Optimal H2O2 preconditioning to improve BMSCs’ engraftment in wounds healing

Ling Guo  
Army Medical University

Ya Zhang  
Army Medical University

Juan Du  
Army Medical University

Dan Feng Yuan  
Army Medical University

Shu Zhang  
Army Medical University

Hua Cai Zhang  
Army Medical University

Jun Wei Mi  
Army Medical University

Ya Lei Ning  
Army Medical University

Min Jia Chen  
Army Medical University

Da Lin Wen  
Army Medical University

Jian Hui Sun  
Army Medical University

Di Liu  
Army Medical University

Ling Zeng  
Army Medical University

An Qiang Zhang  
Army Medical University

Jian Xin Jiang  
Army Medical University

Hong Huang (✉ huanghongcq@126.com)  
Army Medical University https://orcid.org/0000-0001-7688-3506
Research

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

DOI: https://doi.org/10.21203/rs.3.rs-28765/v1

License: ☑️ ☑️ This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License
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.

The optimal protocol for BMSCs preconditioned with $H_2O_2$ was investigated, and the therapeutic efficacy of preconditioned BMSCs in wounds was evaluated.

Methods.

Mouse BMSCs were exposed to various concentrations of $H_2O_2$, and their functions were assessed; The $H_2O_2$-preconditioned BMSCs were transplanted into mice with full-thickness excisional wounds. Wound analysis was performed to assess the transplantation efficacy.

Results.

Treatment BMSCs with 50 µM $H_2O_2$ for 12 h could enhance their proliferation, migration and survival by maximizing up-regulation the 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-BMSCs apoptosis was significantly attenuated by an obviously decreased ratio of Bax/Bcl-2 and cleaved caspase-9/3 expression. After transplantation of BMSCs, the migration of $H_2O_2$ preconditioned-BMSCs into the wounds was dramatically increased compared to un-preconditioned-BMSCs, and had an increased microvessel density and wound closure speed.

Conclusions.

The findings suggested that 50 µM $H_2O_2$ pretreated for 12 h is the optimal precondition for the transplantation of BMSC, 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.

1. Introduction

Growing evidence indicates that BMSCs based therapy for cutaneous wound healing hold great therapeutic value via differentiating into specialized cell types, producing a large variety of growth
factors, and promoting wound closure and angiogenesis\[1–3\]. 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 \[4, 5\] and enhance the cell migration capacity and survival rate. Reportedly, the injured tissue expresses 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 cells surface of MSCs and promotes stem cells homing and survival in the injured tissue, respectively \[6, 7\]. However, during in vitro culture expansion, BMSCs lose their CXCR4 receptor \[8\], and reduce their binding capacity to SDF-1 resulting in \[9, 10\] 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 \[11–13\].

Reactive oxygen species (ROS)-mediated-oxidative stress is an important cause for apoptosis and death of donor MSCs following engraftment \[4, 5, 11, 12\]. Mechanistically, The PI3K/AKT signaling pathway plays central regulatory roles in MSC survival, proliferation, migration, angiogenesis, cytokine production, and differentiation \[14–16\]. SDF-1 could promote MSC survival and migration by the activation of the PI3K/AKT pathway \[8, 9, 17–20\]. Several studies demonstrated that H\(_2\)O\(_2\) preconditioning protects cells against oxidative damage \[21–23\]. In fact, H\(_2\)O\(_2\) at certain low level acts as essential cellular messengers by playing a growth factor-like role in regulating physiological stem cellular processes \[24–26\]. Therefore, we speculated low dose H\(_2\)O\(_2\) functioned as a growth factor-like molecule to activate the PI3K/AKT pathway. Although multiple studies have confirmed the cytoprotective effect of H\(_2\)O\(_2\) preconditioning on MSCs against oxidative damage, neither the optimal concentration of H\(_2\)O\(_2\) used in preconditioning nor underlying molecular mechanism has been fully established \[21–23\].

In this study, the optimal concentration of H\(_2\)O\(_2\) on BMSCs preconditioning was first determined. Then the molecular mechanisms of H\(_2\)O\(_2\) preconditioning BMSCs was explored by focusing on PI3K/AKT pathway due to its role in MSCs survival and migration responding to SDF-1 and oxidative stress \[27\]. The ultimate therapeutic potential of H\(_2\)O\(_2\)-preconditioned BMSCs was evaluated in a mouse full-thickness wound model.

2. Materials And Methods

2.1 Ethics Statement

Animal studies were performed using 4-week-old 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. Primary BMSCs were isolated and cultivated as previously described[10].

