Optimal H₂O₂ preconditioning to improve bone marrow mesenchymal stem cells’ engraftment in wound healing

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Abstract

Background: The transplantation of bone marrow mesenchymal stem cells (BMSCs) is a promising therapeutic strategy for wound healing. However, the poor migration capacity and low survival rate of transplanted BMSCs in wounds weaken their potential application.

Objective: To identify the optimal protocol for BMSCs preconditioned with H₂O₂ and improve the therapeutic efficacy using H₂O₂-preconditioned BMSCs in wound healing.

Methods: Mouse BMSCs were exposed to various concentrations of H₂O₂, and the key cellular functional properties were assessed to determine the optimal precondition with H₂O₂. The H₂O₂-preconditioned BMSCs were transplanted into mice with full-thickness excisional wounds to evaluate their healing capacity and tissue engraftment.

Results: Treatment BMSCs with 50 μM H₂O₂ for 12 h could significantly enhance their proliferation, migration, and survival by maximizing the upregulation of cyclin D1, SDF-1, and its receptors CXCR4/7 expressions, and activating the PI3K/Akt/mTOR pathway, but inhibiting the expression of p16 and GSK-3β. Meanwhile, oxidative stress-induced BMSC apoptosis was also significantly attenuated by the same protocol pretreatment with a decreased ratio of Bax/Bcl-2 and cleaved caspase-9/3 expression. Moreover, after the identification of the optimal protocol of H₂O₂ precondition in vitro, the migration and tissue engraftment of transfused BMSCs with H₂O₂ preconditioning were dramatically increased into the wound site as compared to the un-preconditioned BMSCs. The increased microvessel density and the speedy closure of the wounds were observed after the transfusion of H₂O₂-preconditioned BMSCs.

Conclusions: The findings suggested that 50 μM H₂O₂ pretreated for 12 h is the optimal precondition for the transplantation of BMSCs, which gives a considerable insight that this protocol may be served as a promising candidate for improving the therapeutic potential of BMSCs for wound healing.

Keywords: Mesenchymal stem cells, Hydrogen peroxide, Preconditioning, Oxidative stress, Wound healing
Introduction

Growing evidence indicates that BMSC-based therapy for cutaneous wound healing holds great therapeutic value via differentiating into specialized cell types, producing a large variety of growth factors, and promoting wound closure and angiogenesis [1–4]. Therefore, transplantation of BMSCs is a promising therapeutic strategy for wound healing [1]. However, the poor migration capacity and low survival rate of transplanted BMSCs in wounds weaken their potential application.

Preconditioning has become the most powerful and effective cytoprotective strategy for initiating cell survival signaling to counter the rigorous harsh microenvironment [5, 6] and enhance the cell migration capacity and survival rate [5, 7]. Reportedly, the injured tissue expresses a high level of stromal cell-derived factor (SDF-1), a stem cell homing signal factor which binds to its receptor CXC chemokine receptor 4 (CXCR4) and CXCR7 on cell surface of MSCs and promotes stem cells homing and survival in the injured tissue, respectively [7, 8]. However, during in vitro culture expansion, BMSCs lose their CXCR4 receptor [9] and reduce their binding capacity to SDF-1 resulting in [10, 11] attenuation of their migration capacity. Moreover, various stress conditions including ex vivo isolation, in vitro expansion of MSCs, and confronting the harsh microenvironment (ischemia, hypoxia, and inflammation) caused oxidative stress injury to BMSCs following engraftment at injured sites, resulting in reducing survival of transplanted BMSCs [12–14].

Reactive oxygen species (ROS)-mediated-oxidative stress is an important cause for apoptosis and death of donor MSCs following engraftment [5, 6, 12, 13]. Mechanistically, the phosphatidylinositol 3-kinases (PI3K)/protein kinase B (Akt) signaling pathway plays central regulatory roles in MSC survival, proliferation, migration, angiogenesis, cytokine production, and differentiation [15–17]. SDF-1 could promote MSC survival and migration by the activation of the PI3K/Akt pathway [9, 10, 18–21]. Several studies demonstrated that H₂O₂ preconditioning protects cells against oxidative damage [22–24]. In fact, H₂O₂ at certain low level acts as essential cellular messengers by playing a growth factor-like role in regulating physiological stem cellular processes [25–27]. Therefore, we speculated low-dose H₂O₂ functioned as a growth factor-like molecule to activate the PI3K/Akt pathway. Although multiple studies have confirmed the cytoprotective effect of H₂O₂ preconditioning on MSCs against oxidative damage, neither the optimal concentration of H₂O₂ used in preconditioning nor the underlying molecular mechanism has been fully established [22–24].

In this study, the optimal concentration of H₂O₂ on BMSC preconditioning was first determined. Then, the molecular mechanisms of H₂O₂ preconditioning BMSCs were explored by focusing on the PI3K/Akt pathway due to its role in MSC survival and migration responding to SDF-1 and oxidative stress [28]. The ultimate therapeutic potential of H₂O₂ preconditioned BMSCs was evaluated in a mouse full-thickness wound model.

Materials and methods

Ethics statement

Animal studies were performed using male Balb/C mice provided by the Animal Breeding Center of Third Military Medical University. All procedures in the present study were performed in accordance with the “Guide for the Care and Use of Laboratory Animals” (National Institutes of Health Publication no. 86-23, revised 1985) and were approved by our hospital’s ethics committee.

MSC isolation, culture, and stimulation

Primary BMSCs were isolated from the long bones of 4-week-old male Balb/C mice as previously described [11]. For this purpose, the femurs and tibiae were aseptically removed from the euthanized animals and dissected clean of attached muscles. The bones were flushed with 2 mL of cold PBS and washed 1 time with PBS. Cells were suspended in DMEM/F12 (Hyclone, Logan, UT) and supplemented with 10% (vol/vol) fetal bovine serum (FBS, Invitrogen), 100 U/mL penicillin, and 100 μg/mL streptomycin, and cultured in 25-cm² tissue culture flasks (Falcon) in a humidified atmosphere with 5% CO₂ at 37 °C. After 24 h, non-adherent cells were depleted and 3 mL of fresh culture medium was added. After 7 to 10 days of initiation, BMSCs growing clone-like were passaged at 70 to 80% confluence using TrypLE™ Express (Gibco) digesting. Cells were washed and seeded at a density of 1 × 10⁶ in tissue culture flasks. BMSCs harvested between 3 and 7 passages were used for follow-up experiments.

