Protective effect of berberine against oxidative stress-induced apoptosis in rat bone marrow-derived mesenchymal stem cells

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Abstract. Bone marrow-derived mesenchymal stem cells (BMSCs) have the potential to be used for the treatment of delayed union, nonunion or persistent bone defects in MSC-based cell therapy. However, implantation of BMSCs into the fracture site is confronted with apoptosis on account of harsh conditions and oxidative stress. In the present study, the anti-apoptotic effects of berberine (BBR) on BMSCs subjected to hydrogen peroxide (H_2O_2) are investigated, and the potential underlying mechanisms are explored. Oxidative injury was induced by exposure to H_2O_2, and cell viability was assessed using a cell counting kit-8 assay. The apoptosis of BMSCs was measured by Hoechst 33258 and Annexin V-fluorescein isothiocyanate/propidium iodide assay. Reactive oxygen species staining and superoxide dismutase (SOD) assay were applied to assess the anti-oxidative effect of BBR. Finally, western blot was performed to measure the expression levels of phosphorylated (p)-Akt, B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax) and cleaved caspase-3. In the present study, it was identified that BBR remarkably attenuated H_2O_2-induced apoptotic cell death via quenching ROS production and increasing SOD activity. Further studies indicated that BBR can reduce apoptosis by upregulating the expression level of p-Akt and Bcl-2, and downregulating the expression levels of Bax and cleaved caspase-3. Taken together, the results of the present study demonstrate that pretreatment with BBR could alleviate H_2O_2-induced apoptosis in rat BMSCs *in vitro*.

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Introduction

Delayed union and nonunion with or without defects are common complications of traumatic fractures (1). Fortunately, mesenchymal stem cell (MSC)-based therapy has recently emerged as an appealing and potential therapeutic strategy for the treatment of delayed union, nonunion or persistent bone defects (2). Bone marrow-derived MSCs (BMSCs), one type of MSC cell, have the properties of plasticity and the ability to differentiate into chondrocytes, osteocytes and adipocytes (3). The transplantation of BMSCs in damaged tissues has been an innovation used in tissue engineering, particularly in the field of skeletal regenerative medicine (4,5). Cultured BMSCs have been injected or combined with biomaterials into fracture sits where the BMSCs differentiate into osteoblasts to repair the fracture (2,6,7). Therefore, multiple stem cell-based products and techniques to enhance the efficacy of local implantation of BMSCs are currently being investigated to optimize bone healing in various animal models and clinical trials (3,5).

The therapeutic application of BMSCs is limited due to their susceptibility to oxidative stress which results in the engrafted BMSCs' apoptosis in the injured bone area (8-10). Previous studies have demonstrated that BMSCs injected into fracture sites are confronted with its apoptosis within a few days on account of harsh microenvironment conditions with oxidative stress, which is a state of imbalance between reactibe oxygen species (ROS) generation and intracellular antioxidants, resulting in cellular damage, and eventually leading to cell death (11-13). Therefore, strategies to protect the implantation of BMSCs from apoptosis and to improve their survivability in oxidative stress are therapeutically attractive in MSC-based therapy for delayed union, nonunion and persistent bone defects.

Plants used in Traditional Chinese Medicine have been regarded as a wide source of antioxidants with potential pharmacological and biological effects (14). Berberine (BBR), 5,6-dihydro-9,10-dimethoxy-benzo(g)-1,3-benzodioxolo(5 ,6-a) quinolizinium-, chloride, a natural isoquinoline quaternary alkaloid, is a well-known constituent of the Chinese herb Huanglian (15). The compound possesses a variety of pharmacological and biochemical properties, such as anti-inflammatory (16), anti-microbial (17), anti-cancer (18)

and and antioxidant (19-21) properties. A previous study demonstrated that BBR could attenuate H_2O_2-induced oxidative injury in motor neuron-like cells (22), smooth muscle cells (19), endothelial cells and mesangial cells (23), but whether BBR exerts any protective effects against H_2O_2 in rat (r)BMSCs is still unknown.

The present study, to the best of our knowledge, demonstrated for the first time that BBR is capable of protecting primary cultural rBMSCs against H_2O_2-induced apoptosis via enhancing resistance to oxidative stress *in vitro*, which could be a promising approach to improve stem cell survival during transplantation in MSC-based therapy for traumatic fractures.

Materials and methods

Animals and BBR. Male Sprague-Dawley (SD) rats, weight 80-90 g, were obtained from the animal center of Guangzhou University of Traditional Chinese Medicine (Guangzhou, China; certificate no. 44005900001722). Twenty-two rats were specific pathogen-free animals housed with *ad libitum* access to food and water at a constant temperature of 24±1˚C in climate-controlled conditions with a 12 h light/dark cycle and humidity of 55±5%. All animals received human care in accordance with the guideline set by the Care of Experiment Animals Committee of Guangzhou University of Chinese Medicine. BBR (>99.0% purity) was provided by the Department of Pharmacology & Toxicology of Sun Yat-Sen University (Guangzhou, China). BBR was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) and kept in -20˚C away from the dark. The final concentration of DMSO per well was 0.1%.

