + Akt inhibitor group. $p < 0.01 (n = 18) versus the control group. Values are mean ± SEM.
the conditioned medium of HO-1-overexpressing BMSCs can promote the proliferation and migration of HUVECs, and these processes were dependent on the Akt signaling pathway, whereas the Erk signaling pathway was not involved.

3.4. The effect of HO-1 on the proliferation of BMSCs in biomaterials

To make the cells remain in the ulcer surface, we need a suitable carrier dressing. In this experiment, we chose a solid collagen. We
observed that the number of BMSCs activated by HO-1 significantly increased as described above (Fig. 4); it indicates that the solid collagen is suitable for BMSCs growth and HO-1 can promote BMSCs to grow better on collagen biomaterials.

3.5. The contribution of the new complex of stem cell and biomaterials to wound healing

To study the impact of the new complex of stem cells and collagen on wound healing, we produced wounds and limb ischemia in diabetic mice. The wound was covered with collagen alone or collagen containing $2 \times 10^4$ BMSCs or $2 \times 10^4$ HO-1-overexpressing BMSCs or $2 \times 10^4$ HO-1-lowexpressing BMSCs. We found that collagen with HO-1-overexpressing BMSCs was more effective for wound healing ($n = 6$ per group) than the other treatments either on day 7 or day 14 ($^*p < 0.01$, $^#p < 0.01$). The healing rate of control group (collagen alone) was slowest speed on day 7, but there was little difference among the three groups (control, BMSCs and BMSCs + Snpp) on day 14. It indicates that the complex of collagen and HO-1-overexpressing BMSCs play an important role in treating intractable diabetes ischemic ulcers (Fig. 5).

3.6. Effect of the new complex of stem cell and biomaterials on blood vessel restoration and angiogenesis

Vascular repair and angiogenesis play an important role in wound healing process. In vivo, experiment results indicate that

![Fig. 4. HO-1 promoted BMSCs proliferation in collagen biomaterials. (a–c) BMSCs cultured for 72 h in collagen biomaterials and collagen biomaterials supplemented with high glucose (a) or high glucose + Copp (b) or high glucose + Snpp (c), scale bar = 50 μm (d–f) Synthesized maps composed of collagen biomaterials with the control, Copp and Snpp, scale bar = 50 μm. (g) The cell count of BMSCs cultured for 72 hours in the biological material. $^*p < 0.01$ ($n = 12$) compared with the control group; $^#p < 0.01$ ($n = 12$) compared with the Snpp group. The values are the mean ± SEM.](image)
BMSCs + Copp group can significantly accelerate the wound healing, so we speculate that HO-1-overexpressing BMSCs can promote wound vascular repair and regeneration. To determine the relationship between exogenous BMSCs and the number of vascular in wound, wounds surrounding skin were analyzed on day 14 post-operative when animal sacrificed by an overdose of anesthetic. Exogenous BMSCs were traced by green fluorescence. Capillary profiles were recognized by immuno-histochemical staining using rabbit von Willebrand Factor (vWF, 1:200, over night; BOSTER Corp., Wuhan, China) followed by Rhodamine-conjugated goat anti-rabbit IgG (1:100; BOSTER Corp., Wuhan, China). Results show that there is some green fluorescence (survival BMSCs) in BMSCs + Copp group, and part of those BMSCs have differentiated into endothelial cells identified by vWF (Fig. 6j, shown by the arrows), which indicates that HO-1 can improve BMSCs survival in wound microenvironment, and BMSCs can differentiate into endothelial cells. We can not see any transplanted BMSCs (green fluorescence) in BMSCs group or BMSCs + Snpp group on day 14 (Fig. 6g and o). These results show that HO-1 can protect BMSCs from injury induced by high glucose, ischemia and hypoxia in wound microenvironment. HO-1-overexpressing BMSCs can accelerate the repair and regeneration of the local wound capillary through differentiation and paracrine mechanisms.

In order to evaluate the function of BMSCs to vascular repairation and angiogenesis, the capillary density was determined in a blind manner [14,18]. Results showed that the capillary density in BMSCs + Copp group was 33 n/mm², the number was significantly higher than any other group. It indicates that the HO-1-overexpressing BMSCs can promote wound healing by improving the restoration of blood vessels and angiogenesis (Fig. 6q).