Cell ATP assay

Because mitochondrial activity can better reflect cell vitality, the cell viability was assessed using the ATP assay. A luciferase-based kit (Beyotime Biotechnology, Shanghai, China) was used to measure the ATP level according to the manufacturer’s instruction. Briefly, BMSCs were seeded into 96-well plates at a density of 1 × 10⁴ cells/well. After 24 h, cells were treated with various concentrations of H₂O₂ (0, 25, 50, 100, 200 μM, Sigma, St. Louis, MO, USA) for 24 h at 37 °C in a cell culture incubator. After that, the culture medium was removed and the cells in each well were lysed by lysis buffer and centrifuged (1200g, 4 °C, 5 min). Next, the supernatant was quickly mixed with the working solution at equal volumes and then transferred into a standard opaque well 96-well plate before light recording a microplate reader (Thermo, Varioskan LUX, USA) using the chemiluminescence method.
Cell Counting Kit-8 assay

Cell Counting Kit-8 (CCK-8, Beyotime Biotechnology, Shanghai, China) was applied for the evaluation of cell proliferation. BMSCs were trypsinized and seeded in 96-well plates at a density of 1 × 10^4 cells/well. After overnight incubation, the cell culture medium was replaced and simultaneously added with various concentrations of H_2O_2 (25, 50, 100, 150, 200, 300, and 500 μM). H_2O_2 was diluted with DMEM/F12 and used immediately. After that, 10 μL of CCK-8 solution was added into each well. Cells were incubated at 37 °C for 2 h in the dark. Absorbance values at the wavelength of 450 nm were detected using a microplate reader (Thermo, Varioskan LUX, USA). Each experiment was repeated 3 times.

Scratch wound-healing migration assay

BMSCs were seeded in 6-well plates at a density of 1 × 10^5 cells/well. When BMSCs grew to 90% confluence, scratches were made with a standard 200-μm plastic pipette tip to make a line-shaped wound across the diameter of each well. The medium was then aspirated, and the cells were washed thoroughly with PBS. The PBS was then aspirated. And BMSCs were incubated further for 12 h and 24 h with or without 50 μM H_2O_2. Images were captured at 0, 12, and 24 h using the phase-contrast microscope with ×20 magnification (Olympus, Japan). Five images were taken per scratch which represents a technical replicate of 5 and biological replicate of 3 for the stem cells. The cells within the scratch zone per field were counted using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Cell migration

Migration assays were carried out in a 24-well transwell migration system with an 8-μm pore size polycarbonate membrane at a density of 2 × 10^4 cells/well. BMSCs, preconditioned or not with 50 μM H_2O_2 for 12 h after incubating with or without CXCR4 antibody for 2 h, were plated in the top Boyden chamber (Corning Costar). The cells were allowed to migrate for 12 h in the presence of 50 ng/mL SDF-1 (Sigma, US) in the bottom chamber. The migrating cells underside of the membrane were fixed in 4% paraformaldehyde for 15 min, followed by staining with 0.5% crystal violet. The number of migrating cells was determined by counting five random fields per membrane under the microscope at ×400 magnification (Olympus, Japan) and represented as the average of three independent experiments.

Flow cytometry analysis of BMSC apoptosis

Cell apoptosis was detected with Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) Apoptosis Detection Kit (KeyGen Biotech, Nanjing, China) as described by the manufacturer’s instructions. Briefly, BMSCs were seeded in 6-well plates at a density of 1 × 10^5 cells/well, and treated with various concentrations of H_2O_2 (25, 50, 100, 150, 200, 300, and 500 μM) for 24 h or treated with high concentrations of H_2O_2 (200, 250, 300, and 500 μM) for 24 h after preconditioned with or without 50 μM H_2O_2 for 12 h, or cells were exposed to 12-h preconditioning with H_2O_2 after a 2-h pre-incubation with or without PI3K inhibitor LY294002 (Sigma, US), followed by treatment with 300 μM H_2O_2 for 24 h (Table 1). Thereafter, cells were collected and washed with ice-cold PBS twice, gently resuspended in the binding buffer and incubated with annexin V-FITC and propidium iodide (PI) for 15 min in the dark. Cells were analyzed by flow cytometry (NovoCyte™, ACEA Biosciences, San Diego, USA). The total apoptosis rate of cells was calculated as the sum of the rates of cells observed in the lower-right quadrant and the upper-right quadrant.

Osteogenic, chondrocytic and adipogenic differentiation

BMSCs were pretreated with 50 μM H_2O_2 for 12 h before the original medium was changed to a differentiation medium. StemPro Osteogenesis Differentiation Kit, StemPro Chondrogenesis Differentiation Kit, and StemPro Adipogenesis Differentiation Kit (Gibco) were used for osteogenesis, adipogenesis, and chondrogenesis, respectively. Differentiation was assessed using alkaline phosphatase, Toluidine blue, Alcian blue, and Oil Red O stain, respectively.

Mitochondrial superoxide O_2^- assessment

The generation of mitochondrial O_2^- was determined using MitoSOX Red (Invitrogen, Carlsbad, CA, USA), a selective fluorescent indicator for detecting O_2^- generation within mitochondria. Briefly, BMSCs were seeded in 24-well tissue culture plates (2.0 × 10^4 cells per well) and randomly divided into 4 groups: the control group, 50 μM H_2O_2 group, 300 μM H_2O_2 group, and 50 μM H_2O_2 plus 300 μM H_2O_2 group, in which cells were incubated for 24 h with medium only, 50 μM H_2O_2, 300 μM H_2O_2, and 300 μM H_2O_2 before preconditioned with 50 μM H_2O_2 for 12 h, respectively. Thereafter, the MitoSOX Red reagent stock solution was diluted with HBSS/Ca/Mg buffer to make a working solution of 5 μM. And cells were incubated with 5 μM MitoSOX Red reagent in the dark at 37 °C for 10 min. Cells were washed gently three times with warm buffer. Fluorescence intensities of mtROS were determined using a microplate reader (Thermo, Varioskan LUX, USA), and images were visualized by fluorescence microscopy (Olympus, Japan) with the emission wavelength of 579 nm and the excitation wavelength of 510 nm.
Table 1 The BMSC-treated protocol for the flow cytometry analysis of apoptosis

| Cell density (cells/well) | H2O2 (μM) precondition/time | LY294002 (μM) pre-incubation/time | H2O2 (μM) treatment/time |
|--------------------------|----------------------------|----------------------------------|-------------------------|
| 1 × 10⁵                   | No                         | No                               | 25, 50, 100, 150, 200, 300, 500/24 h |
| 1 × 10⁶                   | 50 μM/12 h                  | No                               | 200, 250, 300, 500/24 h   |
| 1 × 10⁵                   | 50 μM/12 h                  | 50 μM/2 h                        | 300/24 h                |

