

LPS-stimulated Macrophage Exosomes Inhibit Inflammation by Activating the Nrf2 / HO-1 Defense Pathway and Promote Wound Healing in Diabetic Rats

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Research

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Abstract

Background: Impaired wound healing is one of the important complications of diabetes. However, the specific pathogenesis is still unclear, and there is no effective treatment. Macrophages pretreated with chemical or biological factors may increase the biological activity of macrophage-derived exosomes, which is expected to become a new effective treatment method. The purpose of this study was to investigate whether the exosomes secreted by macrophages pretreated with lipopolysaccharide (LPS) have better anti-inflammatory and angiogenic abilities in the treatment of diabetic wound healing and their underlying molecular mechanisms.

Methods: In this study, macroscopical, biochemical, histological, immunofluorescence and molecular biology methods were used to evaluate the potential protective mechanism and effect of lipopolysaccharide stimulated macrophage exosomes (LPS-Exos) on wound healing in streptozotocin induced hyperglycemia rats. In the in vivo experiment, the percentages of wound closure and contraction were compared and analyzed on the 7th, 14th, and 21st day after treatment by grouping treatments with different concentrations of LPS-Exos. At the same time, hematoxylin and eosin staining (HE), Masson staining, immunofluorescence staining and Western blotting (WB) were used for histological analysis of the wound tissue on the 14th day after injury to evaluate the impact of different treatment methods on wound healing. In in vitro experiments, the ability of endothelial cells related to proliferation, migration, tube formation and the expression of vascular endothelial growth factor (VEGF) were tested. At the same time, in vivo and in vitro experiments, the effect of Nrf2/HO-1 signaling pathway on LPS-Exos in inhibiting inflammation and promoting angiogenesis was evaluated by using exosome-specific inhibitors.

Results: LPS-Exos reduced the content of ROS and MDA in the wound tissue of hyperglycemic rats, increased the activity of SOD and the production of GSH-Px, activated the Nrf2/HO-1 pathway, inhibited the expression of inflammation-related proteins, and promoted blood vessels generate. The group given exosome inhibitors reversed this phenomenon.

Conclusions: LPS-Exos may activate the Nrf2/HO-1 defense pathway, improve endothelial cell function, inhibit oxidative damage and inflammation, and promote wound healing in diabetic rats, thereby having the potential to treat diabetic skin defects.

Introduction

Diabetes mellitus (DM) is a chronic metabolic disease with hyperglycemia as the main manifestation, in which the impaired wound healing is one of the main complications of diabetes[1, 2]. There are many reasons for the delayed healing of diabetic wounds, including neuropathy, peripheral vascular disease and immune system damage, which ultimately lead to the prolongation of inflammatory phase, proliferation and shortening of remodeling phase of skin wounds[3]. At the same time, the production of a large number of reactive oxygen species (ROS) aggravates the inflammatory response of cells, promotes the apoptosis of injured tissues, and delays the healing of skin wounds[4]. Therefore, reducing the

production of reactive oxygen species and the continuous effect of inflammation in skin wound can be an important strategy to improve the wound healing of diabetes[5].

Exosomes are double-layer nanospheres with 40–150 nm diameter, which contain miRNAs, mRNAs and proteins and can be secreted by different cell types[4, 6]. Exosomes can enter the target cells and release the contents of the inclusions through recognition of specific antibodies on the target cell membrane, direct fusion, endocytosis, etc., and mediate information exchange between cells, so as to play their biological functions [7]. A large number of studies have shown that the exosomes secreted by macrophages stimulated by lipopolysaccharide (LPS) contain more cytokines and miRNAs, and regulate the inflammatory response by transforming early pro-inflammatory gene transcription into anti-inflammatory gene transcription[8, 9]. Meanwhile, according to reports, LPS stimulated exosomes secreted by macrophages are involved in the pathogenesis of various diseases and play a protective role by inhibiting inflammatory response, such as cerebral ischemia / reperfusion injury, pulmonary fibrosis and various metabolic diseases[10–12].

The delayed healing of diabetic wound is related to inflammation and oxidative stress[13]. Hyperglycemia in diabetes usually leads to endothelial damage, and oxygen cannot be transported to tissues through microvessels and cells[14]. A large number of inflammatory factors, such as interleukin-1 β , interleukin-6 and so on, are produced, resulting in tissue ischemia, hypoxia and collagen synthesis blocked, and delayed wound healing of diabetic skin[15]. Nrf2 is a key transcription factor in the immune system[16, 17]. It plays an important role in the regulation of cellular inflammatory response by responding to oxidative and inflammatory stress in the cell defense system. However, the relationship between LPS stimulated exosomes of macrophages and inflammation and oxidative stress in diabetic skin defects has not been elucidated[18–20]. This study aims to investigate the inhibitory effect of LPS-stimulated exosomes on inflammation and oxidative stress in diabetic skin defects through in vivo and in vitro experiments. Furthermore, the effect and potential mechanism of exosomes secreted by LPS-stimulated macrophages on wound healing in diabetic rats were revealed.

Materials And Methods

Cell culture and sample treatment

Macrophage line RAW264.7 (cat. No: tcm13; Shanghai, China) was purchased from cell bank of Chinese Academy of Sciences. RAW264.7 cells were cultured in DMEM containing 10% fetal bovine serum, 100 U / ml penicillin and 100 μ g / ml streptomycin. Culture at 37 °C with 5% CO₂ and 95% humidified atmosphere, change the medium every three days. Human umbilical vein endothelial cells (HUVECs, Shanghai Institute of cell research, Chinese Academy of Sciences) were cultured in F12 medium containing 10% FBS, 25 U / ml heparin, 2 ml glutamine, 1.5 g / L sodium bicarbonate and 100 U / L penicillin and streptomycin. Meanwhile, in order to simulate the diabetic environment in vitro, HUVECs cells were cultured in F12 medium containing 30 mm / L glucose (sigma Aldrich) for 24 hours, as previously described[6]. The cells pretreated with high glucose (HG, 30 mm / L) were used as

experimental control group. At the same time, in order to better explore the role of LPS stimulated macrophage exosomes, the experimental group used the combination of exosomes inhibitor (GW4869)[5, 21–23] and different concentrations of exosomes to treat cells.

Preparation and purification of exosomes from LPS-stimulated macrophages

The specific steps for preparing and purifying Exos and LPS-Exos in vitro are as previously described [6, 11]. When the content of RAW264.7 cells reached 90% in a 150 mm cell culture dish, the culture medium was replaced by the FBS deleted by exosomes (1 x DMEM, 10% heat inactivated FBS deleted by ultrafiltration). In order to prepare LPS-Exos, LPS (100 ng / ml) was added to the cell culture dish to stimulate the growth of RAW264.7 cells, and the culture continued for 24 hours. The supernatant of cell culture was collected by centrifuge tube, The mixture was then centrifuged at 4 ° C for 10 minutes at 2000 g and at 10000 g for 30 minutes to remove cells and debris, then filtered using a 0.22 μ M filter. Finally, the collected supernatant was transferred to a new ultracentrifuge (Hitachi, Koki, Co., Ltd, Japan), centrifuged at 4 ° C for 1.5 h at 120000 g, and then the supernatant was discarded to collect the transparent sediment at the bottom of the centrifuge tube to obtain the exosomes from LPS stimulated macrophages.

The collected exons from LPS-stimulated macrophages were examined for their morphology and shape using a transmission electron microscope (JEM 1200EX; JEOL, Tokyo, Japan). At the same time, their particle size and surface charge were measured with Zetasizer Nano Z S (Malvin Instruments, Malvin, UK). In order to discuss the identification of Exos and LPS-Exos, Western blot method was used to detect CD81, CD9, TSG101 and Alix (known exosome markers) in their components.

In Vitro Cellular Uptake Analysis

The specific steps of the cell uptake analysis of LPS-Exos using confocal laser scanning microscope (CLSM) are as described previously [4, 11]. To evaluate the targeting of LPS-Exos, HUVECs cells were first plated at a density of 1×10^5 cells per well overnight. When the cell density reaches 70% -80%, 1,1'-dioctadecyl-3,3,3', 3'-tetramethylindocarbocyanine (DiI) labeled LPS-Exos (200 μg / ml) will be incubated with HUVECs cells 4 hour. Then rinse the cells three times with PBS (3 minutes each time), remove LPS-Exos attached to the cell membrane, then add 2.5% paraformaldehyde to fix the cells for 0.5 hours, and stain the nuclei with 5 μg / mL DAPI for 20 minutes. Then use laser confocal scanning microscope to observe the cell distribution of DiI-labeled LPS-Exos.

