Bone marrow mesenchymal stem cell-derived exosome accelerate diabetic rat's wound healing through inhibiting pyroptosis

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Abstract

Diabetic wounds are a type of wound that is characterized by protracted and refractory. In recent years, the use of mesenchymal stem cell-derived exosome(MSC-Exo)in wound treatment has made certain progress, especially bone marrow mesenchymal stem cell exosome (BMSC-Exo also have get achievement in wound treatment, and this paper aims to study whether bone marrow mesenchymal stem cell-derived exosomes can promote the healing of diabetic wound and its mechanism. We conducted in vivo and in vitro experiments to verify whether BMSC-Exo can promote wound healing and promote the proliferation and migration of fibroblasts after high glucose treatment in diabetic rats. The expression of NLRP3, IL-18, IL-1β, caspase-1,and GSDMD proteins in rats and cells in each group was detected to verify the relationship between the mechanism of BMSC-Exo promoting wound healing and cell pyroptosis. The results showed that BMSCs-Exo can significantly improve the wound healing rate in diabetic rats and promote the proliferation and migration of fibroblast under high glucose conditions. At the same time, we confirmed that the pyroptosis in diabetic rat wound and fibroblast in high sugar was significantly increased, and BMSCs-Exo could significantly inhibit the pyroptosis in diabetic rat wound and fibroblast. These results suggest that BMSC-Exo inhibits cell pyroptosis through NLRP3/ caspase-1/GSDMD pathway in vivo and in vitro, and promotes wound healing in diabetic rats.

Introduction

Diabetic foot refers to foot infection, ulceration, or deep tissue rupture associated with local nerve abnormalities and/or peripheral vascular disease of the lower extremity in diabetic patients. According to the findings of the International Diabetes Federation, the global prevalence of diabetes reached approximately 451 million individuals in 2017, with projections estimating a rise to 693 million by 2045 [1]. Based on an epidemiological survey conducted in China in 2013, adult type 2 diabetes exhibited a prevalence rate of 10.9% [2]. Diabetic foot represents one of the prevalent chronic complications accompanying diabetes, with statistical data indicating a lifetime probability ranging from 15% to 25% among diabetic patients [3]. Shockingly, every twenty seconds witnesses an individual losing their lower limb due to diabetic foot worldwide [4]. Furthermore, individuals suffering from diabetic foot ulcers face a staggering five-year risk of mortality that is two and a half times higher compared to those without such ulcers [5]. Consequently, research pertaining to diabetic foot treatment has become increasingly urgent and remains at the forefront of academic investigation.

One of the reasons for the formation of diabetic ulcers is that the stimulation of long-term high sugar environment leads to the change of cell function and thus affects wound healing. Fibroblasts are important wound repair cells, and the proliferation of fibroblasts in diabetic patients is significantly inhibited. Because the exosomes of various stem cells have rich biological functions similar to those of MSCs, and have their own unique advantages, they have become a research hotspot in recent years. BMSCs are multilineage progenitors with self-renewal, multidirectional differentiation, and pleiotropic paracrine functions [22]. BMSCs can repair damaged tissues and are employed in many different

regenerative therapies. Purified BMSC-derived exosomes (BMSC-exos) have more specific distinct benefits in damaged tissue repair than BMSCs themselves, including superior stability, tissue permeability, excellent biocompatibility, and immunomodulatory properties. Whether BMSC-Exo promotes the healing of diabetic wounds is one of the objectives of our study. Pyroptosis is closely related to inflammation. Diabetic wounds are chronic inflammatory wounds, so the relationship between pyroptosis and diabetic wounds needs to be further verified. Another purpose of this study is to confirm whether pyroptosis occurs on diabetic wounds through in vivo and in vitro experiments, and to explain the relationship between the promotion effect of BMSC-Exo on diabetic wound healing and pyroptosis. It provides a new idea for the treatment of diabetic wound.

Materials & Methods

Ethic statement

All the animal experiments were conducted in conformity with a method approved by the Ethics Committee of Gansu Provincial Hospital (Ethical Number: 2023-737).

Isolation and identification of BMSC-Exo

Extraction of exosomes by precipitation method. The supernatant of fresh rat bone marrow mesenchymal stem cells was removed into a new sterile enzyme-free 15 ml centrifuge tube, samples were centrifuged at 2000×g for 30min at 4℃ to remove cells and debris, and the samples were filtered by 0.22μm filter membrane. The supernatant sample was transferred to a new 15ml centrifuge tube and left to rest on the ice. Add 0.5 times the volume of exosome precipitation reagent (cell supernatant sample) to the sample, and mix thoroughly with pipette. The sample was rotated and mixed at 2-8℃ for 30min, then the sample was centrifuged at 10000×g for 10min at 25℃, the supernatant was removed carefully. Exosome samples were precipitated with 100μl of 1×PBS and stored in a -80℃ refrigerator. The presence of BMSC-Exo was detected by transmission electron microscopy and particle size was assessed by nanoparticle tracking analysis (NTA). The surface markers CD9, CD63 and CD81 were analyzed by western blot analysis.