2.2 Cell viability assay

Cell viability was assessed using the MTT assay. BMSCs were seeded in 96-well plates and treated with various concentrations of H$_2$O$_2$ for 24 h in a cell culture incubator, 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Sigma Aldrich, USA) was added and incubated for 4 hours at 37 °C. The optical density of solubilized formazan was measured at 570 nm using an automatic microplate reader.

2.3 Scratch wound-healing migration assay

Scratch assays were performed with BMSCs grown to 90% confluence in six-well plates. Scratches were made on the plates with a standard 200 µL pipette tip to make a line-shaped wound. And BMSCs were incubated in culture medium with or without 50 µmol/L H$_2$O$_2$ for 12 h and 24 h at 37°C. The cells were allowed to migrate and images were acquired under an inverted microscope (Nikon, Tokyo, Japan).

2.4 Cell migration

Migration assays were carried out in a 24-well Transwell migration system with an 8-µm pore size polycarbonate membrane. BMSCs, preconditioned or not with H$_2$O$_2$ (50 µmol/L) for 12 hours after incubating with or without CXCR4 antibody for 2 hours, 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 was 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), and represented as the average of three independent experiments.

2.5 Flow cytometry analysis of BMSCs apoptosis

Apoptotic cells were also detected by Annexin V-FITC/PI Kit (KeyGen Biotech, Nanjing, China). Briefly, BMSCs were treated with various concentrations of H$_2$O$_2$ for 24 h before preconditioned with or without 50 µmol/L of H$_2$O$_2$ for 12 h; Or cells were exposed to 12-hour preconditioning with H$_2$O$_2$ after a 2-hour pre-incubation with or without PI3K inhibitor LY294002 (Sigma, US), followed by treatment with 300 µmol/L H$_2$O$_2$ for 24 h. The flow cytometry assay referenced kit instructions.

2.6 Osteogenic, chondrocytic and adipogenic differentiation

BMSCs pretreated with 50 µmol/L H$_2$O$_2$ 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.
2.7 Wound healing model and BMSCs transplantation

The full-thickness excisional skin wounds in Balb/C mice were made as previously reported [8]. Mice were randomly divided into three groups, which received an injection of 100μL PBS, 1 × 10^6 BMSCs in 100μL PBS and 1 × 10^6 H_2O_2-proconditioned (for 12 h) BMSCs in 100μL PBS, respectively. BMSCs were pre-labeled with chloromethyl-1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate (CM-Dil, Sigma) before injection. Mice post-wounding at once were injected with DiI-labeled BMSCs or PBS via the tail vein.

2.8 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 Image Propus 5.0. 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 were collected for histological and protein analysis. The photos were taken by randomly selecting 5 high-power fields (hpf, × 200), and analyzed using Image Propus 5.0 to calculate the total number of migrated BMSCs with CM-Dil label (red cells) at the wound site.

2.9 Microvessel density of the wound area

Microvessel density was determined by immunohistochemical staining with anti-CD31 antibody (Santa Cruz), according to manufacturer’s instructions and a method previously described [10].

2.10 Western blot analysis

Total protein lysates of cultured cells and wound tissues were prepared using RIPA lysis buffer, and 50 mg total protein samples were separated by SDS-PAGE on 10% gels. Immunoblotting was performed with the following 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, UK). Band density was analyzed using LabWorks 4.6 analysis software.

2.11 Statistical analysis

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

3. Results
3.1 Pretreatment BMSCs with 50 µM H₂O₂ significantly enhanced cell viability/proliferation and migration by augmenting SDF-1/CXCR4/CXCR7 axis

To define the optimal concentration of H₂O₂ in preconditioning BMSCs, we selected five different concentrations between 20-200µM based on previous reports[28] and investigated its effects on cellular properties of viability, proliferation, migration and differentiation. We found that H₂O₂ at 25 µM and 50 µM significantly promoted the proliferation of BMSCs, accompanying by increase in cyclin D1 expression and decrease in p16 expression, whereas 150 and 200 µM H₂O₂ showed a significant inhibitory effect compared with that of the control. 50 µM H₂O₂ caused a peak cyclin D1 expression and a low p16 expression, which represented the optimal concentration of H₂O₂ for BMSCs preconditioning in terms of cell proliferation/viability from this study (Fig. 1A, B).