Cell proliferation determination

Cell count kit 8 (CCK-8; dojindu, Kyushu Island, Japan) was used to detect the proliferation activity of HUVECs. First, the cells were inoculated in 96 well plates (5 repeat holes) with a density of 5×10^4 cells / hole for 24 hours, and cultured overnight in a medium containing 0.5% fetal bovine serum. The medium was replaced by serum-free medium in the presence of low LPS-Exos concentrations (LPS-Exos low, 200 μg/mL), high LPS-Exos concentrations (Exos high, 400 μg/mL), and the combination of LPS-Exos at

400 µg/mL and GW4869 at 10 µM (Exos high + GW4869), respectively. A group without cells served as the blank. After 24 hours, CCK-8 solution (10 ml per well) was added to HUVECs and cells were incubated at 37 °C for 1 hour. Finally, the quantitative detection is carried out on the micro board reader with the wavelength of 450 nm.

Cell scratch wound healing assay

The specific steps for in vitro migration through the scratch wound test are as previously described[24, 25]. Briefly, HUVECs cells are cultured in 96-well petri dishes and in a 37 °C incubator until they reach 100% confluence in a single layer. Then gently scrape the confluent cell monolayer with the pipette suction head to cover the entire diameter of the dish. After scratching, wash with culture medium for 3 times, remove the separated cells, and then supplement with fresh culture medium. The wells were then treated with LPS-Exos of different concentrations and incubated with the cells at 37 °C. After incubation for 24 hours, the image was obtained by inverted Leica fluorescence microscope and quantified by measuring the scratch healing distance.

Tube formation assay

HUVECs (5×10^4 cells per well) were inoculated on the 96 well plate coated with matrix coating. The cells were incubated in serum-free F12 medium with different concentrations of LPS-Exos or PBS at 37 °C for 4 hours, and then the formation of the tubes was examined by inverted microscope (Leica dmi6000b, Germany).

Determination of intracellular ROS accumulation

Using fluorescent marker DCFH2-DA to measure the accumulation of ROS in HUVECs treated under different conditions as previously described[26]. Briefly, HUVEC (1×10^5 cells / well) was seeded in a 6-well plate and treated with HG (30 mM) for 24 hours in the presence of different concentrations of LPS-Exos. After the treatment, the medium supernatant was removed, and the cells were washed 3 times with PBS (3 minutes at a time). Then, DCFH2-DA (10 µM) was mixed with 500 µL F12 medium and added to the culture plate.

Finally, after incubating for 30 minutes, the relative fluorescence intensity was measured at 485 / 535 nm (A485 / 535) using a fluorescence spectrophotometer (Hidex Oy).

Determination of Oxidative Stress

Wound skin tissue homogenates (intact skin around the wound on the 14th day after injury) were prepared with cold phosphate buffered saline. Glutathione peroxidase (GSH Px), malondialdehyde (MDA) and superoxide dismutase (SOD) activities were evaluated with GSH Px, SOD and MDA test kits (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China) respectively according to the manufacturer's instructions.

Diabetic skin wound animal model and treatment

In this experiment, adult female Sprague Dawley (SD) rats (180–200 g) were selected and fed under standard pathogen free conditions. All procedures are approved by the animal research committee of Jinzhou Medical University. Streptozotocin (sigma, St. Louis, Mo, USA) was intraperitoneally injected to SD rats at a dose of 80 mg / kg / body weight to induce experimental diabetes. Blood glucose level was monitored 72 hours later, and rats with blood glucose level > 300 mg / dL were selected for further study. Then the rats were anesthetized by intraperitoneal injection of pentobarbital (50 mg / kg). The back of the rats was shaved off and a round full-thickness wound with a diameter of 1.5 cm was formed on the back of the rats.

The rats were randomly divided into four groups: control group, LPS-Exos low group, LPS -Exos high group, LPS-Exos high + GW4869 group. The diabetic wound sites were treated with 1 ml PBS, 1 ml of low-concentration LPS-Exos (500 µg/mL), high-concentration LPS-Exos (1 mg/mL) and the combination of high-concentration LPS-Exos (1 mg/mL) and GW4869 (1.5 mg/kg), respectively, per wound by subcutaneous injection. The wound reduction rate was calculated by the following formula: wound reduction rate (%) = (AO at) / AO × 100, in which Ao was the initial wound area and at was the wound area at 7, 14 and 21 days after injury. On the 14th day after operation, the peripheral skin of the wound was taken for protein extraction (WB) and staining.

Histology Analysis

First, the excised tissue composed of wound bed and surrounding healthy skin on the 14th day after the wound was fixed in 4% paraformaldehyde solution for 72 hours, then dehydrated with graded alcohol series, embedded in paraffin, and cut into 4-micron slices perpendicular to the wound surface. Then hematoxylin and eosin (H & E) staining were used to evaluate the healing stage, and Masson trichrome (MT) blue staining (Solarbio) was used to study the degree of collagen deposition in the healing tissue. Finally, Leica camera (model DFC 295) was used to take tissue pictures on Leica microscope, and Image Pro Plus 6.0 analysis software was used for quantitative analysis.

Western blot (WB) analysis

HUVECs cells after 24 hours of drug treatment and intact skin around the wound of diabetic rats on the 14th day after injury were collected for cytoplasmic and nucleoprotein analysis, and Western blot was performed as previously described [6, 26]. The main antibodies used in the experiment are as follows: anti-CD81 (1:1000, abcam, cambridge, uk); anti-CD9 (1:2000, abcam, cambridge, uk); anti-TSG101 (1:1000, abcam, cambridge, uk); anti-Alix (1:1000, abcam, cambridge, uk); anti-GAPDH (1:10000, abcam, cambridge, uk);

anti-Nrf2 (1:500, abcam, cambridge, uk); anti-HO-1 (1:1000, abcam, cambridge, uk); anti-NQO-1 (1:1000, abcam, cambridge, uk); anti-IL-1β (1:1000, abcam, cambridge, uk); anti-IL-6 (1:1000, Cell Signaling Technology, Inc., Danvers, MA, USA); anti-TNF-α (1:1000, abcam, cambridge, uk); anti-VEGF (1:1000, Cell Signaling Technology, Inc.,

Danvers, MA, USA); anti-CD31 (1:500, abcam, cambridge, uk); anti-SMA (1:1000, Cell Signaling Technology, Inc., Danvers, MA, USA); anti-MMP-9 (1:500, abcam, cambridge, uk).

Finally, enhanced chemiluminescence reagents (Thermo Fisher Scientific) were used to visualize the results. ImageJ software (NIH, Bethesda, MD, USA) was used to analyze the density of protein bands.

Immunofluorescence Analysis

VEGF and SMA were stained with immunofluorescence to determine the degree of angiogenesis in granulation tissue on the 14th day after injury. In short, the slices were dewaxed in xylene and rehydrated with a gradient ethanol solution. After washing with PBS, the tissue sections were sealed in 1% BSA at room temperature for 30 minutes, incubated overnight at 4 °C, and then stored at room temperature for 1 hour with their secondary antibodies (goat rabbit or goat rat). In addition, the nuclei were stained with 2-(4-aminophenyl) - 6-indoleaminoformamide (DAPI) (Invitrogen, Carlsbad, CA, USA).

Finally, all sections were observed under the fluorescence microscope (Olympus, Hamburg, Germany). The number of vessels in different groups was determined by using Image-Pro Plus 6. software to count 4 random fields in each segment between the wound edges.

Statistical analysis

All data were expressed as mean \pm standard error of mean (SEM). Independent sample t-test was used to compare the mean between two different groups. Using GraphPad Prism software (GraphPad, La Jolla, CA, USA), one-way ANOVA was used to determine the significance level, and P value < 0.05 was considered statistically significant.