The internalization of BMSC-Exo in fibroblasts

PKH26 (Mao-kang Biotechnology Co., LTD, China) was prepared into 100× mother liquor, 50μl exosome solution was taken, and 5μl PKH26 dye solution was added. After the reaction at 37℃ for 2 hours, 100μl 1mg/ml BSA protein was added to terminate the reaction, and exosomes were extracted. Exosomal protein quantification to 1mg/ml. The extracted exosomes were transfected according to the amount of 2ml cells adding 5μl exosomes. The nucleus was re-stained with DAPI and the cells were stained with Phalloidin (Yeasen, China). Observed by fluorescence microscope.

Diabetic model induction

The male SD rats weighing between 100–150g were purchased and bred in Laboratory Animal Center of Lanzhou University Medical School, Gansu, China. A week was spent for rats to adapt to their new surroundings. After adaptation, they were initially fed with a high-fat diet (Dingguo Changsheng Biotechnology Co., LTD, China) for 8 weeks, followed by intraperitoneal injection with STZ (Sigma-Aldrich, China) solution (140mg/kg, dissolved in 0.1 M sodium citrate buffer). Following the injection, the rats were given an HF diet for an additional four weeks. The level of blood glucose (BG) was measured from the mice's tail tip. Once the levels of BG greater than 11.2 mmol/L were achieved, the development of the diabetic mice model was judged effective.

Animal experiments

54 male SD rats were randomly divided into 3 groups: Normal control group (Control), diabetes model + normal saline group (DM+NS), and diabetes model+ exosome group (DM+Exo). After the successful modeling of diabetes mellitus, full-thickness excisional wound measuring 2cm×2cm were constructed on the back of rats in each group, and normal saline was injected into the wounds of Control group and DM+NS group, and exosomes were injected into the wounds of DM+Exo group. The appearance of the wound was observed on the 3rd, 5th and 7th day and take photos. Wound healing rate was calculated using ImageJ. Wound healing rate = (original wound area - open wound area)/original wound area ×100%. Edges of wound margin were cut at the 3rd, 5th and 7th day respectively and stored at -80℃ refrigerator.

Apoptosis assays

Precool PBS at 4℃ and dilute the Binding Buffer appropriately for use. The cells in the six-well plate were washed once with PBS, and 400μL 0.25% pancreatic enzyme (Genom, China) digestion cells were added. When the cells became round and some cells fell off, the digestion was terminated by adding complete medium. Collect in 1.5mLEP tube, centrifuge 300g for 5 minutes, discard supernatant. Add 1mL PBS to re-suspend the cells, centrifuge 300g for 5 minutes, discard the supernatant. The precipitates were re-suspended with 200μL Binding Buffer. Add 5μL Annexin V-FITC (SUNGENE BIOTECH, China), mix and incubate away from light for 10 minutes, add 5μL PI and mix and incubate away from light for 5 minutes.

Cell proliferation assay

The skin fibroblasts in the logarithmic growth phase were digested with trypsin and prepared into a cell suspension with a concentration of 1×10^5 cells /ml, and 10,000 cells/Wells were inoculated on the 96-well plate. The medium was divided into 3 groups: fibroblast (Control) group, fibroblast + hyperglycemia (Hy) group and fibroblast + hyperglycemia + exosome (Hy+Exo) group. At 48h, 10μLCCK-8 solution was added to each well, and after incubation in the incubator for 2 h, the light absorption value was determined at 450 nm. The outcomes are articulated as the mean ± standard deviation from 3 separate experiments. from 3 separate experiments.

Cell scratch assay

Use a marker pen to draw evenly spaced parallel straight lines behind the 6-hole plate, approximately 0.5-1cm apart, crossing each hole with five lines. Each well should be supplemented with 2mL containing about 5×10^5 cells. Once the cells have covered the bottom of the pores, use a yellow pipette tip to create a straight line by scratching through the single layer of fibroblasts, followed by cleaning the cells with PBS. The culture medium was divided into three groups: fibroblast (Control) group, fibroblast + hyperglycemia (Hy) group, and fibroblast + hyperglycemia + exosome (Hy+Exo) group, which were then continued to be cultured at 37℃. Photographs of scratch wounds were taken at both 0 and 24 hours, and ImageJ software was used for analyzing the area of scratches.

Protein extraction and Western blot

Extraction of total proteins from the wound edge tissue and skin fIbroblast was done using Radio immune-precipitation assay buffer (RIPA) buffer (Aspen Biotechnology, China) as per the protocol stipulated by the manufacturer. The protein concentration was ascertained utilizing the BCA protein assay kit (Aspen Biotechnology, China). Extracted proteins (40 μg) were isolated by a 10 percent SDS polyacrylamide gel (Aspen Biotechnology, China). The membrane was blocked in TBS buffer containing 5% buttermilk for 1 h and incubated with primary antibodies at 4 ° C overnight. The membrane was washed 3 times with TBST and incubated with the corresponding reactive second antibody for 30 minutes. Wash four times with TBST on a shaker at 25° C for 5 minutes each time. Protein visualization was done by ECL kit (Aspen Biotechnology, China). The AlphaEaseFC software processing system analyzes the optical density value of the target strip. Antibodies information is in Table 1.

Table 1. Antibodies information.