Mitochondrial membrane potential (ΔΨm) determination
Mitochondrial membrane potential (ΔΨm) was detected using the fluorescent, lipophilic dye, JC-1 (Beyotime Biotechnology, Shanghai, China). JC-1 can easily penetrate cells with healthy mitochondria. A green fluorescent JC-1 probe exists as a monomer at low membrane potentials. However, at higher potentials, JC-1 forms red-fluorescent “J-aggregates.” BMSCs were trypanized and seeded in 24-well tissue culture plates (2.0 × 10⁴ cells/well), randomly divided into 4 groups, and treated like as 2.9. Thereafter, the cells were incubated with JC-1 staining solution for 20 min, then cells were washed twice with JC-1 staining buffer (1×). When the JC-1 monomer was detected, the excitation wavelength was set to 490 nm and the emission wavelengths was set to 530 nm. When JC-1 polymer was detected, the excitation wavelength was set to 525 nm and the emission wavelength was set to 590 nm using a microplate reader (Thermo, Varioskan LUX, USA). The ΔΨm was represented as the ratio of red to green fluorescence intensity. Then, these cells were observed and taken a photograph by fluorescence microscopy (Olympus, Japan).

Wound healing model and BMSC transplantation
Animal studies were performed using 6-week-old male BALB/C mice (21–23 g) provided by the Animal Center of The Third Military Medical University. Animals were individually housed in plastic cages at 25 °C with access to food and water ad libitum. Animals were kept on a 12-h light/dark cycle. The full-thickness excisional skin wounds in Balb/C mice were made as previously reported [11]. Animals were weighed and anesthetized by intraperitoneal injection of the appropriate dose (60 mg/kg) of 2% pentobarbital sodium. After shaving the dorsal surfaces of mice, full-thickness excisional skin wounds (8 mm in diameter) were created aseptically on the midline of the mice’s back. Mice were randomly divided into three groups, which received an injection of a suspension of 100 μL PBS, 1 × 10⁶ BMSCs in 100 μL PBS and 1 × 10⁶ H2O2-preconditioned BMSCs (for 12 h) in 100 μL PBS, respectively. BMSCs were pre-labeled with chloromethyl-1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate (CM-DiI, Sigma) before injection. BMSCs were incubated with 10 μM Dil for 20 min at 37 °C. Mice post-wounding at once were injected with Dil-labeled BMSCs or PBS via the tail vein.

Wound analysis
Wound analysis began at 1 day after wounding. Digital photographs of wounds were taken on days 0, 1, 3, 5, 7, 10, 13, and 15 and analyzed using the ImageJ software. The wound closure rate was calculated as follows: [(area of original wound − area of actual wound)/area of original wound] × 100%.

Mice were sacrificed at days 0, 3, 5, 7, and 10 post-wounding, and the skin samples including the wound and a 4-mm marginal unwounded skin from each wound were collected for biochemical and histological analyses. Half of each sample was fixed in 4% paraformaldehyde for 5 h and then preserved in 20% sucrose at 4 °C or fixed in 4% paraformaldehyde for 12 h and embedded in paraffin for hematoxylin and eosin (H&E) staining, Masson’s trichrome stain, and CD31 immunohistochemical staining. These samples (preserved in 20% sucrose) were cut into 10-μm frozen sections and observed with a fluorescence microscope. The photos were taken by randomly selecting 6 high-power fields (hpf, × 200). We used the ImageJ software to calculate the total number of migrated BMSCs with CM-DiI label (red cells) at the wound site. The other half of the same sample was snap-frozen in liquid nitrogen and stored at –80 ºC until protein analysis using Western blot assay. Three animals were collected from each experimental time point.

Western blot analysis
BMSCs seeded in 6-cm tissue culture dishes (1 × 10⁶/well) in the logarithmic growth phase were treated with various low concentrations of H2O2 (0, 25, 50, 100, and 200 μM) for 24 h or were treated with 50 μM for different time courses (0, 3, 6, 12, 24, and 36 h). For another set of experiments, either a 12-h pre-incubation with 50 μM H2O2 or with or without a 2-h pre-incubation with 50 μM PI3K inhibitor LY294002 (Sigma), followed by treatment with 300 μM H2O2 for 24 h, cultured cells were then collected. Total protein lysates of cultured cells and wound tissues were prepared using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China), and the protein concentrations were determined using a BCA Protein Assay Kit (Keygen, Nanjing, China). Equal volumes of proteins (50 μg) from each sample were separated on SDS-PAGE (4–20%) gels and transferred to PVDF membranes (Millipore, Billerica, MA). Then, the membranes were blocked with 5% non-fat dry milk in

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Tris-buffered saline for 60 min at room temperature and incubated with primary antibodies: cyclin D1, p16, SDF-1, CXCR4/7, Bcl-2, Bax, caspase3/9, cleaved-caspase3/9, PI3K/Phospho-PI3K, Akt/Phospho-Akt (pAktThr308), mTOR/Phospho-mTOR (Ser 2481), VEGF (Cell Signaling Technology, USA), β-actin (Sigma-Aldrich), and GAPDH (Abcam, Cambridge, UK) overnight at 4°C, followed by sequential incubation with HRP-conjugated secondary antibodies for 2 h at room temperature. Lastly, the bands were visualized by an enhanced chemiluminescence detection kit (ECL, Amersham Arlington Heights, IL, USA) and exposed to the gel imaging system (Bio-Rad, USA). Band density was analyzed using LabWorks 4.6 analysis software.

**Microvessel density of the wound area**

Microvessel density was determined by immunohistochemical staining with anti-CD31 antibody (Santa Cruz), according to the manufacturer’s instructions and a method previously described [11]. In brief, antigen retrieval was performed in a 0.01-mol/L citrate buffer (pH 6.0) with a heating interval—keeping the temperature at 92–98°C by using a microwave oven for 15 min and then cooling for 30 min. The sections were permeabilized with 0.1% Triton X-100 (Sigma) for 10 min and incubated with 3% H2O2 in methanol for 20 min. After blocking with goat serum at room temperature for 1 h, the sections were incubated overnight at 4°C with anti-mouse CD31 antibody diluted in 0.1% BSA in PBS. Subsequently, the sections were incubated with biotin-labeled secondary antibody for 30 min and then with streptavidin peroxidase for 30 min at 37°C. The cell nucleus was counterstained with hematoxylin. The microvessels that were lined with the endothelium and had a patent lumen were counted. The mean number of microvessels, defined as CD31 positive, and GAPDH (Abcam, Cambridge, UK) overnight at 4°C, followed by sequential incubation with HRP-conjugated secondary antibodies for 2 h at room temperature. Lastly, the bands were visualized by an enhanced chemiluminescence detection kit (ECL, Amersham Arlington Heights, IL, USA) and exposed to the gel imaging system (Bio-Rad, USA). Band density was analyzed using LabWorks 4.6 analysis software.