Results

Characterization of LPS-exos

First, the morphology, size, and related marker proteins of the exosomes secreted by LPS-stimulated macrophages are identified and described. The morphology of exosomes secreted by macrophages stimulated by LPS was observed by transmission electron microscopy (TEM). It was found that the exosomes had typical spherical characteristics. The size of them is analyzed and calculated, and it is found that their diameter is distributed between 110 ~ 150 nm, as shown in Fig. 1A, B. The exosomes secreted by macrophages (Exos) and the exosomes secreted by LPS stimulated macrophages (LPS-Exos) were analyzed by Western blot technology. The results showed that the particulate components of both groups of exosomes were enriched in CD81, CD9, Alix, and TSG101 (known exosomal markers), but were not detected in the supernatant, indicating that the two groups of exosomes were completely collected from the supernatant. It was also found that the exosomes secreted by LPS stimulated macrophages contained more marker protein than the exosomes secreted by macrophages, and all the features met our design requirements (Figs. 1D-G).

Effects of LPS-Exos on the angiogenic effect of vascular endothelial cells

Angiogenesis is mainly through the proliferation and migration of endothelial cells and the formation of capillary like structure, which plays an important role in diabetic wound healing. Therefore, we tested the effect of LPS-Exos on HUVECs in vitro. First of all, we determined that LPS-Exos could enter the cell through cell uptake experiments. After labeling LPS-Exos with red fluorescent dye (DIL) and incubation with HUVECs cells for 4 hours, it was found that the DIL labeled LPS-Exos was transferred to the perinuclear region of HUVECs (Fig. 2A), indicating that LPS-Exos was absorbed into HUVECs cells. Then we used CCK8 kit to test the effect of LPS-Exos with different concentrations on the proliferation of HUVECs cells. Obviously, from the experimental results, we found that the proliferation ability of cells treated with high concentration and low concentration LPS-Exos was significantly improved, and the enhancement ability was positively correlated with the concentration. However, the proliferation ability of GW4869 treated cells decreased significantly (LPS-Exos high vs. Control, $P < 0.001$; LPS-Exos high vs. LPS-Exos high + GW4869, $P < 0.001$; Fig. 2B). Then, we measured the effect of LPS-Exos on HUVECs cell migration by scratch method. The results showed that after 24 hours of LPS-Exos treatment, HUVECs cell mobility was significantly enhanced, and after high concentration of LPS-Exos treatment, HUVECs migration was further enhanced, but this phenomenon was reversed after GW4869 treatment (LPS-Exos high vs. Control, $P < 0.001$; LPS-Exos high vs. LPS-Exos high + GW4869, $P < 0.01$; Figs. 2C,D). In order to further investigate the effect of LPS-Exos on endothelial cell angiogenesis, HUVECs cells were first seeded on matrix gel and incubated with PBS or different concentrations of LPS-Exos for 4 hours. Then the total tube length of HUVECs cells after 4 hours was measured to determine the ability of HUVECs cells to form tubes under different conditions. Results as shown in Figs. 2E, F, the total tube length of LPS-Exos high group was higher than that of the control group, while that of LPS-Exos high + GW4869 group was significantly lower than that of LPS-Exos high group ($P < 0.001$). A series of experiments show that LPS-Exos is helpful to endothelial cell angiogenesis, and the ability of high dose LPS-Exos is more obvious.

LPS-Exos can inhibit oxidative damage of skin wound in diabetic rats

Through further experiments to explore the inhibitory effect of LPS-Exos on the oxidative damage of diabetic rat skin wounds. First, the effect of different concentrations of high concentration glucose on HUVECs cell viability was measured by the cck8 kit. The results showed that when the high glucose concentration was < 30 mM, the cells could proliferate normally after 24 hours of incubation with the cells. But when the concentration of high sugar increased to 50 mM, the cell activity decreased to 24% (Fig. 3A). Through in vitro experiments, according to the manufacturer's plan, using DCFDA cell active oxygen detection kit (Abcam, Cambridge, MA, USA) staining for exogenous reactive oxygen species. Simply put, first, HUVECs cells were exposed to 30 mM high glucose and incubated together for 24 hours under different concentrations of LPS-Exos treatment. Then, DCFH2-DA (10 μ M) was mixed with 500 μ L F12 medium and added to the culture plate, and incubated for another 30 minutes. Finally, use

fluorescence spectrophotometer to analyze the generation of ROS. It can be found that the active oxygen content of the LPS-Exos group decreased significantly ($P < 0.001$, Figs. 3B, C). Then, the wound skin tissue homogenate was prepared with cold phosphate buffer solution, and the content of superoxide dismutase (SOD) and malondialdehyde (MDA) and the activity of glutathione peroxidase (GSH-PX) were detected. The results showed that compared with the control group, the GSH-PX activity and SOD content in the wound skin tissue of the LPS-Exos high group were significantly increased ($P < 0.001$). The LPS-Exos high + GW4869 group was lower than the LPS-Exos high Group ($P < 0.05$, Figs. 3D, F). Compared with the control group, the level of MDA in the LPS-Exos high group decreased ($P < 0.001$), and the LPS-Exos high + GW4869 group was higher than the LPS-Exos high group ($P < 0.05$, Fig. 3E).

LPS-Exos can inhibit the high glucose induced inflammatory response of HUVECs cells through Nrf2 / HO-1 pathway

In order to further explore the mechanism of LPS-Exos regulating endothelial cell function, western blot was used to detect some proteins related to inflammation and angiogenesis. We first examined the effects of different concentrations of LPS-Exos on the expression levels of Nrf2, HO-1, NQO-1 and inflammation-related proteins expressed in HUVECs cells after a period of high glucose (30 mM) treatment. The analysis of the results shows that the LPS-Exos high group can significantly increase the expression of nuclear Nrf2, HO-1, NQO-1 related proteins compared with the control group. Interestingly, this phenomenon was reversed in the GW4869 treatment group. These results indicate that the anti-inflammatory and antioxidant effects of LPS-Exos on the wound surface of diabetic rats are related to the activation of Nrf2 / HO-1 pathway. At the same time, the results also showed that under different concentrations of LPS-Exos treatment, inflammation-related proteins also decreased, and the expression of vascular endothelial growth factor (VEGF) increased (Figs. 4A-I). As shown in Figs. 4J-K, on this basis, we performed double-staining of VEGF immunofluorescence on HUVECs cells treated with different concentrations of LPS-Exos. The results showed that compared with the control group, the LPS-Exos high group significantly increased the proportion of VEGF-positive cells ($P < 0.001$). At the same time, the proportion of VEGF-positive cells in the LPS-Exos high + GW4869 group was lower than that in the LPS-Exos high group ($P < 0.001$). These results indicate that LPS-Exos can inhibit inflammation and promote endothelial cell angiogenesis through the Nrf2 / HO-1 pathway in vitro.

LPS-Exos promotes STZ induced skin wound healing in diabetic rats

In order to evaluate the effect of LPS-Exos on wound healing in diabetic rats, we first established a diabetic skin defect rat model through in vivo experiments, then subcutaneously injected different concentrations of LPS-Exos for 21 consecutive days, and then evaluated each group of rats by calculation. The wounds healed on the 7th, 14th, and 21st days after injury. What is interesting is that the experimental results show that, compared with the control group, the wound healing rate of the LPS-Exos treatment group with different concentrations is generally higher, and the wound healing rate is positively correlated with the concentration of LPS-Exos. These experimental results fully prove that LPS-Exos can

promote wound healing in diabetic rats (Figs. 5A, B). As shown in Figs. 3C-F, next, we took the wound skin tissue on the 14th day after wounding for HE staining and Masson staining, and evaluated the degree of wound healing and regeneration by analyzing the healing length and collagen formation of each group of wounds. According to the analysis, the wound healing degree of the LPS-Exos high group was higher than that of the control group ($P < 0.001$), but the LPS-Exos high + GW4869 group reversed this phenomenon compared with the LPS-Exos high ($P < 0.05$). All the results show that the treatment of LPS-Exos can accelerate the wound healing of diabetic rats.