In a scratch assay, BMSCs with 50 µM H₂O₂ preconditioned showed a significantly higher migration capacity compared to that of non-preconditioned BMSCs (Fig. 1C). The Boyden chamber assay was further demonstrated that the number of migrating BMSCs in 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. 1D, E). Simultaneously, the expression of SDF-1, CXCR4 and CXCR7 in H₂O₂ group was significantly upregulated at 25 µM and 50 µM compared to that of the control (Fig. 1F). These results suggested the 50 µM H₂O₂ treatment for 12 h could significantly enhance the BMSCs viability 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 (Toluidine blue, Alcian blue), as well as osteoblastic (alkaline phosphatase) lineages. This results showed that 50 µM H₂O₂ treatment did not affected the differentiation capacity of BMSCs.

3.2 Optimal H₂O₂ preconditioning suppressed cell death of BMSCs via mitochondria mediated mechanism

Oxidative stress induces apoptosis through the intrinsic mitochondrial pathway [29]. The ratio of anti-apoptotic Bcl-2 and pro-apoptotic Bax (Bcl-2/Bax) are the key to the mitochondrial apoptotic pathway [29]. The flow cytometric (FCM) analysis showed that exposure to 25–100 µM H₂O₂ did not cause BMSCs apoptosis (Fig. 2A). However, exposure to 150–500 µM H₂O₂ induced an obvious increase in apoptotic rate of BMSCs in a dose-dependent manner (Fig. 2A, B), compared with the control. The results showed that H₂O₂ exceeding 100 µmol/L might induce oxidative stress thereby leading to cell apoptosis. Moreover, the result demonstrated that the apoptotic rate of BMSCs was very significantly lower in H₂O₂ preconditioned each group than in corresponding high doses of H₂O₂ groups (Fig. 2C, D). 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, B, C). Subsequently, after treatment of BMSCs with 50 µM H$_2$O$_2$ at different times, it was found that when H$_2$O$_2$ treated for 12 h, the Bcl-2/Bax ratio reached a peak, while the expression of Bax and caspase-3 was not affected, however, the two pro-apoptosis proteins were increased when the action time exceeds 12 h (Fig. 3D, E). The data also confirmed that treatment with 50 µM H$_2$O$_2$ for 12 h might be the optimal pretreatment condition for BMSCs preconditioning in vitro in terms of expression ratio of anti- and pro-apoptotic genes.

In addition to increasing the Bcl-2/Bax expression ratio, BMSCs preconditioned with 50 µM H$_2$O$_2$ for 12 h significantly decreased level of cleaved caspase-3 without having effects on caspase-9 and -3, and the level of cleaved caspase-9 (Fig. 3F-H). This was in contrast to the effects by 300 µM H$_2$O$_2$ treatment. These results indicated that preconditioning BMSCs with 50 µM H$_2$O$_2$ for 12 h could suppress oxidative stress-induced activation of mitochondrial apoptosis pathway.

### 3.3 Optimal H$_2$O$_2$ 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 H$_2$O$_2$ induced an obvious increase in the phosphorylation levels of PI3K, AKT, mTOR and GSK-3β in BMSCs, but they were significantly deceased at 200 µM H$_2$O$_2$, inversely, the GSK-3β expression was up-regulated at higher H$_2$O$_2$ (Fig. 4A-D). The results indicated that low doses of H$_2$O$_2$ could activate the PI3K/Akt/mTOR pathway and the inhibit GSK-3β activity in BMSCs, whereas high dose of H$_2$O$_2$-induced oxidative stress inhibited this pathway and enhanced GSK-3β activity. Further, the results showed that 50 µM H$_2$O$_2$ preconditioning could largely prevent 300 µM H$_2$O$_2$-induced inactivating PI3K/Akt/mTOR pathway and activating GSK-3β. This effect was significantly blocked by PI3K inhibitor LY294002 (Fig. 4E), simultaneously, the protective effect of 50 µM H$_2$O$_2$ preconditioning was counteracted by LY294002 with a remarkable increase in the apoptotic rate of BMSCs (Fig. 4F, G). These results showed that the protective effect of 50 µM H$_2$O$_2$ against oxidative stress-induced BMSCs apoptosis was mediated, at least in part, by activation of PI3K/Akt/mTOR pathway and inhibition of GSK-3β activity.