**Analysis of collagen deposition and H&E staining in the wound area**

To determine the extent of collagen deposition in the granulation tissue of the wounds at days 7 after injury, collagen was stained with Masson’s trichrome stain. We used the ImageJ software to quantify collagen deposition from the Masson’s trichrome stained images. Each image was captured under ×40 magnification by a blinded individual. Under the analyze function in ImageJ, we used the RedGreen Blue (RGB) histogram to determine the mean value for the blue color intensity, in which our trichrome images represent collagen. In addition, these sections of the granulation tissues of the wounds in each group at days 5 post-wounding were analyzed using H&E staining.

**Statistical analysis**

We obtained all the results from at least three independent experiments. All statistical analyses were performed using the SPSS13.0 software. Differences between multiple groups were analyzed by analysis of variance (ANOVA), whereas differences between the two groups were analyzed using a t test. A value of P < 0.05 was considered to be statistically significant.

**Results**

**Treatment of BMSCs with 50 μM H2O2 significantly enhanced cell viability, proliferation, and migration by augmenting the SDF-1/CXCR4/CXCR7 axis**

To define the optimal concentration of H2O2 in preconditioning BMSCs, we selected five different concentrations between 25 and 200 μM based on previous reports [29] and investigated its effects on cellular properties of viability, proliferation, migration, and differentiation. By using the ATP assay and CCK-8 assay, we found that H2O2 at 25 μM and 50 μM significantly increased the viability of BMSCs (Fig. 1a) and promoted the proliferation of BMSCs (Fig. 1b), accompanied by an increase in cyclin D1 and p16 expression, whereas 150 and 200 μM H2O2 showed a significant inhibitory effect compared with that of the control. Fifty-micromolar H2O2 caused a peak cyclin D1 expression and a low p16 expression, which represented the optimal concentration of H2O2 for BMSC preconditioning in terms of cell proliferation/viability from this study (Fig. 1c).

Next, the effect of 50 μM H2O2 on the migration ability of BMSCs was evaluated using a scratch assay. The results showed that 50 μM H2O2 treated enhanced cell migration significantly compared with control cells untreated with H2O2 (Fig. 1d, e). The Boyden chamber assay was further demonstrated that the number of migrating BMSCs in the H2O2 group was much more than in that of the control group; however, the enhanced migration capacity was attenuated by pretreatment with anti-CXCR4 antibody (Fig. 1f, g). Simultaneously, we found that the expression of SDF-1, CXCR4, and CXCR7 in the H2O2 group was significantly upregulated at 25 μM and 50 μM compared to that of the control (Fig. 1h). These results suggested that the 50 μM H2O2 treatment for 12 h could significantly enhance the MSC viability, proliferation, and migration capabilities, which may be through activating the SDF-1/CXCR4/CXCR7 pathway.

Additionally, we found that the BMSCs still have maintained the multipotent differentiation capacity of stem cells even after pretreated with 50 μM H2O2 for 12 h (Supplement Fig. 1). They could differentiate into adipocytic (Oil red O staining), chondrocytic
Fig. 1 Optimization of H$_2$O$_2$ concentrations for preconditioning BMSCs. 

a. BMSCs were treated with the indicated concentrations of H$_2$O$_2$ (0–200 μM) for 12 h. The cell viability was measured by ATP assay. Values represent the mean ± standard errors of the means (SEM) (n = 3).

b. The effect of H$_2$O$_2$ on BMSC proliferation determined by the CCK-8 assay. H$_2$O$_2$ at 25 and 50 μM significantly promoted the proliferation of BMSCs compared with the control (n = 3).

c. Western blot analysis of cyclin D1 and p16 expressions in BMSCs exposed to different low concentrations H$_2$O$_2$ for 12 h. Values represent the mean ± SEM (n = 3). *P < 0.05, **P < 0.01 vs. 0 μM H$_2$O$_2$.

d. Representative images of the scratch assay showed the migration of BMSCs with or without 50 μM H$_2$O$_2$ treatment for 12 h and 24 h. Scale bar, 100 μm.

e. Quantification of the number of cells which have migrated into the scratch zone; 50 μM H$_2$O$_2$-treated cells had a significantly increased migration compared with control untreated cells (n = 3). f Images of transmigrated BMSCs (stained with crystal violet) are shown (× 200 magnification). Scale bar, 100 μm.

g. Quantitative analysis of migrated cells. Data is presented as mean ± SEM of the six independent experiments (n = 6).

h. Western blot analysis of SDF-1 and CXCR4 expressions in BMSCs exposed to different low doses of H$_2$O$_2$ for 12 h. Values represent the mean ± SEM (n = 3). *P < 0.05, **P < 0.01 vs. the control group (0 μM H$_2$O$_2$); #P < 0.05 vs. the 50 μM H$_2$O$_2$ group.
(Toluidine blue, Alcian blue), and osteoblastic (alkaline phosphatase) lineages. These results showed that 50 μM H₂O₂ treatment did not affect the differentiation potential of BMSCs.

**Fig. 2** Optimal H₂O₂ preconditioning provides anti-apoptotic protection to BMSCs. 

- **a** Representative images of flow cytometric analysis by annexin V-FITC/PI dual staining after BMSCs treated for 24 h with various concentrations of H₂O₂ (25, 50, 100, 150, 200, 300, and 500 μM). The bottom right quadrant represents annexin V-FITC-stained cells (early-phase apoptotic cells), and the top right quadrant represents PI- and annexin V-FITC-dual-stained cells (late-phase apoptotic/necrotic cells). 
- **b** Apoptotic cells are represented as the percentage of annexin V single-positive plus annexin V-FITC/PI double-positive cells (n = 3). 
- **c** Representative images of flow cytometric analysis by annexin V-FITC/PI dual staining. Confluent BMSCs were pretreated with or without 50 μM of H₂O₂ for 12 h. After removing the supernatants, cells were incubated with fresh medium in the presence of various concentrations of H₂O₂ (200, 250, 300, and 500 μM) for an additional 12 h. 
- **d** Apoptotic cells are represented as the percentage of annexin V single-positive plus annexin V-FITC/PI double-positive cells (n = 3). *P < 0.05, **P < 0.01 vs. the control groups (0 μM H₂O₂) or the H₂O₂ un-pretreatment group.