LPS-Exos inhibits skin wound inflammation in diabetic rats by activating Nrf2 / HO-1 pathway

Through appealing a series of in vivo experiments, we found that LPS-Exos can indeed promote wound healing in diabetic rats. Combined with in vitro experiments, we speculate that this therapeutic effect may be related to the anti-inflammatory and antioxidant effects of LPS-Exos. In order to ascertain the specific mechanism, we carried out the following experiments. As shown in Figs. 6A-I, First, after the successful establishment of the skin defect model of diabetic rats, we treated each group of rats with different concentrations of LPS-Exos once a day for 14 consecutive days. Then, the skin wound tissues of each group were taken for Western blot analysis. The results showed that compared with the control group, Nrf2, HO-1 and NQO-1 related proteins increased, IL-6, IL-1 β , TNF - α decreases. However, it is interesting that the LPS-Exos high + GW4869 group reversed this phenomenon compared to the LPS-Exos high group. At the same time, we found that LPS-Exos could also reduce the protein expression of MMP-9, which was lower in LPS-Exos high group compared with the control group ($P < 0.001$), and higher in LPS-Exos high + gw4869 group compared with LPS-Exos high group ($P < 0.001$). These results indicate that LPS-Exos can reduce the expression of inflammatory protein and MMP-9 in local diabetic wound by activating Nrf2 / HO-1 pathway, thus accelerating the healing process of diabetic wound.

LPS-Exos promotes angiogenesis in the wound site of diabetic rats

Angiogenesis plays a key role in wound healing. In order to explore whether LPS-Exos can promote the formation of new blood vessels in the skin defects of diabetic rats, we conducted the following experiments. First, we extracted the wound tissue from the rats that had been successfully modeled and received treatment for 14 days, and performed Western blot analysis and tissue fluorescence double staining. As shown in Figs. 7A-D, first, we analyzed from Western blot results that after treatment with different concentrations of LPS-Exos, angiogenesis-related proteins such as CD31, VEGF, and SMA all increased to varying degrees. Compared with the control group, the LPS-Exos high group was significantly higher ($P < 0.001$), but the LPS-Exos high + GW4869 group was lower than the LPS-Exos high group ($P < 0.05$). Immediately afterwards, we stained VEGF and SMA by immunofluorescence double staining technique. The analysis of the staining results showed that LPS-Exos treatment can significantly increase the number of wound blood vessels, and the increase rate is positively correlated with the concentration of LPS-Exos, but in The GW4869 treatment team did reverse this phenomenon (Figs. 7G,

H).In conclusion, from the above results, LPS-Exos can promote angiogenesis of wounds in diabetic skin defects rats and promote skin wound healing.

Discussion

In this experiment, we found that lipopolysaccharide stimulated exosomes secreted by macrophages (LPS-Exos) can promote the healing of diabetic rats' skin wounds, and further verified that LPS-Exos can inhibit the inflammation and oxidative stress of diabetic rats' skin wounds through in vitro and in vivo experiments. Chronic inflammation and oxidative stress play an important role in the pathogenesis of diabetes mellitus[27]. Previous studies have shown that exosomes do have a protective effect on skin defects in diabetic rats[28, 29]. At the same time, our experimental team found that LPS-Exos had superior anti-inflammatory effect in the disease of cerebral ischemia-reperfusion[11]. Therefore, we speculate whether LPS-Exos has a better inhibitory effect on chronic inflammation and oxidative stress of diabetic skin wounds. Fortunately, we have proved through a series of experiments in vivo and in vitro that LPS-Exos can inhibit inflammation and accelerate wound healing in diabetic rats by activating Nrf2 / HO-1 pathway.

In the current treatment, the wound healing of diabetes can not be improved[30–32]. After the trauma, it will lead to a series of diseases, leading to chronic inflammation and the production of a large number of reactive oxygen species[33, 34]. At the same time, it will lead to a large number of endothelial cell apoptosis and collagen synthesis reduction, and the formation of blood vessels is blocked[4]. So we are eager to find a new treatment. At the same time, a large number of studies show that exosomes secreted by macrophages have positive protective effects in various diseases[35–37]. For example, Takeda Y et al reported that exosomes secreted by macrophages in pancreatic cancer can inhibit the proliferation of cancer cells and the development of pancreatic cancer[1, 38]. Gebraad A et al. Reported that exosomes secreted by macrophages can promote the differentiation of stem cells into osteoblasts[39]. Fortunately, on the basis of previous studies, our experimental team found that lipopolysaccharide stimulated exosomes secreted by macrophages can induce microglia polarization and inhibit the development of inflammation in cerebral ischemia-reperfusion disease[11]. It has also been reported that the exosomes stimulated by LPS contain more anti-inflammatory factors than those secreted by macrophages[8, 12]. These provide the possibility for LPS-Exos to promote wound healing in diabetic rats by inhibiting inflammation.

Inflammation plays an important role in the pathogenesis of diabetic skin defects. It is mainly due to the damage of the immune system in the wound of diabetes mellitus, and the increase of a large number of pro-inflammatory cytokines, such as TNF- α , IL-1 β , IL-6[40]. At the same time, all kinds of matrix metalloproteinases (such as MMP-9) also increased[41].

This will cause the collagen synthesis of skin wounds to be blocked, at the same time hinder endothelial cell angiogenesis, limiting the effect of wound closure[42]. In this experiment, through in vivo and in vitro experiments, we found that after the treatment of different concentrations of LPS-Exos in diabetic rats,

the inflammatory factors in the area of skin injury decreased in varying degrees, and promoted the vascular formation ability of endothelial cells, and increased collagen synthesis, which indicated that LPS-Exos had anti-inflammatory effect in the skin wound of diabetic rats.

Reactive oxygen species (ROS) are produced in large quantities in skin wounds due to chronic inflammation, which will further aggravate the inflammatory response and toxic effects of cells[43]. The reduction of antioxidant active substances such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) and the increase of free radicals can all lead to delayed healing of diabetic wounds[44]. This is consistent with our experimental results. Through extracorporeal experiments, we can find that LPS-Exos can significantly reduce the reactive oxygen species induced by endothelial cells under high glucose treatment. At the same time, different concentrations of LPS-Exos can increase the antioxidant substances in the wounds of diabetic rats to different degrees. This indicates that LPS-Exos can inhibit the production of ROS in diabetic skin wounds, and at the same time inhibit oxidative damage to promote skin wound healing.

Nrf2 (NF-E2-related factor) is an important transcription factor that maintains redox homeostasis in cells, participates in the development of various diseases, and plays an important role in the regulation of oxidative stress[16, 42, 45]. At the same time, related reports indicate that Nrf2 is involved in the proliferation, differentiation, migration and apoptosis of various types of cells[5, 46]. The exosomes of adipose-derived stem cells that highly express Nrf2 can promote the healing of diabetic foot ulcer skin wounds in rats[47, 48]. In vivo and in vitro experiments, we found that LPS-Exos can activate Nrf2 / HO-1 pathway-related proteins, while reducing inflammation-related proteins, promoting endothelial cell vascularization and collagen synthesis. Interestingly, after treatment with exosome-specific inhibitors (GW4869), the symptoms improved by LPS-Exos were all reversed. The above results indicate that LPS-Exos can play its therapeutic role in diabetic skin defect rats by activating the Nrf2 / HO-1 pathway.

Objectively speaking, this experiment has certain limitations in studying the mechanism of LPS-Exos in promoting wound healing in diabetic rats. The role of LPS-Exos in the body may be affected by a variety of cytokines and pathways. At the same time, further In clinical trials, we should consider the differences in pathology and physiology between human and animal models. However, from the other side, we verified that LPS-Exos can inhibit inflammation and promote wound healing in diabetic rats by activating the Nrf2 / HO-1 pathway. It provides a new treatment strategy for the treatment of diabetic wound healing.

Conclusion

In summary, our research shows that LPS stimulates exosomes secreted by macrophages can improve endothelial cell function, activate Nrf2/HO-1 signaling pathway, inhibit oxidative damage and inflammation, and promote angiogenesis and diabetic rat wounds heal. In short, our experimental results provide a new treatment strategy for the treatment of diabetic wound healing disorders.

Abbreviations

LPS

Lipopolysaccharide;STZ:Streptozotocin;Exos:Exosomes secreted by macrophages;

LPS-Exos

LPS stimulates exosomes secreted by macrophages;SOD:Superoxide dismutase;

MDA

Malondialdehyde;ROS:Reactive oxygen species;GSH-Px:Glutathione peroxidase;VEGF:Vascular endothelial growth factor;SMA:Smooth muscle actin;MMP-9:Matrix Metalloproteinase-9;HUVEC:Human umbilical vein endothelial cells;TEM:Transmission electron microscope

Declarations

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Authors' contributions

Daoyong Li and Chao Wu mainly conceived, designed and wrote the manuscript. Liang Mao and He Tian mainly participated in the collection and analysis of experimental data. Zhanshan Gao and Nan Xia collected samples and analyzed the data. Xifan Mei and Chang Liu conceived and designed the entire experiment, and provided financial support, and finally approved the manuscript.