### 3.4 Optimal H$_2$O$_2$-preconditioning enhanced BMSCs’ tissue engraftment and wound healing in mice

In order to further confirm whether H$_2$O$_2$ preconditioning enhanced the target homing efficiency of tail vein infused BMSCs towards wounds and improved the therapeutic potential of BMSCs in wounds, the full thickness wound model in Balb/C mice was established. The number of Dil-labeled BMSCs in the H$_2$O$_2$-preconditioned group at 1 day and 3 day 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 H$_2$O$_2$-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. 5A, 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. The wound closure rate was markedly higher in two BMSCs groups than in the PBS control group and was the highest in H$_2$O$_2$-preconditioned group from day 5 to day 15 post-wounding (Fig. 5C, D). Therefore, H$_2$O$_2$ preconditioning not only increased migration of BMSCs into wound areas, but also accelerated wound closure. Further, the results showed that the level of VEGF in the two BMSCs groups were significantly higher compared to that of the PBS group (Fig. 5E, F), and the microvessel density were the highest in H$_2$O$_2$-preconditioned group at 5, 7 and 10 day post-wounding (Fig. 5G, H). Similarly, compared with the other two groups, HE staining showed the greatest increase in cell density and blood vessel density in the granulation tissue of H$_2$O$_2$-preconditioned group at 5 days post-wounding (Supplement Fig. 2). These results suggested that H$_2$O$_2$-preconditioned BMSCs improved tissue engraftments, and local angiogenesis that resulted in accelerated wound healing.

4. Discussion

Although cell-based therapy plays an important role in the field of the 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 survival of MSCs [21–23]. 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 biological functions of stem cells [24–26]. 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 [15, 24, 30]. 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, severe increase of H$_2$O$_2$ induces senescence and oxidative stress in MSCs [15, 26]. Thus, exploring the optimal concentration of H$_2$O$_2$ for MSCs 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 H$_2$O$_2$, we identified that H$_2$O$_2$ at 25 µM and 50 µM significantly facilitated the proliferation of BMSCs, accompanied by increase in expression of the cell cycle-promoting proteins cyclin D1 and decrease in the cell cycle blocker p16 expression. 100 µM H$_2$O$_2$ was not impacted cell growth, Over 150 µM H$_2$O$_2$ induced cell growth arrest with down-regulating cyclin D1 and up-regulating p16 expressions, accompanying by BMSCs apoptosis. Importantly, we found 50 µM H$_2$O$_2$ optimally promoted BMSCs proliferation and maximized the promotion of cyclin D1, while the greatest degree of suppression of p16 expression, which was suggested 50 µM H$_2$O$_2$ may be an optimal concentration to promote stem cell growth.

Simultaneously, we confirmed that 50 µM H$_2$O$_2$ maximized the promotion of SDF-1 and its receptors CXCR4/7 expression, which was also conducive to the enhancement of cell migration and survival,
respectively [6, 7]. In fact, we observed that 50 μM H₂O₂ treatment could markedly promote migration capability of BMSCs in vitro, however, the effect was eliminated by 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 biological characteristics of stem cells, and also confirmed that 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[22, 23], but there's no sufficient basis for deciding which H₂O₂ concentration to choose, and we believed that 50 μM H₂O₂ represent the optimal concentration of stem cells preconditioning in our study.