Name of antibody	Antibody dilution	Company	Catalogue number
GSDMD-N	1:1000	abclonal	A22523
Cleaved caspase-1	1:500	affbiotech	AF4005
IL-1β	1:2000	Proteintech Group	26048-1-AP
IL-18	1:1000	abcam	ab191860
NLRP3	1:1000	Proteintech Group	68102-1-Ig
CD9	1:1000	Proteintech Group	20597-1-AP
CD63	1:500	affbiotech	AF5117
CD81	1:1000	abcam	ab109201

Electron microscope

The tissues of each group were fixed overnight with 2.5% glutaraldehyde solution at 4 ° C, cleaned with PBS 4 times, standing for 15min each time, dehydrated with 30%, 50%, 70%, 80% and 90% ethanol gradient for 15min each, then dehydrated with 100% ethanol 3 times, standing at 4 ° C for 20min each

time, and the samples were dried with CO2 critical point dryer. The sample was glued to the sample table with conductive tape, and 10nm gold film was plated with ion sputtering instrument. The samples were observed under scanning electron microscope, and the images were collected and analyzed.

Results

Isolation and identification of BMSC-Exo

NTA was used to determine the size of exosomes, which are 50-250nm in diameter (Fig. 1A). The main morphology of exosomes is elliptical or cupped as measured by transmission electron microscopy (Fig. 1B). Western blot was used to detect the positive markers CD9,CD63 and CD81 of exosomes in BMSCs-Exo (Fig. 1C).

The internalization of BMSC-Exo in fibroblasts

The dermal cytoskeleton was green after Phalloidin cyclic peptide staining, and the nucleus was blue after DAPI staining. After co-culture of PKH67-labeled BMSCs-Exo (red) with skin fibroblasts for 24 h, the fibroblasts showed red fluorescence around the nucleus (Fig. 2). This suggests that BMSCs-Exo was ingested into fibroblasts.

Animal experiments

It can be seen from the wound photos of each group of rats that the wound healing rate of DM + NS group was significantly lower than that of Control group, while that of DM + Exo group was significantly higher than that of DM + NS group (Fig. 3), indicating that the wound healing rate of diabetic rats was significantly reduced, and BMSCs-Exo could significantly promote the wound healing of diabetic rats. It was observed that the expressions of NLRP3, IL-18, IL-1β, caspase-1 and GSDMD proteins in wound tissue of DM + NS group were significantly higher than those in Control group, while the expressions of NLRP3, IL-18, IL-1β, caspase-1 and GSDMD proteins in DM + Exo group were significantly lower than those in DM + NS group (Fig. 4A,B,C,D,E). These results indicated that the level of pyroptosis in diabetic rats was significantly increased, and BMSCs-Exo could significantly inhibit the level of pyroptosis in diabetic rats.

Effects of BMSC-Exo on proliferation and migration of fibroblasts

The effect of Exo on fibroblast proliferation was verified by CCK-8 method, the effect of Exos on fibroblast migration was verified by scratch test, and the effect of Exo on fibroblast apoptosis was detected by flow cytometry. The results showed that the proliferation and migration of Hy fibroblasts were significantly inhibited compared with the control group, and Hy + Exo group significantly promoted the proliferation and migration of fibroblasts compared with Hy group (Fig. 5). Compared with the control group, the

apoptosis level of Hy component fibroblasts was significantly increased. Compared with the Hy group, Hy + Exo group significantly inhibited the apoptosis of fibroblasts(Fig. 6).

Vitro experiment results of Western Blot

The contents of NLRP3, IL-18, IL-1β, caspase-1 and GSDMD in each group were detected by Western Blot. The results showed that the expressions of NLRP3, IL-18, IL-1β, caspase-1 and GSDMD in Hy group were significantly increased compared with those in Control group. The expression of NLRP3, IL-18, IL-1β, caspase-1 and GSDMD in Hy + Exo fibroblast decreased significantly compared with the Hy group (Fig. 7). These results indicated that pyroptosis of rat skin fibroblasts was significantly increased under high glucose environment, and BMSCs-Exo could significantly inhibit pyroptosis of rat fibroblasts.

Electron microscope result

In the control group, the cell membrane exhibited intact morphology without any discernible globular protrusions. Conversely, in the HY group subjected to high sugar treatment, an increased number of perforations and globular protrusions were observed on the cell membrane surface, indicative of compromised integrity and subsequent pyroptotic demise. Remarkably, upon supplementation with exosomes in the HY + Exo group, a significant amelioration in pyroptosis was evident.

Discussion

Wound healing in diabetic patients poses a significant challenge, primarily due to diabetes-related vascular and neuropathic complications; however, the precise underlying mechanism remains incompletely understood. Prolonged exposure to a high-sugar environment induces alterations in cellular function, thereby impairing wound healing and leading to the development of chronic ulcers [6–9]. The chronic hyperglycemic milieu results in reduced keratinocyte proliferation and migration [10]. Fibroblasts play a crucial role in wound repair as they secrete collagen, fibronectin, and other extracellular matrix components to fill tissue defects. Recent studies have demonstrated that within a specific concentration range, higher levels of blood glucose and advanced glycation end products (AGEs) exert more pronounced inhibitory effects on fibroblast proliferation [11]. In our study, we observed significantly impaired wound healing rates in diabetic rats compared to those of normal rats. Furthermore, under high-glucose conditions, rat fibroblasts exhibited diminished proliferation and migration abilities when compared with the control group.