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Availability of data and materials

All data and analysis supporting this research are included in this article.

Ethics approval and consent to participate

All animal experiments were approved by the Animal Research Committee of Jinzhou Medical University.

Consent for publication

Not applicable.

Competing interests

In this experiment all authors declare that they have no competing interests.

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Figures

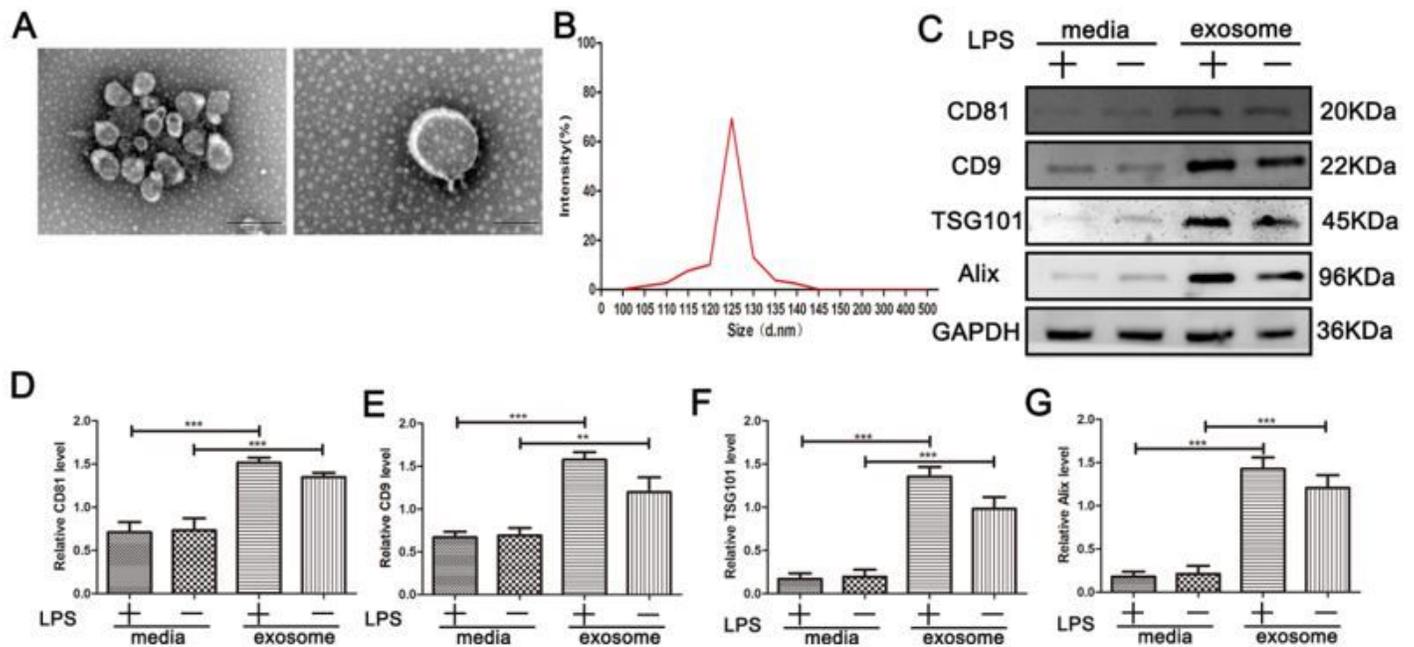


Figure 1

Characterization of LPS-Exos. (A) The ultrastructure of LPS-Exos was observed by transmission electron microscopy. Scale bar, 100 nm. (B) Particle size and distribution analysis of LPS-Exos. (C-G) Western blotting was used to detect the protein expression of LPS exos markers CD81, CD9, TSG101 and Alix. The data are expressed as means \pm SD, ** $P < 0.01$, *** $P < 0.001$.

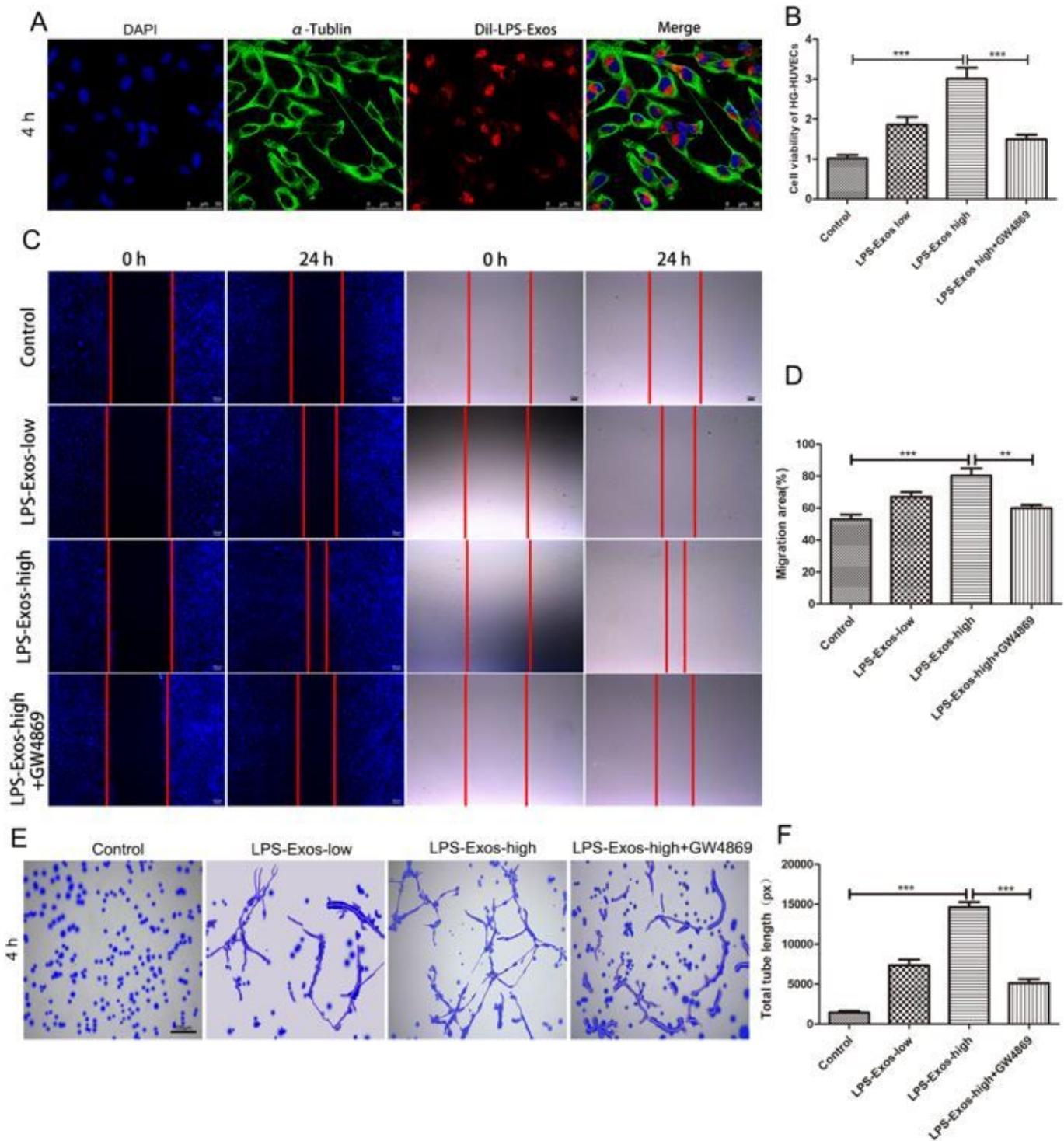


Figure 2

Pro-angiogenic effects of LPS-Exos on endothelial cells. (A) The uptake of DiI-LPS-Exos by HUVECs in vitro was analyzed by fluorescence microscopy. Scale bar: 50 μ m. (B) The CCK-8 method was used to analyze the effect of LPS-Exos treatment at different concentrations for 24 hours on HUVECs cell viability. (C,D) The migration ability of HUVECs treated with LPS exos of different concentrations was analyzed by cell scratch test. Scale bar: 100 μ m. (E,F) Tube formation experiments were used to detect

changes in the tube-forming ability of HUVECs cells treated with different concentrations of LPS-Exos after 4 hours. Scale bar: 100µm. Data are expressed as means ± SD, **p < 0.01, ***p < 0.001.