It is well known that oxidative stress induces apoptosis through the intrinsic mitochondrial pathway [28]. The expression ratio of anti-apoptotic Bcl-2 and pro-apoptotic Bax (Bcl-2/Bax) are 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 [28]. 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 [29]. In the present study, we similarly confirmed that 50 μM H₂O₂ could maximize the up-regulation of Bcl-2 and Bcl-2/Bax expression ratio with no changes in pro-apoptotic proteins Bax and cleaved-caspase-3 expressions, when using different concentrations of H₂O₂ to stimulate stem cells for 12 hours. 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-caspase-3 expressions. Lastly, we suggested that stem cell preconditioning with 50 μM H₂O₂ for 12 h is the optimal protocol for resistance to oxidative stress apoptosis of BMSCs through maximizing increasing the Bcl-2/Bax expression ratio, in addition, we also confirmed that 50 μM H₂O₂ could markedly promote 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 BMSCs survival under oxidative stress and suppress mitochondrial apoptotic pathway through increase the expression ratio of Bcl-2/Bax and inhibition of apoptosis executive proteins cleaved caspase-9 and − 3 expressions. However, LY294002 abolishes of the cytoprotective effect of 50 μM H₂O₂ preconditioning. The data suggested that the PI3K/Akt pathway was involved in 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 [15, 20, 31]. The activation of PI3K/Akt causes a cascade of a downstream responses from mTOR, Bcl-2, Bax and GSK-3β, which all regulate cellular functions [15, 20]. The PI3K/Akt pathway regulates cell migration via activation of mTOR, increases cell survival via Bcl-2/Bax, and increases cell proliferation and against apoptosis via inhibition of GSK-3β [32, 33]. Akt directly phosphorylates GSK-3β at Ser9 which negatively regulates its kinase activity [34], phosphorylated GSK-3β, a key molecules 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 [34]. And also, Akt induced GSK-3β phosphorylated leads to the accumulation of β-catenin in the nucleus [33], which is a transcriptional factor that increases the expression of c-Myc to promote cell proliferation through up-regulation 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 of PI3K/Akt/mTOR pathway and inhibiting GSK-3β activity, the effect of 50 µM H₂O₂ is the strongest. These results further demonstrated that low dose of H₂O₂ possesses growth factor-like characteristics, which could activate the PI3K/Akt pathway. The growth hormones and cytokines are the upstream stimulators of this pathway [16, 24]. SDF-1 could promote MSC survival and migration also by the activation of the PI3K/AKT pathway [8, 9, 17–20]. In addition, low dose H₂O₂ directly activated this pathway through diffusion into the cytoplasm as a second messenger may be another activation pathway [16, 24]. Inversely, we confirmed that high concentration of H₂O₂ (300 µM)-induced oxidative stress not only inhibited the PI3K/Akt/mTOR pathway and increased the GSK-3β activity by inhibiting the phosphorylated of these proteins in this pathway, but also significantly increased apoptotic rate of BMSCs. Hence, oxidative stress-induced apoptosis was mediated by inactivation of the PI3K/Akt/mTOR pathway and increased GSK-3β activity, which was consistent with literature reports [15, 20, 31]. However, we found that 50 µM H₂O₂ preconditioning could reduce the inhibition of PI3K/Akt pathway induced by oxidative stress through promoting the phosphorylation of PI3K, Akt, mTOR, and GSK-3β of this pathway and increasing in Bcl-2 expression in BMSCs, finally, resulting in inhibition of 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 [35, 36]. Therefore, enhancing the migration ability and survival of BMSCs are the key to optimize stem cell therapy in wounds. Based on the vitro experiment, in which we confirmed that 50 µM H₂O₂ maximized the migration and viability of stem cells, thereby hypothesizing that H₂O₂ preconditioning may enhance BMSCs homing toward wounds and improved their therapeutic efficacy. As we expected, 50 µM H₂O₂ preconditioning for 12 h contributed to more efficient engraftment of transplanted BMSCs into wounds via enhancement of their migration capacity, and increasing in their survival by prolonging their retention time in wounds. This result was consistent with the outcomes of in vitro migration studies where 50 µM H₂O₂ pretreated BMSCs displayed enhancement of migration and survival.

Although some studies showed that transplanted MSCs could promote wound healing [37], we observed that the therapeutic effect of H₂O₂-preconditioned BMSCs was significantly better than that of un-preconditioned BMSCs in terms of increase in the speedy wound closure rate. The higher VEGF levels and the microvessel density in wound healing tissues indicated the critical role of H₂O₂-preconditioned BMSCs in elevating angiogenesis for accelerated wound healing. Taken together, these results indicated
that the preconditioning of BMSCs with $\text{H}_2\text{O}_2$ 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.

5. Conclusions

This study first demonstrated that pretreated BMSCs with 50 µM $\text{H}_2\text{O}_2$ for 12 hours 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 BMSCs apoptosis. Finally, the data confirmed that BMSCs preconditioned with this protocol could significantly improve therapeutic potential of transplanted stem cells, which might represent an attractive intervention strategy to promote wound healing.

Abbreviations

CM-Dil
Chloromethyl-1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate
BMSCs
Bone marrow mesenchymal stem cells
MSCs
Mesenchymal stem cells
$\text{H}_2\text{O}_2$
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

Declarations

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

Conflicts of Interest
The authors declare that they have no conflicts of interest.

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

Fundings
This work was supported by the National Natural Science Foundation of China (No. 81571912 and 81372059), the Foundational and Cutting-edge Research Plan of Chongqing Special Projects for Academicians (No. cstc2017zdcy-yszxX0002), Open fund topic of State Key Laboratory of Trauma, Burns and Combined Injury (No.200717), and the National Natural Science Foundation of China for key project (81530063). And It was also supported by Innovation Team Building at Institutions of Higher Education in Chongqing (CXTDX201601005).

Authors’ Contributions
HH conceived and designed the study, conducted experiments, interpreted data, and prepared the manuscript. LG, JD, SZ and HZ carried out the experiment. HH, LZ, and AZ 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. DY 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.

Acknowledgment
We thank Xinli Zhang and Alan Chien at University of California Los Angeles for the excellent assistance in English editing, We also thank Drs Zhan Li and Wei Qiu in our Center for their technical assistance.