MSCs possess various physiological functions, such as self-renewal, clonal cell population generation, and multilineage differentiation [12]. Exosomes are small vesicles secreted by cells with a diameter ranging from 30 to 150 nm. They exhibit non-immunogenic and non-tumorigenic properties while containing diverse bioactive substances including mRNA, microRNA, lipids, proteins, etc. These exosomes exert anti-inflammatory effects, promote angiogenesis and cellular proliferation/growth. As paracrine mediators of MSCs' function, exosomes offer advantages in wound treatment due to their low

immunogenicity, high safety/stability, small size facilitating easy utilization through biofilm application and storage convenience [13]. In the proliferative stage of skin wound healing, the proliferation and migration of epidermal cells to the wound center are fundamental for wound coverage and repair, while fibroblasts play an indispensable role in granulation tissue formation, wound contraction, and extracellular matrix (ECM) deposition. Previous studies have demonstrated that ADSCs-Exo can regulate AKT/HIF-1α, Wnt/β-catenin, and other signaling pathways [14–15], thereby promoting keratinocyte proliferation and migration. Additionally, Wang et al. [16] discovered that exosomes derived from fetal dermal mesenchymal stem cells (FDMSCs) activate the Notch signaling pathway to enhance fibroblast proliferation, migration, and ECM synthesis in mice models of skin wound healing acceleration. Gao et al. [17] identified miR-135a within human amniotic mesenchymal stem cell (hAMSCs)-derived exosomes as a promoter of fibroblast migration and rat skin wound healing by inhibiting LATS2 through targeted regulation. miR-126-3p overexpressed synovial mesenchymal stem cell-derived exosomes stimulated the proliferation of human dermal fibroblasts in a dose-dependent manner, resulting in accelerated epithelial regeneration in vivo and promoting collagen maturation [18]. Exosomes derived from adipose mesenchymal stem cells can be absorbed and internalized by fibroblasts to promote fibroblast proliferation and migration and optimize collagen deposition through the PI3K/Akt signaling pathway [19].Furthermore, variations exist in growth factor content mediating wound healing among exosomes secreted by MSCs from bone marrow, adipose tissue, and umbilical cord tissue; notably higher expression levels of VEGF-A, fibroblast growth factor 2 (FGF-2), and platelet-derived growth factor BB (PDGF-BB) were observed in exosomes derived from bone marrow mesenchymal stem cells (BMSCs) [20]. Our study demonstrates that BMSCs-Exo significantly enhances diabetic rat wound healing rate while promoting rat fibroblast proliferation and migration.

Pyroptosis is an inflammatory type of programmed cell death. One of the mechanisms of diabetic wound healing difficulty is excessive inflammatory response, which is related to pyroptosis. Gasdermin family proteins mediate pyroptosis, characterized by pore formation, cell lysis, and release of pro-inflammatory cytokines [21]. In the classical pyroptosis pathway, caspase-1 is activated by the NLRP3 inflammasome, and active caspase-1 can promote the cleavage of IL-1β and IL-18 precursors to form mature IL-1β and IL-18. On the other hand, GSDMD can also be cut to form GSDMD-N terminal and GSDMD-C terminal. The GSDMD-N terminal can bind with phosphatidylinositol phosphate on eukaryotic cells, and after forming pores, the osmotic pressure inside and outside the cells will be unbalanced, resulting in cell lysis and death. At the same time, mature IL-1β and IL-18 are released outside the cell, recruiting immune cells and triggering inflammatory response, and finally forming cell pyroptosis. Therefore, pyroptosis and inflammatory response are closely related. Long-term high glucose environment can lead to continuous inflammatory response. In this study, we found that the expressions of GSDMD, caspase-1, IL-1β, IL-18 and NLRP3 proteins were significantly increased in the peri-traumatic tissues of diabetic rats and in rat fibroblasts treated with high glucose. It was confirmed that pyroptosis significantly increased in peripheral wound cells and fibroblasts in diabetic rats under the condition of high glucose, and the increase of pyroptosis would lead to the aggravation of inflammatory response, so pyroptosis may be one of the important reasons for the sustained inflammatory response on diabetic wounds. The

experimental results showed that BMSCs-Exo could significantly inhibit the scorch death of diabetic wound cells and rat fibroblasts in the condition of high glucose, indicating that BMSCs-Exo could inhibit pyroptosis and reduce inflammatory response, thus promoting wound healing in diabetic rats.

Conclusions

In this research, we investigated the effects of BMSCs-Exo on wound healing and pyroptosis in diabetic rats. We also examined the impact of BMSCs-Exo under high glucose conditions on fibroblast proliferation, apoptosis, migration, and pyroptosis in rats. Our findings revealed a significant decrease in wound healing rate in diabetic rats compared to normal rats, along with reduced fibroblast proliferation and migration ability under high glucose environment. However, treatment with BMSCs-Exo significantly improved wound healing rate in diabetic rats and promoted fibroblast proliferation and migration. Additionally, we observed a significant increase in fibroblast pyroptosis at the wound site of diabetic rats as well as under high glucose conditions; however, BMSCs-Exo effectively inhibited fibroblast pyroptosis in both scenarios. These results suggest that BMSCs-Exo suppresses cellular pyroptosis through the NLRP3/caspase-1/GSDMD pathway both in vitro and in vivo, thereby facilitating wound healing in diabetic rats. The specific mechanism by which BMSCs-Exo reduces cellular pyroptosis levels requires further investigation.

Declarations

Acknowledgements

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Funding Declaration

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Competing Interest declaration

The authors declare no Competing Interests.