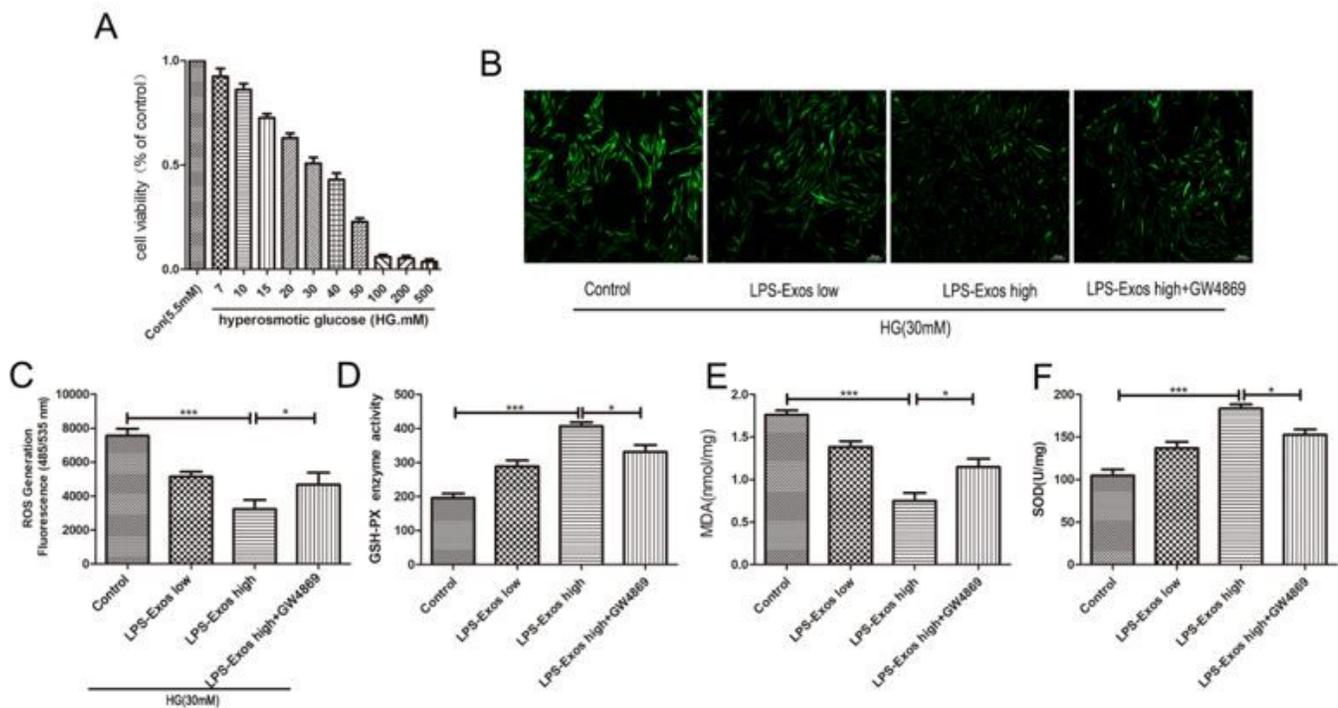


Figure 3

Effects of different concentrations of LPS-Exos on oxidative damage in diabetic skin defect rats. (A) The CCK8 kit was used to detect the effect of different concentrations of high glucose on HUVECs cell viability. (B,C) The ROS kit was used to detect the inhibitory effect of different concentrations of LPS-Exos on reactive oxygen species induced by high glucose in HUVECs cells. The formation of ROS in the LPS-Exos group was significantly inhibited. (D-F) GSH-PX activity, SOD and MDA content in the skin tissue of diabetic skin defect rats in each group. Data are expressed as means ± SD, *p < 0.05, ***p < 0.001.

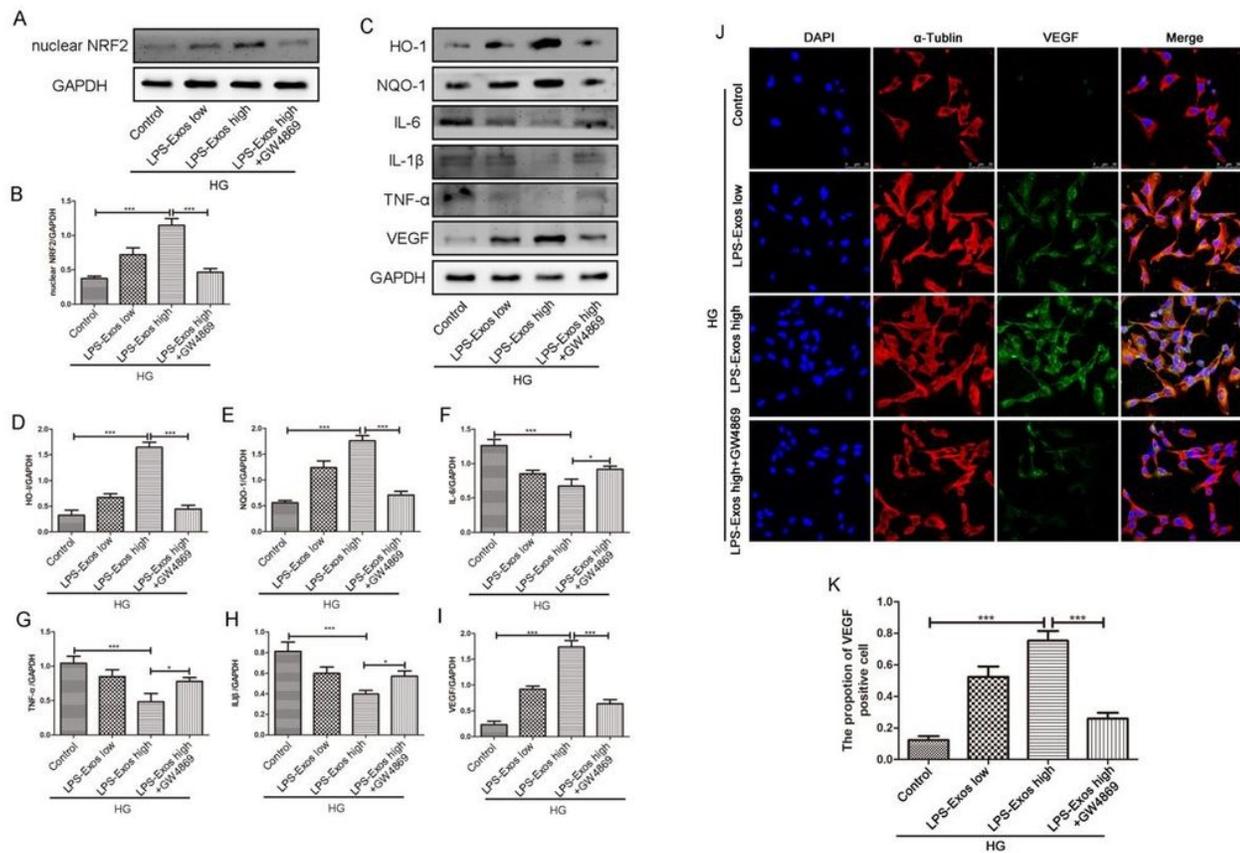


Figure 4

LPS-Exos can inhibit the inflammation of HUVECs cells through the Nrf2 / HO-1 pathway. (A-I) The protein expression of Nrf2 antioxidant-related protein, inflammation-related protein and VEGF in HUVECs cells of different groups treated with different concentrations of LPS-Exos for 24 hours was detected by Western blot. (J-K) Immunofluorescence double staining method was used to detect the expression of VEGF positive cells in HUVECs cells. Compared with the control group, the expression of VEGF-positive cells in the LPS-Exos high group was significantly increased, but the expression of VEGF-positive cells in the LPS-Exos high + GW4869 group was reduced compared with the LPS-Exos high group. Data are expressed as means \pm SD, ***p < 0.001.