References
1. Caplan, A.I. and D. Correa, *The MSC: an injury drugstore*. Cell Stem Cell, 2011. 9(1): p. 11-5.

2. Isakson, M., et al., *Mesenchymal Stem Cells and Cutaneous Wound Healing: Current Evidence and Future Potential*. Stem Cells Int, 2015. 2015: p. 831095.

3. Trounson, A. and C. McDonald, *Stem Cell Therapies in Clinical Trials: Progress and Challenges*. Cell Stem Cell, 2015. 17(1): p. 11-22.

4. Haider, H. and M. Ashraf, *Preconditioning and stem cell survival*. J Cardiovasc Transl Res, 2010. 3(2): p. 89-102.

5. Peart, J.N. and J.P. Headrick, *Sustained cardioprotection: exploring unconventional modalities*. Vascul Pharmacol, 2008. 49(2-3): p. 63-70.

6. Askari, A.T., et al., *Effect of stromal-cell-derived factor 1 on stem-cell homing and tissue regeneration in ischaemic cardiomyopathy*. Lancet, 2003. 362(9385): p. 697-703.

7. Cencioni, C., M.C. Capogrossi, and M. Napolitano, *The SDF-1/CXCR4 axis in stem cell preconditioning*. Cardiaco Rev, 2012. 94(3): p. 400-7.

8. Marquez-Curtis, L.A. and A. Janowska-Wieczorek, *Enhancing the migration ability of mesenchymal stromal cells by targeting the SDF-1/CXCR4 axis*. Biomed Res Int, 2013. 2013: p. 561098.

9. Ceradini, D.K., Anita & Callaghan, Matthew & Tepper, Oren & Bastidas, Nicholas & Kleinman, Mark & Capla, Jennifer & Galiano, Robert & Levine, Jamie & Gurtner, Geoffrey, *Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1*. Nature medicine, 2004. 10: p. 858-64.

10. Xu X, Z.F., Zhang M, Zeng D, Luo D, Liu G, Cui W, Wang S, Guo W, Xing W, Liang H, Li L, Fu X, Jiang J, Huang H, *Stromal cell-derived factor-1 enhances wound healing through recruiting bone marrow-derived mesenchymal stem cells to the wound area and promoting neovascularization*. Cells, Tissues, Organs, 2013. 197(2): p. 103-113.

11. Amiri, F., A. Jahanian-Najafabadi, and M.H. Roudkenar, *In vitro augmentation of mesenchymal stem cells viability in stressful microenvironments : In vitro augmentation of mesenchymal stem cells viability*. Cell Stress Chaperones, 2015. 20(2): p. 237-51.

12. Haider, H. and M. Ashraf, *Strategies to promote donor cell survival: combining preconditioning approach with stem cell transplantation*. J Mol Cell Cardiol, 2008. 45(4): p. 554-66.

13. Toma, C., et al., *Fate of culture-expanded mesenchymal stem cells in the microvasculature: in vivo observations of cell kinetics*. Circ Res, 2009. 104(3): p. 398-402.

14. Chen, J., et al., *The key regulatory roles of the PI3K/Akt signaling pathway in the functionalities of mesenchymal stem cells and applications in tissue regeneration*. Tissue Eng Part B Rev, 2013. 19(6): p. 516-28.

15. Le Belle JE, O.N., Paucar AA, Saxe JP, Mottahedeh J, Pyle AD, Wu H, Komblum HI, *Proliferative neural stem cells have high endogenous ROS levels that regulate self-renewal and neurogenesis in a PI3K/Akt-dependant manner*. Cell Stem Cell, 2011. 8(1): p. 59-71.

16. Liu, J. and T. Finkel, *Stem cells and oxidants: too little of a bad thing*. Cell Metab, 2013. 18(1): p. 1-2.
17. De Falco, E., et al., SDF-1 involvement in endothelial phenotype and ischemia-induced recruitment of bone marrow progenitor cells. Blood, 2004. 104(12): p. 3472-82.

18. Liu, X., et al., SDF-1/CXCR4 axis modulates bone marrow mesenchymal stem cell apoptosis, migration and cytokine secretion. Protein Cell, 2011. 2(10): p. 845-54.

19. Rosova, I., et al., Hypoxic preconditioning results in increased motility and improved therapeutic potential of human mesenchymal stem cells. Stem Cells, 2008. 26(8): p. 2173-82.

20. Srijaya, T.C., T.S. Ramasamy, and N.H. Kasim, Advancing stem cell therapy from bench to bedside: lessons from drug therapies. J Transl Med, 2014. 12: p. 243.