**Optimal H₂O₂ preconditioning suppressed cell death of BMSCs via the mitochondria-mediated mechanism**

Based on the above results, further, the protective effect of 50 μM H₂O₂ preconditioning for 12 h on
BMSCs against oxidative stress-induced apoptosis was detected by the flow cytometric (FCM) analysis. The results showed that exposure to 25–100 μM H₂O₂ did not cause BMSC apoptosis (Fig. 2a). However, exposure to 150–500 μM H₂O₂ induced an obvious increase in the apoptotic rate of BMSCs in a dose-dependent manner (Fig. 2a, b), compared with the control. The results showed that H₂O₂ exceeding 100 μM might induce oxidative injury thereby leading to cell apoptosis. However, the apoptotic rate of BMSCs was very significantly lower in 50 μM H₂O₂-preconditioned each group than in corresponding high doses of H₂O₂ groups (Fig. 2c, d). This result suggested that 50 μM H₂O₂ preconditioning for 12 h remarkably attenuated high concentration H₂O₂-induced apoptosis in BMSCs.

Oxidative stress induces apoptosis through the intrinsic mitochondrial pathway [30]. The ratio of anti-apoptotic Bcl-2 and pro-apoptotic Bax (Bcl-2/Bax) is the key to the regulation of the mitochondrial apoptotic pathway [30]. Treatment of BMSCs with 50 μM H₂O₂ for 12 h resulted in a peak Bcl-2/Bax expression ratio compared to that of the control, but Bax and caspase-3 were not affected (Fig. 3a). Subsequently, after treatment of BMSCs with 50 μM H₂O₂ for different time intervals (3, 6, 12, 24, and 36 h), it was found that when H₂O₂ treated for 12 h, the Bcl-2/Bax ratio reached a peak, while the expression of Bax and caspase-3 was not

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**Fig. 3** Effect of optimal H₂O₂ on the mitochondria-mediated apoptosis pathway in BMSCs. Western blot analysis of the expressions of Bcl-2, Bax, and caspase-3 proteins in BMSCs exposed to various low concentrations of H₂O₂ (25, 50, and 100 μM) for 12 h. **a** The bar graphs showed the ratio of Bax/Bcl-2 and the quantification of the caspase-3 protein in BMSCs. **b** Western blot analysis of the expressions of Bcl-2, Bax, and caspase-3 in BMSCs exposed to 50 μM H₂O₂ for different time intervals (3, 6, 12, 24, and 36 h). The bar graphs showed the ratio of Bax/Bcl-2 and the quantification of the caspase-3 protein; Western blot analysis of the expressions of Bcl-2, Bax, and caspase-3 in BMSCs exposed to 50 μM H₂O₂ for 12 h after pretreatment with 50 μM H₂O₂ for 12 h. **c** The bar graphs showed the ratio of Bax/Bcl-2 in BMSCs. **d** The bar graphs showed the quantification of the caspase-9 and cleaved caspase-9 protein. **e** The bar graphs showed the quantification of the caspase-3 and cleaved caspase-3 protein. All results are presented as mean ± SEM (n = 3). *P < 0.05, **P < 0.01 vs. the control group; ***P < 0.01 vs. the 300 μM H₂O₂ group.
affected; however, the two pro-apoptosis proteins were increased when the action time exceeds 12 h (Fig. 3b). The data also confirmed that treatment with 50 μM H₂O₂ for 12 h might be the optimal treatment condition for BMSCs preconditioning in vitro in terms of the expression ratio of anti- and pro-apoptotic proteins.

Further, the expressions of apoptosis-related proteins in BMSCs were measured by Western blot after cells were pretreated with 50 μM H₂O₂ for 12 h, and then treated with or without 300 μM for an additional 24 h. As shown in Fig. 3c–e, 300 μM H₂O₂ led to a sharply decreased Bcl-2/Bax expression ratio (Fig. 3c) and increased expressions of caspase-3 and caspase-9, and cleaved caspase-3 and cleaved caspase-9 proteins (Fig. 3d, e), while 50 μM H₂O₂ preconditioning significantly increased the Bcl-2/Bax expression ratio, and decreased the levels of caspase-3, cleaved caspase-3, and cleaved caspase-9 without having effects on caspase-9. These results indicated that preconditioning BMSCs with 50 μM H₂O₂ for 12 h could markedly suppress oxidative stress-induced activation of the mitochondrial apoptosis pathway through inhibiting the expression of pro-apoptotic proteins.

Fifty-micromolar H₂O₂ preconditioning attenuated oxidative stress-induced mitochondrial dysfunction by inhibiting mitochondrial reactive oxygen species (mtROS) generation in BMSCs

Accumulating excessive ROS in the mitochondria is considered to be one of the major causes of oxidative stress in cells, resulting in perturbation of mitochondrial redox homeostasis and mitochondrial dysfunction. Mitochondrial reactive oxygen species (mtROS) homeostasis plays an essential role in preventing oxidative injury in BMSCs [31]. Since mitochondria are the main source of ROS, determining the fate of cells through regulating multiple signal transduction pathways, and mitochondria are also vulnerable targets. The protective effect of 50 μM H₂O₂ preconditioning BMSCs on the regulation of mitochondrial redox homeostasis (mitochondrial redox status) was evaluated using MitoSOX Red, a highly selective fluorescent probe for detection of the O₂⁻ generated within the mitochondria. The MitoSOX Red reagent is a live-cell permeant and is selectively targeted to the mitochondria. Once in the mitochondria, MitoSOX Red is oxidized by O₂⁻ and exhibits red fluorescence [32]. As shown in Fig. 4a, b, 300 μM H₂O₂ significantly increased mitochondrial O₂⁻ generation compared with the control group. However, this effect was dramatically mitigated by 50 μM H₂O₂ preconditioning in BMSCs. Since the mitochondrial membrane potential (Δψm) is significantly affected by the mitochondrial redox status, further, the effect of 50 μM H₂O₂ preconditioning on mitochondrial membrane potential (Δψm) in BMSCs was measured using JC-1 probe. JC-1 can selectively enter the mitochondria and reversibly changes color as the Δψm changes [33]. As shown in Fig. 4b, c, there was a significantly increased green fluorescence in cells exposed to 300 μM H₂O₂, suggesting that the mitochondrial membrane was depolarized. However, 50 μM H₂O₂ pretreatment repressed the changes of Δψm induced by 300 μM H₂O₂, indicated by a reduction of green fluorescence and restoration of red fluorescence. Moreover, these results suggested that 50 μM H₂O₂ preconditioning for 12 h remarkably suppressed oxidative stress-induced collapse of mitochondrial membrane potential (Δψm) in BMSCs, which was further confirmed that 50 μM H₂O₂ preconditioning protected against oxidative stress-induced apoptosis in BMSCs, at least partly, via restoring MMP function.