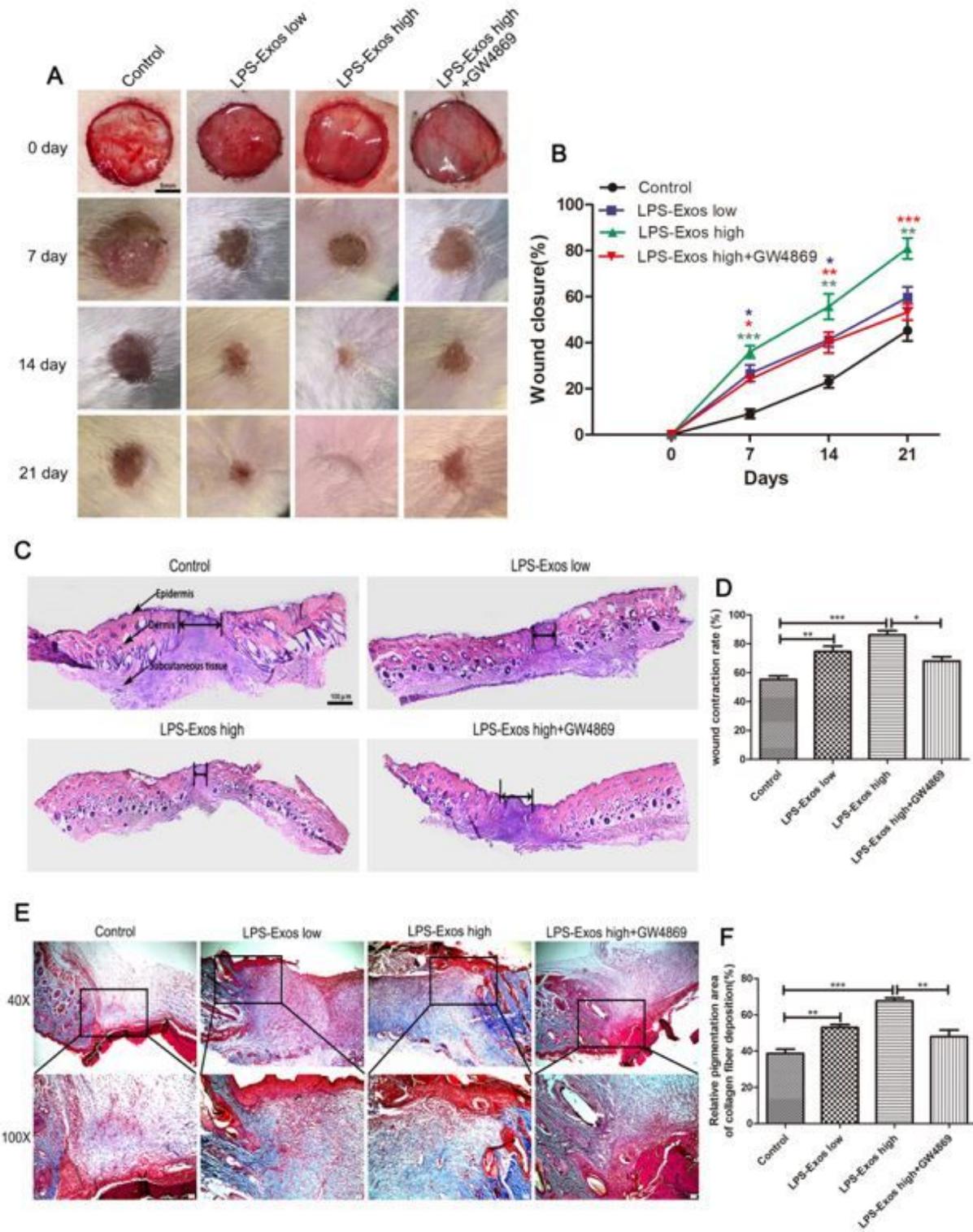


Figure 5

LPS-Exos can promote skin wound healing in diabetic rats. (A,B) Statistical analysis of wound healing of rats in different groups under different concentrations of LPS-Exos treatment at 0, 7, 14, 21 days after trauma. Scale bar: 5mm. (C, D) After 14 days of treatment with different concentrations of LPS-Exos, HE staining and statistical analysis of the wound tissues of rats in each group. The double-headed arrow indicates the edge of the scar. Scale bar: 100 μ m. (E, F) The wound tissues of rats in each group were

stained with Masson 14 days after injury, and the collagen formation in each group was statistically analyzed. Scale bar: 100 μ m. Data are expressed as means \pm SD, * p < 0.05, ** p < 0.01, *** p < 0.001.

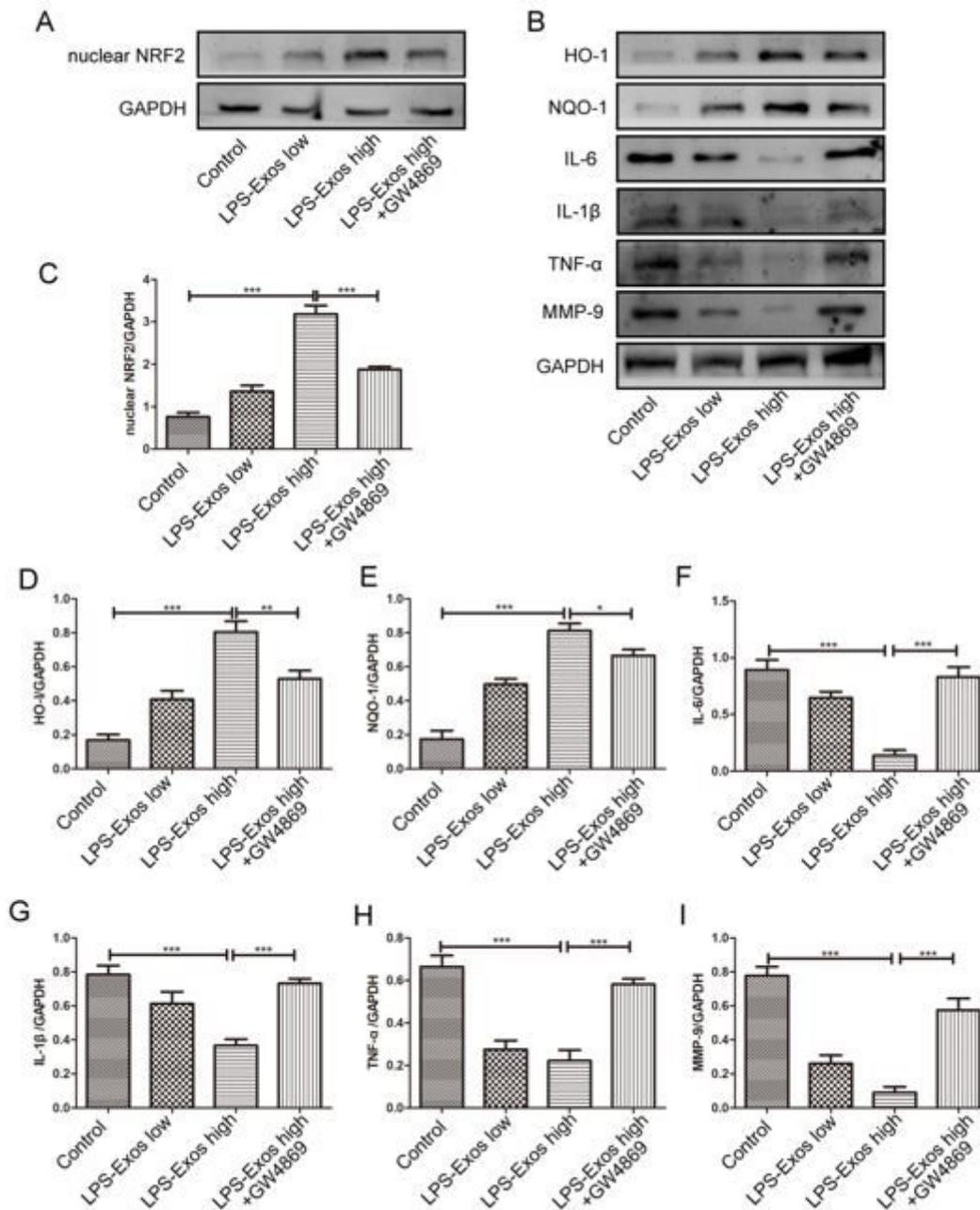


Figure 6

LPS-Exos can inhibit the inflammatory response in the skin wounds of diabetic rats through the Nrf2 / HO-1 pathway. (A-I) Western blotting was used to detect Nrf2, HO-1, NQO-1, IL-6, IL-1 β , TNF- α , MMP-9 and other related proteins in skin wound tissues of diabetic rats 14 days after skin defect expression. Data are expressed as means \pm SD, * p < 0.05, ** p < 0.01, *** p < 0.001.