4. Discussion

Chronic wounds in diabetes are mainly due to the lack of angiogenesis [19]. How to initiate and effectively promote angiogenesis is one efficacious way. Preliminary evidence supports the potential of BMSCs for the healing of skin ulcers [2,20,21]. BMSCs can differentiate and release pro-angiogenic factors to accelerate angiogenesis and promote wound healing; thus, BMSCs have powerful potential in the treatment of diabetic ischemic foot ulcers. As previously mentioned, the local micro-environment in diabetic ischemic ulcers is detrimental to the survival of BMSCs, and exogenous BMSCs can survive for only a short time [2]. Therefore, how to improve the tolerance and vitality of BMSCs is a key concern. HO-1 is one of the most important endogenous antioxidant factors. The over-expression of HO-1 can reduce sugar-mediated cell growth inhibition and apoptosis of human micro-vascular endothelial cells. In addition, the over-expression of HO-1 can increase BMSCs survival in ischemic and infarcted myocardium [22,23]. Relevant studies have also shown that the expression and
activity levels of HO-1 in STZ-induced diabetic rats are significantly decreased compared with non-diabetic control rats [24]. In vitro, our experiment results show that high concentration glucose can promote HO-1 expression in BMSCs, it indicates that HO-1 as an important antioxidant factor can significantly increase in the early stage when cells were stimulated, but the increase can not resist the damage caused by high glucose. On the basis of the above studies, we achieve over-expression of HO-1 in BMSCs through Copp. In addition, collagen is a kind of safe and absorbable biomaterials, so we choose a solid collagen as a carrier of stem cells.

In vivo, we covered the wound with collagen containing different groups of BMSCs. Results show that the complex of collagen and HO-1-overexpressing BMSCs is the most effective for wound closure. On day 14 after cell transplantation, fluorescent tracer results show that there are some survival BMSCs in the wound and BMSCs merged with vascular wall (shown by the arrows), which indicates that HO-1 can improve BMSCs survival in wound microenvironment, and BMSCs can differentiate into vascular endothelial cells. Furthermore, capillary density assay results show that HO-1-overexpressing BMSCs can promote capillary repair and regeneration, accelerate wound healing. According to some reports, BMSCs can affect other nearby cells though the
release of a large number of soluble growth factors and cytokines, and this process is affected by the growth conditions and the vitality of the BMSCs [25–27]. In vitro, our results also confirm that HO-1-overexpressing BMSCs have strong proliferation and paracrine ability than other treatments. These findings indicate that the over-expression of HO-1 can accelerate angiogenesis through promoting BMSCs proliferation and differentiation. Although HO-1-overexpressing BMSCs play an important role in promoting wound healing, further work should be performed to clarify whether the cells can maintain their efficacy over longer experimental periods.

VEGF is the most important pro-angiogenesis factor: it has a strong ability to promote angiogenesis, and it can also stimulate cell proliferation, delay senility, inhibit apoptosis and promote cell survival [28–30]. Our experimental results indicate that the over-expression of HO-1 can significantly promote BMSCs to secrete VEGF and the conditioned medium of HO-1-overexpressing BMSCs can significantly promote migration and proliferation of HUVECs, so we believe that HO-1-overexpressing BMSCs can promote wound healing through not only BMSCs themselves proliferation and differentiation but also promoting surrounding endothelial cell migration and proliferation, then accelerate angiogenesis and restore blood supply in the wound. In addition, previous evidence indicates that hypoxia is vital to the differentiation of MSCs into endothelial cells [31]. Under hypoxic conditions, MSCs can secrete more VEGF by autocrine process, which induces MSC differentiation. In this experiment, the microenvironment of diabetic ischemic ulcers is hypoxic and ischemic, and the viability of HO-1-overexpressing BMSCs is very good. Therefore, we can conclude that the over-expression of HO-1 can protect BMSCs from injury induced by diabetic ischemic wound microenvironment. HO-1-overexpressing BMSCs can promote angiogenesis and wound healing through the following two approaches: one is to increase proliferation and differentiation of BMSCs through autocrine activity, the other is to recruit more endothelial cells to the wound through paracrine activity.

Akt is a cytosolic protein kinase (or alternatively protein kinase B, PKB). It is closely related to VEGF–mediated angiogenesis and cell protective effects [32]. Akt signaling pathway plays an important role in the survival and apoptosis of cells. Many growth factors and survival factors can activate Akt signaling pathway. In addition, extracellular signal-regulated kinase (Erk) is a member of the MAPK family. It is the core of the signal network involved in regulating cell growth, development and division. As these two signaling pathways are very important for angiogenesis and life activity of cells, we wondered whether they were involved in the mechanism of HO-1 action. Our results show that the HO-1 can promote proliferation and paracrine action of BMSCs, the process is mainly dependent on the Akt signaling pathway and not dependent on the Erk signaling pathway.

5. Conclusion

The over-expression of HO-1 can enhance stem cell viability and paracrine activity in diabetic ischemic ulcers micro-environment, the new complex of HO-1–overexpressing BMSCs and collagen biomaterials is effective in stimulating wound cicatrization, and the process is associated with Akt signaling pathway and not dependent on the Erk signaling pathway. These preclinical findings open new perspectives for the treatment of diabetic foot ulcers.

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