Optimal H₂O₂ preconditioning activated PI3K/Akt/mTOR signaling while inhibited GSK-3β activity in BMSCs

The PI3K/Akt/mTOR pathway and GSK-3β protein play a vital role in promoting stem cell survival in response to oxidative stress [17–19, 29]. Exposure to 25–50 μM H₂O₂ induced an obvious increase in the phosphorylation levels of PI3K, Akt, mTOR, and GSK-3β in BMSCs, but they were significantly decreased at 200 μM H₂O₂; inversely, the GSK-3β expression was upregulated at higher H₂O₂ (Fig. 5a–d). The results indicated that low doses of H₂O₂ could activate the PI3K/Akt/mTOR pathway and inhibit GSK-3β activity in BMSCs, whereas a high dose of H₂O₂-induced (200 μM) oxidative stress inhibited this pathway and enhanced GSK-3β activity. Further, the results showed that 50 μM H₂O₂ preconditioning could largely prevent 300 μM H₂O₂-induced inactivating PI3K/Akt/mTOR pathway and activating GSK-3β. This effect was significantly blocked by PI3K inhibitor LY294002 (Fig. 5e); simultaneously, the protective effect of 50 μM H₂O₂ preconditioning was counteracted by LY294002 with a remarkable increase in the apoptotic rate of BMSCs (Fig. 5f, g). These results showed that the protective effect of 50 μM H₂O₂ against oxidative stress-induced BMSC apoptosis was mediated, at least in part, by the activation of the PI3K/Akt/mTOR pathway and inhibition of GSK-3β activity.

Optimal H₂O₂ preconditioning enhanced BMSCs’ tissue engraftment and wound healing in mice

In order to further confirm whether optimal H₂O₂ preconditioning enhanced the target homing efficiency of tail vein-infused BMSCs toward wounds and improved the therapeutic potential of BMSCs in wounds, full-thickness wounds were created at the dorsum of Balb/C mice, followed by the tail vein injection of Dil-labeled BMSCs. The number of Dil-labeled BMSCs in the H₂O₂-preconditioned group at 1 day and 3 days post-
wounding significantly increased compared to that of the un-preconditioned BMSCs group. Compared with the first day post-wounding, the number of BMSCs in the H2O2-preconditioned group decreased only slightly on the third day; however, the number of BMSCs in the un-preconditioned BMSCs group was significantly
reduced on the third day (Fig. 6a, b). The result hinted that the H$_2$O$_2$ preconditioning could not only increase BMSCs migrating and homing toward wounds, but also prolong the residence time of transplanted BMSCs. In addition, the results showed that the original wounds in the H$_2$O$_2$-preconditioning group were significantly smaller compared to the PBS control and BMSC alone groups at days 5, 7, 10, 13, and 15 post-wounding (Fig. 6c) and were completely healed at day 10 post-wounding, but none of the other two groups.

**Fig. 5** The protective effect of optimal H$_2$O$_2$ preconditioning on the activation of PI3K/Akt/mTOR/GSK-3β signaling. a, c Western blot analysis of the expressions of total and phosphorylated PI3K, Akt, mTOR, and GSK-3β proteins in BMSCs exposed to various concentrations of H$_2$O$_2$ (25, 50, 100, and 200 μM) for 24 h. b The bar graphs show the quantitation of phosphorylation of PI3K, Akt, and mTOR protein. d The bar graphs show the quantitation of phosphorylation of GSK-3β protein. Values represent the mean ± SEM (n = 3). *P < 0.05, **P < 0.01 vs. the control group. e Western blot analysis of the expressions of phosphorylated Akt, mTOR, and GSK-3β proteins, and apoptosis-related protein Bcl-2 and cleaved caspase-3 proteins in H$_2$O$_2$-preconditioned BMSCs exposed to 300 μM for 24 h after 2 h pre-incubation with 50 μM LY294002. f Representative images of flow cytometric analysis by annexin V-FITC/PI dual staining. g Apoptotic cells are represented as the percentage of annexin V single-positive plus annexin V-FITC/PI double-positive cells. Values represent the mean ± SEM (n = 3). **P < 0.01 vs. the control group; ***P < 0.01 vs. the 300 μM H$_2$O$_2$ group; ΔΔP < 0.05 vs. the H$_2$O$_2$-preconditioned group.
Fig. 6 Optimal H2O2 preconditioning enhances BMSC tissue engraftment and wound closure. The full-thickness wounds were created at the dorsum of mice, followed by the tail vein injection of PBS and Dil-labeled BMSCs with or without H2O2 preconditioning for 12 h, respectively. a, b Histological analysis of the number of transplanted Dil-labeled BMSCs (red) migrating into newly formed granulation tissue at days 1 and 3 post-wounding. Scale bar, 100 μm. Data are presented as the total cells per field (× 200) ± SEM (n = 5). **P < 0.01 vs. the BMSCs group; ΔΔP < 0.01 vs. the day 1 post-wounding within the same group. c Gross view of excisional wounds at days 0, 5, 7, 10, 13, and 15 post-wounding. d The rate of wound closure after BMSCs engrafting into the wounds. Data are shown as means ± SEM (n = 5). e, f Western blot analysis of the expressions of VEGF of wound tissue after transplantation of BMSCs. Data are shown as means ± SEM (n = 3). *P < 0.05 vs. the PBS group; ΔP < 0.05 vs. the BMSCs group.
Correspondingly, the wound closure rate was the highest in the H$_2$O$_2$-preconditioned group from day 5 to day 15 post-wounding (Fig. 6d). Therefore, the optimal H$_2$O$_2$ preconditioning not only increased the migration of BMSCs into the wound areas, but also accelerated wound closure.

Further, the growth factor detection and histological analysis of wounds were performed to assess the degree of wound healing and regeneration. The results showed that the level of VEGF in the two BMSCs groups was significantly higher compared to that of the PBS group (Fig. 6e, f), and the microvessel density was the highest in the H$_2$O$_2$-preconditioned group at days 5, 7, and 10 post-wounding (Fig. 7a, b). Similarly, compared with the other two groups, H&E staining showed the greatest increase in cell density and blood vessel density in the granulation tissue of the H$_2$O$_2$-preconditioned group at days 5 post-wounding (Fig. 7c). Moreover, a larger and better-organized collagen deposition was observed in the wounds of H$_2$O$_2$-preconditioned BMSC mice at days 7 post-wounding, showing a significantly higher collagen density compared with the PBS control and BMSCs alone groups (Fig. 7d, e). These data indicated that the transplanted BMSCs after optimal H$_2$O$_2$-preconditioning not only augmented tissue engraftments and accelerated wound closure, but also significantly promoted collagen deposition and angiogenesis in wounds, and thus facilitated wound healing.