21. Boopathy, A.V., et al., Oxidative stress-induced Notch1 signaling promotes cardiogenic gene expression in mesenchymal stem cells. Stem Cell Res Ther, 2013. 4(2): p. 43.

22. Khatlani, T., et al., Preconditioning by Hydrogen Peroxide Enhances Multiple Properties of Human Decidua Basalis Mesenchymal Stem/Multipotent Stromal Cells. Stem Cells Int, 2018. 2018: p. 6480793.

23. Pendergrass, K.D., et al., Acute preconditioning of cardiac progenitor cells with hydrogen peroxide enhances angiogenic pathways following ischemia-reperfusion injury. Stem Cells Dev, 2013. 22(17): p. 2414-24.

24. Holmstrom, K.M. and T. Finkel, Cellular mechanisms and physiological consequences of redox-dependent signalling. Nat Rev Mol Cell Biol, 2014. 15(6): p. 411-21.

25. Lee, B.W.L., P. Ghode, and D.S.T. Ong, Redox regulation of cell state and fate. Redox Biol, 2019. 25: p. 101056.

26. Rhee, S.G., Cell signaling. H2O2, a necessary evil for cell signaling. Science, 2006. 312(5782): p. 1882-3.

27. Zhang, H., et al., SDF1/CXCR7 Signaling Axis Participates in Angiogenesis in Degenerated Discs via the PI3K/AKT Pathway. DNA Cell Biol, 2019. 38(5): p. 457-467.

28. Maraldi, T., et al., Reactive Oxygen Species in Stem Cells. Oxid Med Cell Longev, 2015. 2015: p. 159080.

29. Youle, R.J. and A. Strasser, The BCL-2 protein family: opposing activities that mediate cell death. Nat Rev Mol Cell Biol, 2008. 9(1): p. 47-59.

30. Wang, K., et al., Redox homeostasis: the linchpin in stem cell self-renewal and differentiation. Cell Death Dis, 2013. 4: p. e537.

31. Wullschleger, S., R. Loewith, and M.N. Hall, TOR signaling in growth and metabolism. Cell, 2006. 124(3): p. 471-84.

32. Huang, H., et al., Impaired wound healing results from the dysfunction of the Akt/mTOR pathway in diabetic rats. J Dermatol Sci, 2015. 79(3): p. 241-51.

33. Jang, M.W., et al., Cooperation of Epac1/Rap1/Akt and PKA in prostaglandin E(2)-induced proliferation of human umbilical cord blood derived mesenchymal stem cells: involvement of c-Myc and VEGF expression. J Cell Physiol, 2012. 227(12): p. 3756-67.
34. Juhaszova, M., et al., *Role of glycogen synthase kinase-3beta in cardioprotection*. Circ Res, 2009. 104(11): p. 1240-52.

35. Ganju, R.K., et al., *The alpha-chemokine, stromal cell-derived factor-1alpha, binds to the transmembrane G-protein-coupled CXCR-4 receptor and activates multiple signal transduction pathways*. J Biol Chem, 1998. 273(36): p. 23169-75.

36. Zaruba, M.M. and W.M. Franz, *Role of the SDF-1-CXCR4 axis in stem cell-based therapies for ischemic cardiomyopathy*. Expert Opin Biol Ther, 2010. 10(3): p. 321-35.

37. Lee, D.E., N. Ayoub, and D.K. Agrawal, *Mesenchymal stem cells and cutaneous wound healing: novel methods to increase cell delivery and therapeutic efficacy*. Stem Cell Res Ther, 2016. 7: p. 37.

**Figures**
Figure 1

Optimization of H2O2 concentrations for preconditioning BMSCs. (A) BMSCs were treated with the indicated concentrations of H2O2 (0-200 μM) for 12 h. Cell viability was then measured by MTT assay. Values represent the mean ± standard errors of the means (SEM) (n = 3). (B) Western blot analysis of cyclin D1 and p16 expressions in BMSCs exposed to different low concentrations H2O2 for 12 h. Values represent the mean ± SEM (n = 3). * p < 0.05, ** p < 0.01 vs. 0 μmol/L H2O2. (C) Scratch wound-healing migration assay of H2O2-treated BMSCs with or without anti-CXCR4 antibody. Scale bar = 50 μm. (D) Images of transmigrated BMSCs (stained with crystal violet) are shown (×200 magnification). (E) Quantitative analysis of migrated cells. Data is presented as mean ± SEM of six independent experiments. n = 5. **p < 0.01 versus control group, # p < 0.05 versus 50 μmol/L H2O2 group. (F) Western
blot analysis of SDF-1 and CXCR4 expressions in BMSCs exposed to different low doses of H2O2 for 12 h. Values represent the mean ± SEM (n = 3). *p < 0.05, **p < 0.01 vs. 0 μmol/L H2O2.