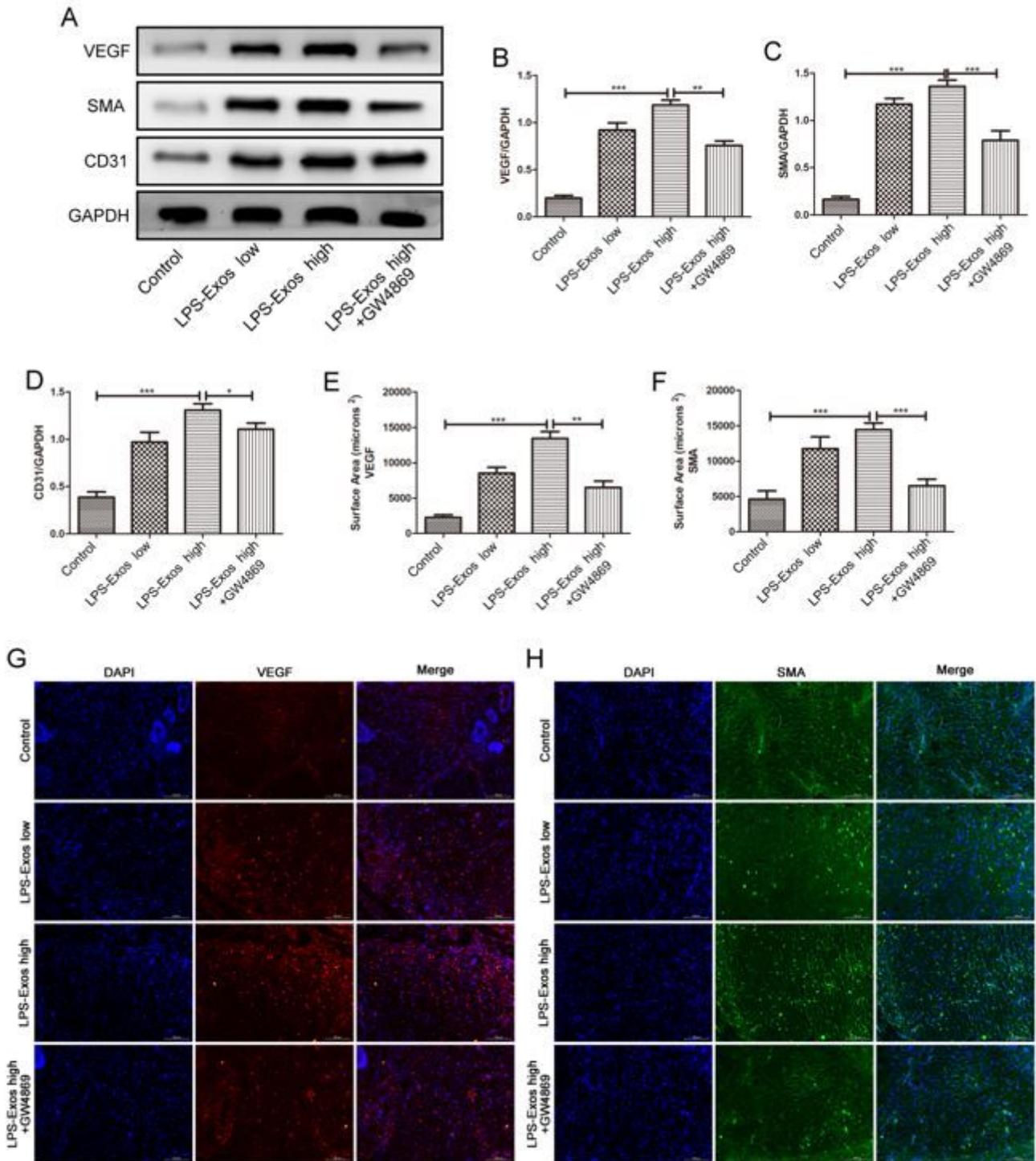


Figure 7

LPS-Exos promotes the formation of new blood vessels in the wound site of diabetic rats. (A-D) Western blotting was used to detect the expression of SMA, CD31, VEGF and other angiogenesis-related proteins in the skin wound tissues of diabetic rats 14 days after the skin defect. (E-H) Immunofluorescence staining was used to detect the expression of VEGF and SMA protein in the skin wound tissues of diabetic rats 14 days after the skin defect. Compared with the control group, the expression of VEGF and

SMA protein was increased in the LPS-Exos high group, and the expression of VEGF and SMA protein was decreased in the LPS-Exos high + GW4869 group compared with the LPS-Exos high group. Data are expressed as means \pm SD, *p < 0.05, **p < 0.01, ***p < 0.001.

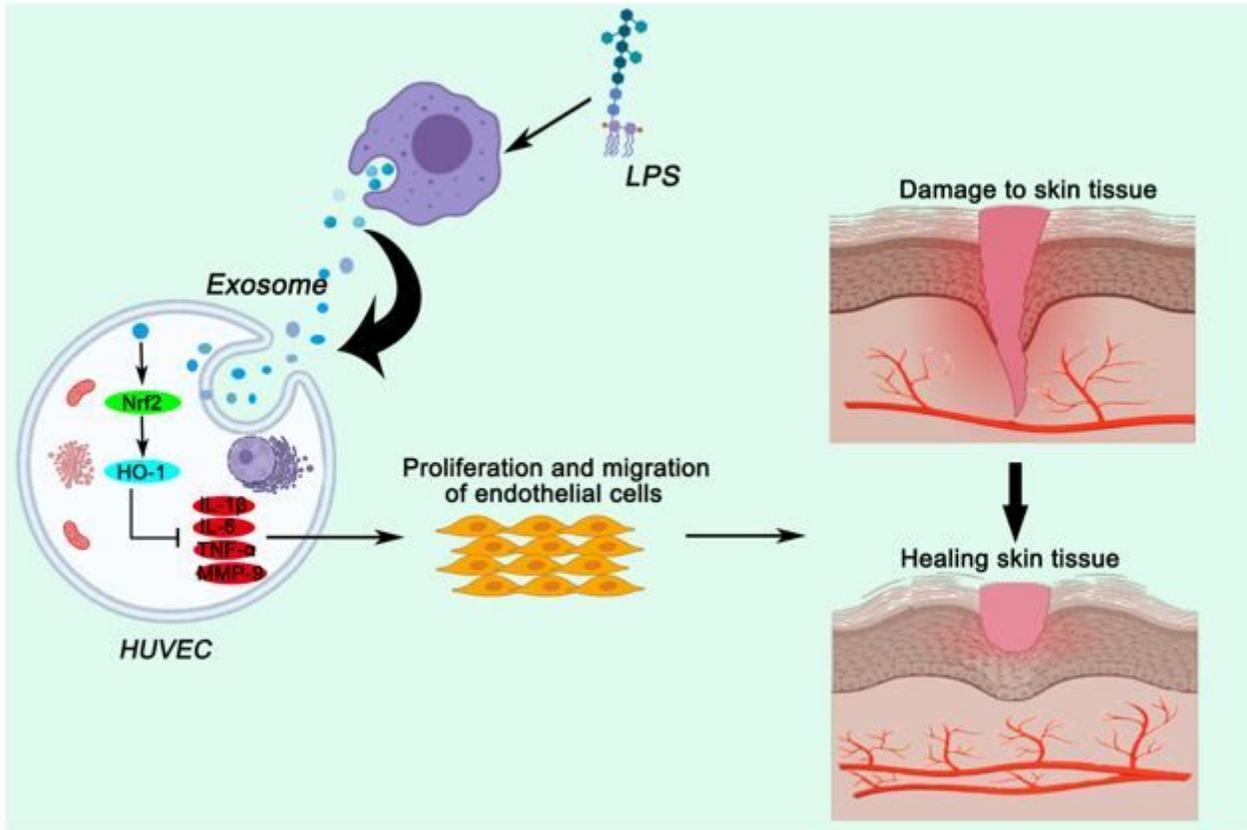


Figure 8

The schematic diagram shows that LPS-Exos promotes wound healing in diabetic rats by activating the Nrf2/HO-1 defense pathway.