**Discussion**

Although cell-based therapy plays an important role in the field of skin tissue regeneration, low engraftment efficiency and poor survival rate of transplanted stem cells triggered by oxidative stress at the wound tissue limit the therapeutic potential of MSCs [2, 3]. Accumulating evidences suggest that H$_2$O$_2$ preconditioning could enhance the migration and survival of MSCs [22–24, 34]. However, optimal H$_2$O$_2$ concentration, suitable treatment time course, and the underlying mechanism remained largely undetermined.

Different concentrations of H$_2$O$_2$ profoundly affect the biological functions of stem cells [25–27]. For a long time, H$_2$O$_2$ was considered deleterious molecules. However, recent studies suggest that H$_2$O$_2$ is necessary for effective self-renewal in stem cells [16, 25, 35]. In general, moderate levels of H$_2$O$_2$ may function as signals to promote cell proliferation and survival, playing a growth factor-like role in cells. However, a severe increase of H$_2$O$_2$ induces senescence and oxidative stress in MSCs [16, 27]. Thus, exploring the optimal concentration of H$_2$O$_2$ concentration, suitable treatment time course, and the underlying mechanism remained largely undetermined.
H₂O₂ for MSC preconditioning in vitro is a prerequisite for improving the therapeutic potential of MSCs. Indeed, during the oxidative stress experiment of BMSCs with various concentrations of H₂O₂, we identified that H₂O₂ at 25 μM and 50 μM significantly facilitated the proliferation of BMSCs, accompanied by an increase in the expression of the cell cycle-promoting protein cyclin D1 and decrease in the cell cycle blocker p16 expression; 100 μM H₂O₂ has not impacted cell growth, and over 150 μM H₂O₂ induced cell growth arrest with downregulating cyclin D1 and upregulating p16 expressions, accompanied by BMSC apoptosis. Importantly, we found 50 μM H₂O₂ optimally promoted BMSC proliferation and maximized the expression of cyclin D1, while the greatest degree of suppression of p16 expression, which was suggested 50 μM H₂O₂, may be an optimal concentration to promote stem cell growth. Simultaneously, we confirmed that 50 μM H₂O₂ maximized the promotion of SDF-1 and its receptors CXCR4/7 expression that were conducive to the enhancement of cell migration and survival, respectively [7, 8]. In fact, we observed that 50 μM H₂O₂ treatment could markedly promote the migration capability of BMSCs in vitro; however, the effect was eliminated by the CXCR4 antibody, which indicated H₂O₂-promoting migration of BMSCs through augmenting the SDF-1/CXCR4 axis. Thus, these results confirmed the important effect of H₂O₂ concentration on the biological characteristics of stem cells and also confirmed that a low concentration of H₂O₂ had a growth factor-like effect to promote stem cell proliferation. Although it has been reported that the concentration of stem cells preconditioned with H₂O₂ is 25 μM, 50 μM, and 100 μM [23, 24], there is no sufficient basis for deciding which H₂O₂ concentration to be chosen, and we believed that 50 μM H₂O₂ represent the optimal concentration of MSC preconditioning.

It is well known that oxidative stress induces apoptosis through the intrinsic mitochondrial pathway [29]. The expression ratio of anti-apoptotic Bcl-2 and pro-apoptotic Bax (Bcl-2/Bax) is the key to maintaining mitochondrial membrane stability, preventing mitochondrial depolarization and the release of cytochrome c into the cytoplasm, and thereby inhibiting the mitochondrial apoptotic pathway [29]. High concentrations of H₂O₂-induced oxidative stress have been shown to induce mitochondrial injury by reducing mitochondrial membrane potential and decreasing the expression ratio of Bcl-2/Bax, subsequently initiating apoptosis [30]. In the present study, we similarly confirmed that 50 μM H₂O₂ could maximize the upregulation of Bcl-2 and Bcl-2/Bax expression ratio with no changes in pro-apoptotic proteins Bax and cleaved-caspase3 expressions, when using different concentrations of H₂O₂ to stimulate stem cells for 12 h. Further, it was found that pretreatment BMSCs for 12 h was the optimal time course when using 50 μM H₂O₂ to treat BMSCs with different time course, because the Bcl-2 and Bcl-2/Bax expression ratio peaked with no changes in Bax and cleaved-caspase3 expressions. Lastly, we suggested that BMSC preconditioning with 50 μM H₂O₂ for 12 h is the optimal protocol for resistance to oxidative stress apoptosis through maximizing increasing the Bcl-2/Bax expression ratio. In addition, we also confirmed that 50 μM H₂O₂ could markedly promote the migration capability of BMSCs through maximizing augmenting the SDF-1/CXCR4/7 axis. Further, the results confirmed that the optimal preconditioning protocol (50 μM H₂O₂ for 12 h) could significantly enhance MSC survival under oxidative stress and suppress the mitochondrial apoptotic pathway through increasing the expression ratio of Bcl-2/Bax and inhibition of apoptosis executive proteins cleaved caspase-9 and cleaved caspase-3 expressions. However, LY294002 abolished the cytoprotective effect of 50 μM H₂O₂ preconditioning. The data suggested that the PI3K/Akt pathway was involved in the preconditioning-induced cytoprotective effect.