**Figure 2**

Optimal H2O2 preconditioning provides anti-apoptotic protection to BMSCs (A, B) The ratio of apoptotic BMSCs at various doses of H2O2 treatment was analyzed by flow cytometry. Each value represents the mean ± SEM of five independent experiments. (C, D) The apoptotic percentage of BMSCs with different doses of H2O2 treatment, before preconditioning with 50 μM for 12 h, was analyzed by flow cytometry. Each value represents the mean ± SEM of three independent experiments. *P<0.05, **P<0.01, compared with the control.
Figure 3

Effect of optimal H2O2 on the mitochondria-mediated apoptosis pathway in BMSCs. (A, B, C) Western blot analysis of the expressions of Bcl-2, Bax, and caspase-3 in BMSCs exposed to various low concentrations of H2O2 for 24 h. Values represent the mean ± SEM (n = 3) *p < 0.05, compared with the control group; (D, E) Western blot analysis of the expressions of Bcl-2, Bax, and caspase-3 in BMSCs exposed to 50 μM H2O2 for 3, 6, 12, 24 and 36 h. (F, G, H) Western blot analysis of the expressions of Bcl-2, Bax, caspase-9/3 and cleaved caspase-9/3 in MSCs exposed to 300 μM H2O2 for 24 h after pretreatment with 50 μM H2O2 for 12 h. Values represent the mean ± SEM (n = 3) *P < 0.05, **P < 0.01, compared with the control group; ## P < 0.01, compared with the 300 μM H2O2 group.
Figure 4

The protective effect of optimal H2O2 preconditioning is mediated by the activation of PI3K/Akt/mTOR/GSK-3β signaling. (A, B, C, D) Western blot analysis of the expressions of total and phosphorylated PI3K, Akt, mTOR and GSK-3β proteins in BMSCs exposed to various low concentrations of H2O2 for 24 h. Values represent the mean ± SEM (n = 3) *p < 0.05, compared with the control group.

(E) Western blot analysis of the expressions for phosphorylated Akt, mTOR, GSK-3β, Bcl-2 and cleaved caspase-3 in H2O2 -preconditioned BMSCs exposed to 300 μmol/L for 24 h after 2 h pre-incubation with 10 μM LY294002. (F, G) The rate of apoptotic cells was determined by flow cytometry. Values represent the mean ± SEM (n = 3). **P<0.01, compared with the control group; ##P<0.01, compared with H2O2 300 μmol/L group. ΔΔP<0.05, compared with the H2O2-preconditioned group.
Figure 5

Optimal H2O2 preconditioning enhances BMSCs tissue engraftment and would closure. (A, B) Histological analysis of the number of transplanted Dil-labeled BMSCs (red) migrating into newly formed granulation tissue 1 and 3 days after wounding. Scale bar = 100 μm. Data are presented as the total cells per section (×200) ± SEM. **P < 0.01, compared with the BMSCs group; ΔΔP < 0.01, compared with the either day one post-wounding, or one day after post-wounding. (C) Photographs showing the macroscopic wound closure at days 0, 3, 5, 7, 13 and 15 after injury. (D) The rate of wound closure after BMSCs engrafting into the wounds. Data are shown as means ± SEM. (n= 5). (E, F) The levels of VEGF of wound tissue after BMSCs transplantation. Data are shown as means ± SEM. (n = 3). (G) Representative images of mouse CD31 staining of wounds in PBS, BMSC, and H2O2-preconditioned groups. Scale bar =
50 μm. (H) Microvessel density in the wounds was assessed by CD31-positive staining for microvessels, expressed as the number of CD31-positive microvessels per field. *P < 0.05, **P < 0.01, compared with the PBS group; ΔP < 0.05, compared with the BMSCs group.

Figure 6

Schematical representation of protective mechanism of H2O2 preconditioning for improving therapeutic potential of BMSCs. Low dose of H2O2 acts in signalling pathways as a second messenger. H2O2 preconditioning-induced BMSCs 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 BMSCs proliferation, migration, angiogenesis and anti-apotosis. The PI3K inhibitor (LY294002) blocked the protective effect induced by 50 μM H2O2 preconditioning.
Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryMaterial.docx