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Figures

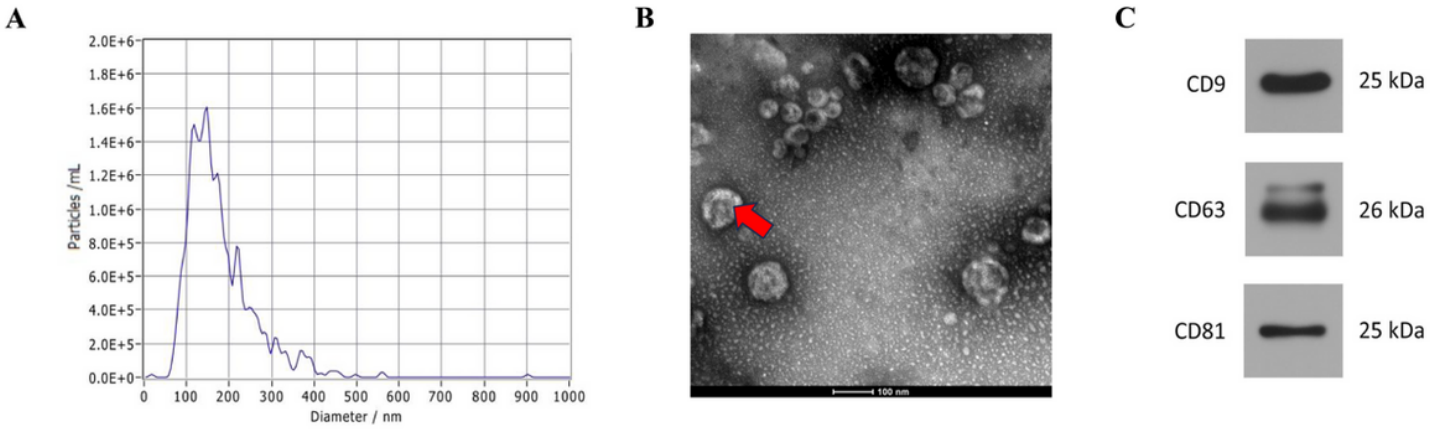

Fig1. Identification results of BMSCs-Exo .(A) The size distribution of the BMSCs-Exo was assessed utilizing dynamic light scattering.(B) Micrographs of transmission electron microscopy of purified BMSCs-Exo, showing a spheroid shape. Scale bar=100 nm. (C) Western blotting results indicated the positive expression of CD9, CD63, and CD81 protein in BMSCs-Exo.

Figure 1

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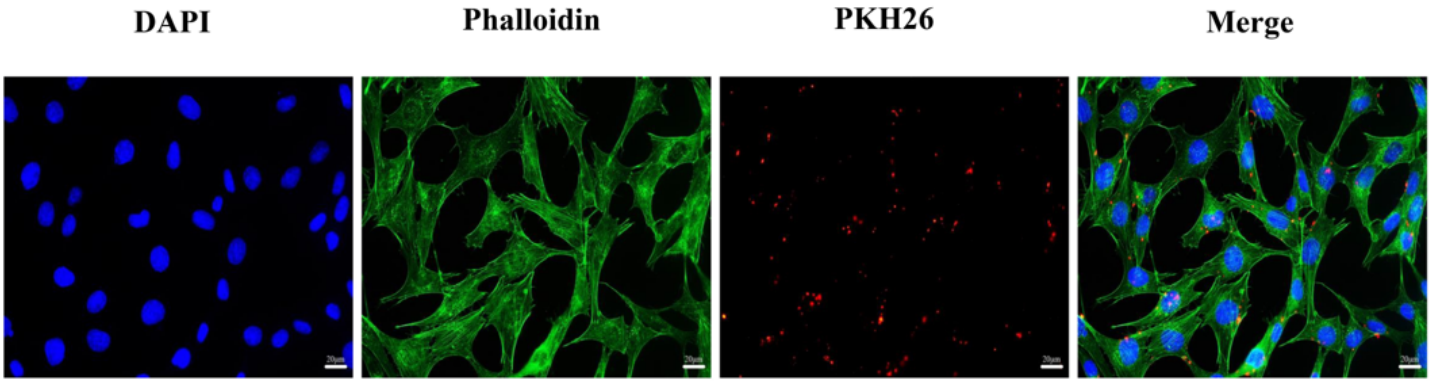