It was reported that the PI3K/Akt pathway has been shown to play a major role in the promotion of cell survival, and prevention of apoptosis in response to oxidative stress [16, 21, 36]. The activation of PI3K/Akt causes a cascade of downstream responses from mTOR, Bcl-2, Bax, and GSK-3β, which all regulate cellular functions, such as cell migration, growth, proliferation, survival, and differentiation [16, 21] (Fig. 8). The PI3K/Akt pathway regulates cell migration via activation of mTOR, promotes cell survival via Bcl-2/Bax, and facilitates cell proliferation, differentiation, and resistance apoptosis via inhibition of GSK-3β [37, 38]. Akt directly phosphorylates GSK-3β at Ser9 which negatively regulates its kinase activity [39]. Phosphorylated GSK-3β, a key molecule, inhibits oxidative stress-induced apoptosis through inhibition of the opening of mitochondrial permeability transition pore (mPTP), thereby suppressing cytochrome C release for mitochondria to cytosol and preventing cell apoptosis [39]. And also, Akt-induced GSK-3β phosphorylated leads to the accumulation of β-catenin in the nucleus [38], which is a transcriptional factor that increases the expression of c-Myc to promote cell proliferation through upregulation of cyclin D1 expression, while inhibiting p16 expression, leading to promoting cell proliferation. In the present study, 25–100 μM H₂O₂ upregulated the phosphorylated levels of PI3K, Akt, mTOR, and GSK-3β, resulting in activating the PI3K/Akt/mTOR pathway and inhibiting GSK-3β activity; the effect of 50 μM H₂O₂ is the strongest. These results further demonstrated that a low dose of H₂O₂ possesses growth factor-like characteristics, which could activate the PI3K/Akt pathway.
pathway. The growth hormones and cytokines are the upstream stimulators of this pathway [17, 25]. SDF-1 could promote MSC survival and migration also by the activation of the PI3K/AKT pathway [9, 10, 18–21]. In addition, low dose H2O2 directly activated this pathway through diffusion into the cytoplasm as a second messenger may be another activation pathway [17, 25] (Fig. 8). Inversely, we confirmed that a high concentration of H2O2 (300 μM) oxidative stress not only inhibited the PI3K/Akt/mTOR pathway and increased the GSK-3β activity by inhibiting the phosphorylated of these key proteins, but also significantly increased the apoptotic rate of BMSCs. Hence, oxidative stress-induced apoptosis was mediated by the inactivation of the PI3K/Akt/mTOR pathway and increased GSK-3β activity, which was consistent with the literature reports [16, 21, 36]. However, we found that 50 μM H2O2 preconditioning could reduce the inhibition of the PI3K/Akt pathway induced by oxidative stress through upregulation of the phosphorylation of PI3K, Akt, mTOR, and GSK-3β, and the expression of Bcl-2 in BMSCs, finally, resulting in inhibiting of the mitochondrial apoptosis pathway. Therefore, the protective effect of 50 μM H2O2 preconditioning might be dependent on the activation of the PI3K/Akt signaling pathway, contributing inhibition of its downstream target GSK-3β activity and upregulation of anti-apoptotic Bcl-2 expression.

The success of stem-cell therapy depends on the migration and survival of the transplanted cells [40, 41]. Therefore, enhancing the migration ability and survival of BMSCs are the key to optimize stem cell therapy in wounds. Based on the vitro experiment, we confirmed that 50 μM H2O2 maximized the migration

**Fig. 8** Schematical representation of the protective mechanism of H2O2 preconditioning for improving the therapeutic potential of BMSCs. A low dose of H2O2 (50 μM) acts in the signaling pathways as a second messenger. H2O2 preconditioning-induced BMSC protection seems to involve augmenting the SDF-1/CXCR4 axis and activation of PI3K/Akt/mTOR and inhibiting GSK-3β activity, leading to enhancing abilities of BMSC proliferation, migration, angiogenesis, and anti-apoptosis. And the PI3K/Akt pathway is also one of the downstream pathways that activated the SDF-1/CXCR4 axis [9, 10, 18–21]. In addition, low-dose H2O2 directly activated this pathway through diffusion into the cytoplasm as a second messenger may be another activation pathway [17, 25]. The PI3K inhibitor (LY294002) could block the protective effect induced by 50 μM H2O2 preconditioning.
and viability of stem cells, thereby hypothesizing that H_2O_2 preconditioning may enhance BMSCs homing toward wounds and improve their therapeutic efficacy. As we expected, 50 μM H_2O_2 preconditioning for 12 h contributed to more efficient engraftment of transplanted BMSCs into the wounds via enhancement of their migration capacity and increasing their survival by prolonging their retention time in the wounds. This result was consistent with the outcomes of in vitro migration studies in which 50 μM H_2O_2-preconditioned BMSCs displayed enhancement of migration and survival.

Numerous studies confirm that transplanted MSCs could promote wound healing [42] and suggest that the differentiation and paracrine signaling have both been implicated as mechanisms by which MSCs improve tissue repair. However, growing evidence indicates that MSC paracrine signaling is the predominant mechanism responsible for enhanced wound repair though accelerating wound closure, enhancing reepithelialization, increasing angiogenesis, and promoting granulation tissue formation [42]. Further studies confirm that the pretreatment of stem cells with cytokines, drugs, and reagents can enhance their paracrine function and therapeutic potential of stem cells thereby improving wound healing [15, 23, 42]. In fact, the therapeutic effect of H_2O_2-preconditioned BMSCs was significantly better than that of un-preconditioned BMSCs in terms of an increase in the speedy wound closure rate. The higher VEGF levels and the microvessel density in wound healing tissues indicated the critical role of H_2O_2-preconditioned BMSCs in elevating angiogenesis for accelerated wound healing. Taken together, these results indicated that the preconditioning of BMSCs with H_2O_2 enhanced their therapeutic potential by increasing their engraftment efficiency in wounds, responsive secretion of VEGF, and facilitating neovascularization, thereby improving the quality of wound healing.

Conclusions
This study first demonstrated that pretreated BMSCs with 50 μM H_2O_2 for 12 h was the optimal preconditioning protocol, which was indicated by maximizing activating the pathways of SDF-1/CXCR4 and PI3K/Akt/mTOR and inhibiting GSK-3β activity, thereby playing a cytoprotective role in oxidative stress-induced BMSC apoptosis. Finally, the data confirmed that BMSCs preconditioned with this protocol could significantly improve the therapeutic potential of transplanted stem cells, which might represent an attractive intervention strategy to promote wound healing.

Supplementary information
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Additional file 1: Supplement Fig. 1. The BMSCs have maintained the multipotent differentiation capacity of stem cells even after pretreated with 50 μM H_2O_2 for 12 h.

Abbreviations
CM-DiI: Chloromethyl-1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate; BMSC: Bone marrow mesenchymal stem cells; MSCs: Mesenchymal stem cells; H_2O_2: Hydrogen peroxide; VEGF: Vascular endothelial growth factor; SDF-1: Stromal cell-derived factor-1; CXCR4: CXC chemokine receptor 4; PI3K: Phosphoinositide 3-kinase; Akt: Protein kinase B; mTOR: Mammalian target of rapamycin; GSK-3β: Glycogen synthase kinase-3β; ROS: Reactive oxygen species; FCM: Flow cytometric; mPTP: Mitochondrial permeability transition pore

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Authors’ contributions
HH conceived and designed the study, conducted the experiments, interpreted the data, and prepared the manuscript. LG, JD, SZ, DY, and HZ carried out the experiment. HH, LZ, AZ, JD, and DY takes responsibility for the integrity of the data and the accuracy of the data analysis. MC, DW, JS, JM, and YZ performed the animal experiments. YN and DL performed the cell culture. JJ and HH performed the histological analysis and reviewed the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The data that support the findings of this study are available from the corresponding author upon request.

Ethics approval and consent to participate
All animal studies were according to the protocols approved by the Laboratory Animal Committee of Army Medical University (Approval No. 2019–103).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no conflicts of interest.

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