Figure 2

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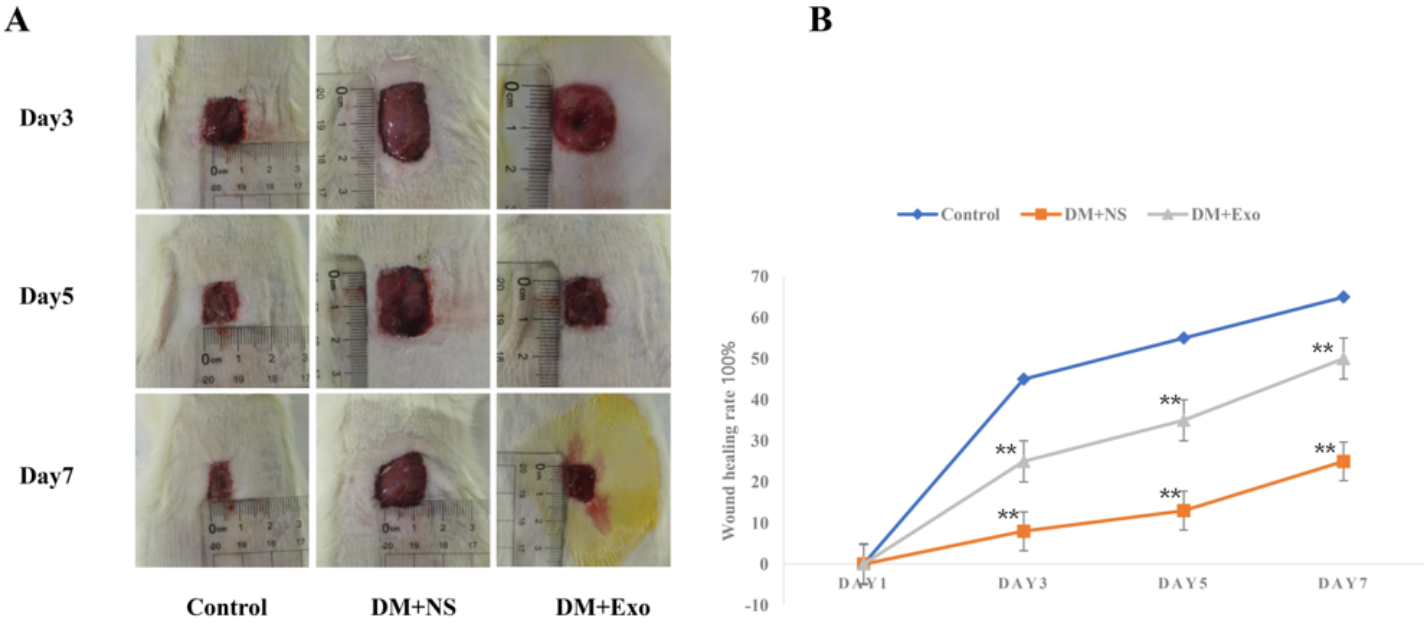

Fig3.Wound closure of mice in control group, DM+NS group and DM+Exo group on the 3th ,5th ,7th day. **P<0.01

Figure 3

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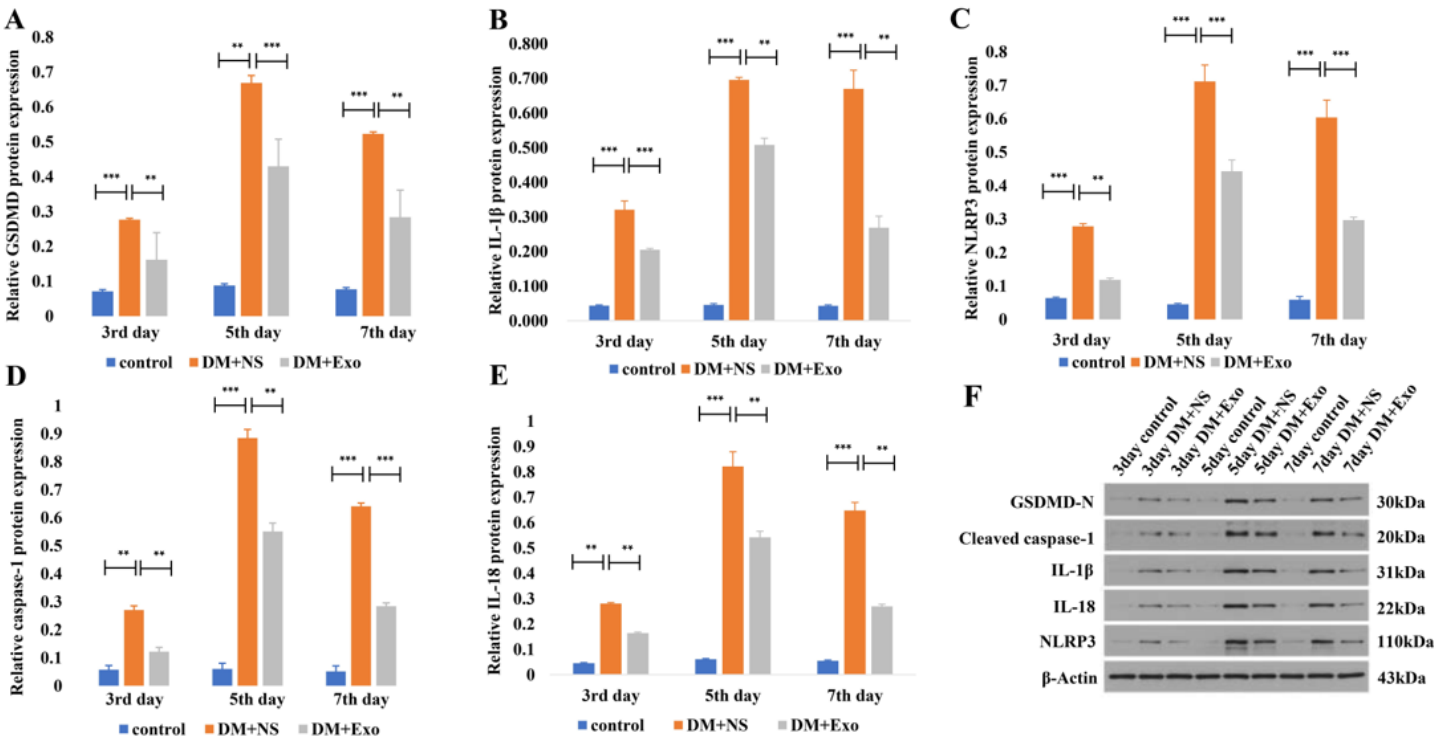

Fig 4. BMSC-Exo inhibited the pyroptosis in diabetic rat wound. Representing three separate experiments (means ± SD) ***P<0.001and **P<0.01.

Figure 4

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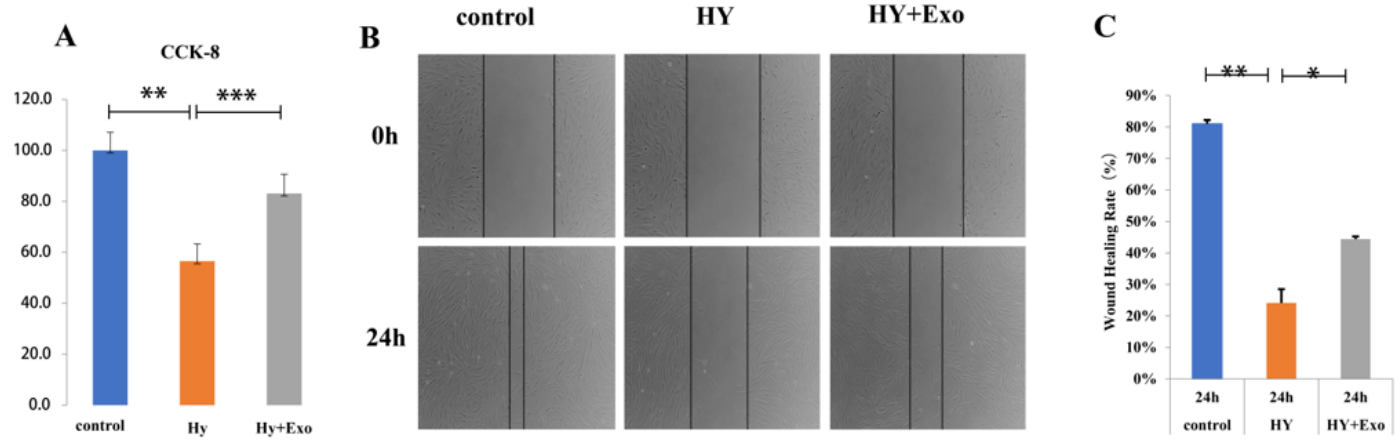

Fig 5. BMSC-Exo promoted proliferation and migration of fibroblast subjected to high glucose conditions. CCK-8 assay findings illustrated that BMSC-Exo promoted proliferation of fibroblast subjected to high glucose conditions(**A**). Cell scratch assay showed that that BMSC-Exo promoted migration of fibroblast subjected to high glucose conditions(**B,C**). Data are articulated as mean±SD (n=6), and the experiment was redone separately three times. *P<0.05 ,**P<0.01, and ***P<0.001.

Figure 5

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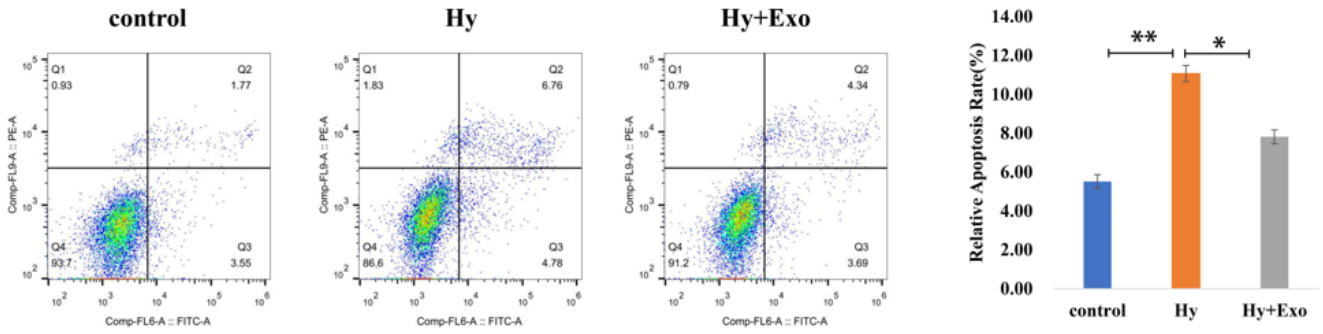

Fig 6.Apoptosis of fibroblasts in each group. Data are articulated as mean ±SD *P<0.05 and **P<0.01.

Figure 6

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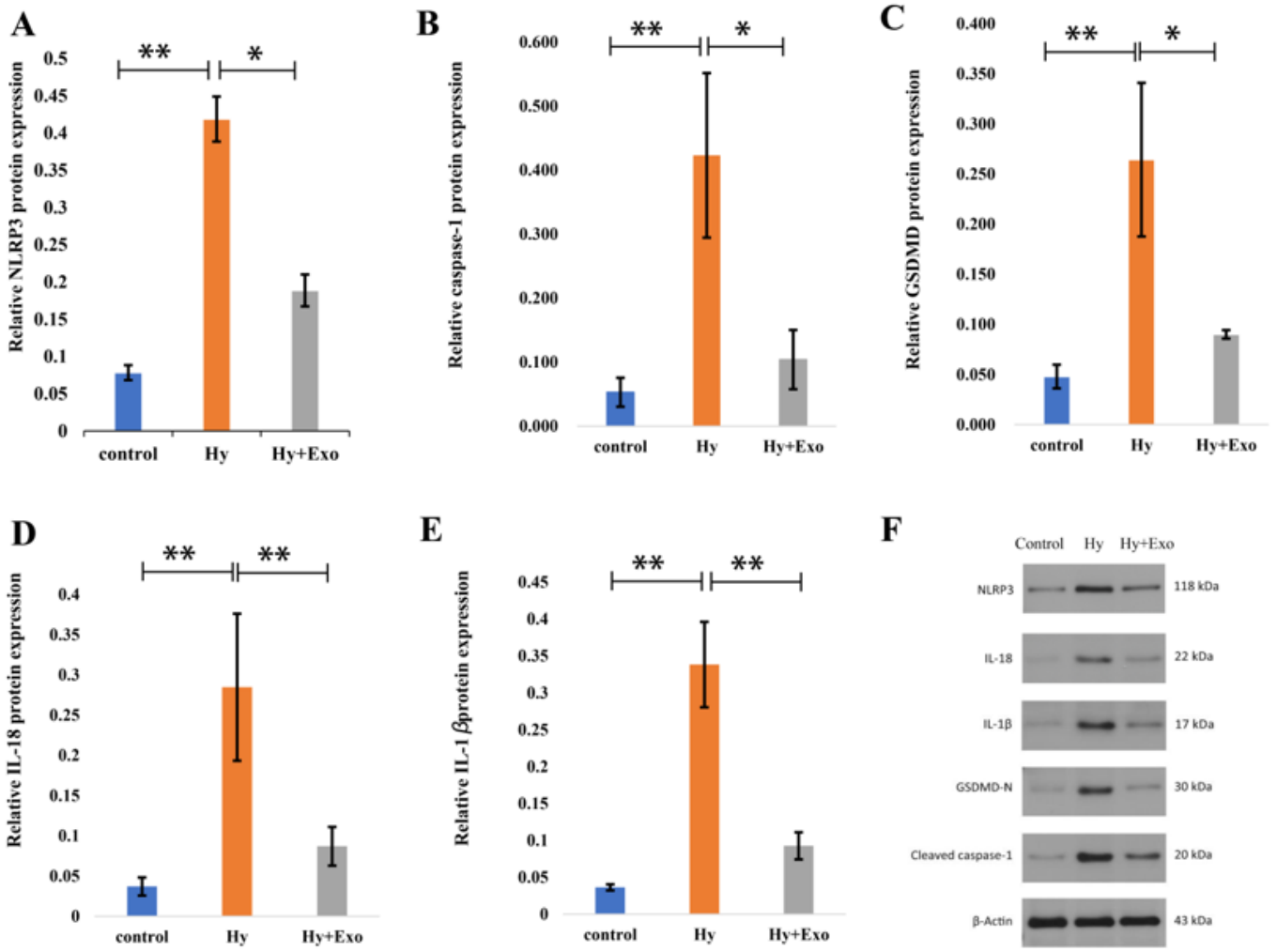

Fig 7. BMSC-Exo inhibited HG-induced pyroptosis of fibroblast . Representing three separate experiments (means ±SD) *P<0.05 and **P<0.01.

Figure 7

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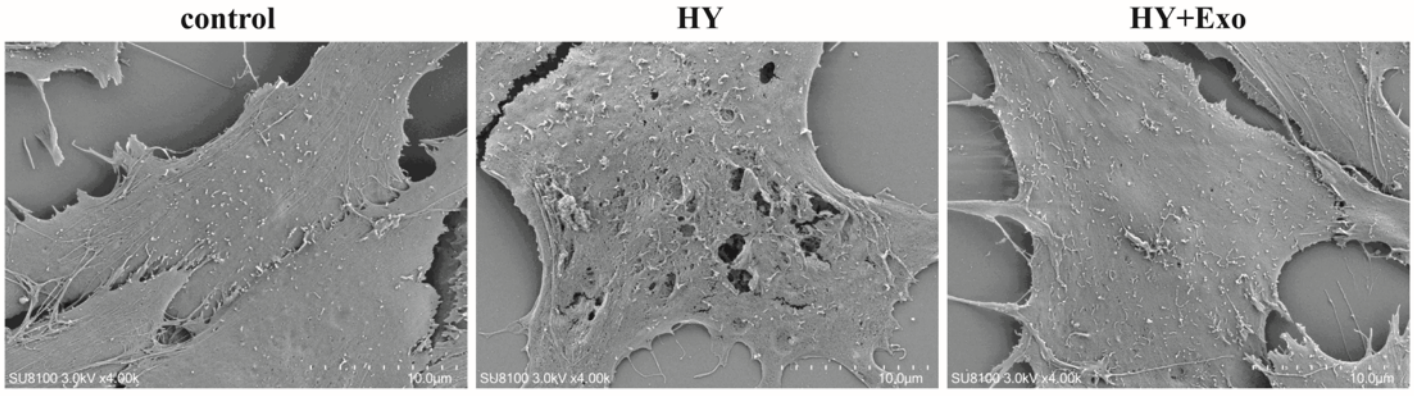

Fig 8. The pyroptosis of each group of cells was observed under an electron microscope

Figure 8

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