Isolation and culture of rBMSCs. MSCs were isolated from the bone marrow of rats as previously reported with minor modification (24). Briefly, the femurs and tibias were clipped from the SD rats under sterile conditions. After removing all the connective tissues and cutting epiphyseal extremities, bone marrow was flushed out with low (L-)glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, YSA) containing 1% penicillin/streptomycin in a sterile petri dish. The cells were centrifuged at 300 x *g* for 8 min and resuspended in L-DMEM (1,000 mg/l glucose) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and a mixture of 1% penicillin/streptomycin. Bone marrow was transferred to a plastic culture flask and incubated at 37˚C with 5% CO_2 in a humidified atmosphere. After 24 h, the medium was changed to remove free-floating cells and replaced every 3 days. At 80-90% confluence, the adherent cells were washed with phosphate-buffered saline (PBS) twice, followed by digestion in 0.25% trypsin (Gibco; Thermo Fisher Scientific, Inc.) and expanded at a 1:2 dilution.

Cell surface phenotype detection. Surface marker analysis was performed by flow cytometry. rBMSCs were collected in passage three, cells were resuspended in 100 μl flow cytometry staining buffer (Cyagen Biosciences, Inc., Guangzhou, China) containing PBS and 0.1% bovine serum albumin (Sigma-Aldrich; Merck Millipore) at a concentration of $3x10^6$ cells/ml and incubated for 30 min at 4˚C with the following specific primary antibodies: Anti-rat cluster of differentiation (CD)90, anti-rat CD34 and anti-rat CD11b/c at a concentration of 0.5 mg/ml, which were from the Cyagen Mesenchymal Stem Cell Characterization kit (cat. no. RAXMX-09011; Cyagen Bioscience Inc.) After incubation, the samples were washed twice in 1 ml flow cytometry staining buffer, then centrifuged at 250 x *g* for 5 min. Then, the supernatant was discarded. Following incubation at 4˚C with phycoerythrin-conjugated goat anti-rat at a concentration of 1 mg/ml for 30 min in the dark, the samples were washed twice with flow cytometry staining buffer, centrifuged at 250 x *g* for 5 min and resuspended in 400 μl of flow cytometry staining buffer for cytometric analysis. Labeled cells were analyzed by flow cytometry (Coulter EPICS XL; Beckman Coulter, Inc., Brea, CA, USA) with standard software (FACSDiva; version 6.1.3; BD Biosciences, Franklin Lakes, NJ, USA).

Cell viability assay. Cell viability was assessed by detecting the optical density (25). Briefly, rBMSCs were seeded at a density of $1x10^5$/ml in 96-well culture plates for 24 h. Subsequently, the cells were pretreated with different concentrations of BBR for 2 h and then exposed to H_2O_2 for 24 h in 100 μl DMEM with 10% FBS and 1% penicillin/streptomycin. Then, 10 μl cell counting kit (CCK)-8 solution (Dojindo Laboratories, Kumamoto, Japan) was added into each well. Following 2 h incubation, the absorbance at a wavelength of 450 nm was detected using a Bio-kinetics reader (PE-1420; Bio-Kinetics Corporation, Sioux Center, IA, USA). Cell viability was presented as the percentage of the control culture value. The results were calculated using three different batches of wells and each experiment was performed in triplicate as independent experiments.

Morphologic changes. rBMSCs were cultured in 12-well plates for 24 h and then pretreated with BBR for 2 h with subsequent exposure to H_2O_2 (Sigma-Aldrich; Merck Millipore) for 24 h. Then, cultured cells were washed twice with PBS and fixed with 4% paraformaldehyde for 10 min and stained with 2 μg/ml Hoechst 33258 (Sigma-Aldrich; Merck Millipore) for 20 min at 37˚C in the dark (26). Then, morphologic changes were observed by phase contrast microscopy and cells images were visualized through a fluorescence microscope.

Apoptosis analysis by flow cytometry. The percentage of apoptotic cells were assessed using Annexin V and propidium iodide (PI) staining (Annexin V-FITC apoptosis detection kit; cat. no. KGA107; Jiancheng Biological Engineering Research Institute, Nanjing, China) and flow cytometry. In brief, rBMSCs were seeded at $5x10^4$ cells/ml in 6-well culture plates and incubated overnight. Following treatment, both adherent and floating cells were harvested, washed twice in cold PBS, and resuspended in 200 μl of binding buffer (Jiancheng Biological Engineering Research Institute). Annexin V-FITC solution (5 μl) was added and the cells were incubated for 30 min at 4˚C in the dark. Subsequently, 10 μl PI was added and the solution was incubated for 15 min at the room temperature. The cell suspension was immediately analyzed by flow cytometry (Coulter EPICS XL) with standard software.

Measurement of ROS production. Production of intracellular ROS was evaluated using a 2'7'-dichlorofluorescein (DCF) assay (27), which was conducted using dichlorofluorescein diacetate (H_2DCF-DA; Sigma-Aldrich; Merck Millipore). Briefly, following treatment, rBMSCs were washed and then incubated with 10 μM H_2DCF-DA in serum-free culture medium (Gibco; Thermo Fisher Scientific, Inc.) for 30 min at 37˚C in the dark. DCF fluorescence was illuminated by visual effect of cell morphology through fluorescence microscopy or analyzed using a fluorescence plate reader (Flex Station3; Molecular Devices, LLC, Sunnyvale, CA, USA) at an excitation and emission wavelength of 490 nm and 533 nm.

Detection of superoxide dismutase (SOD). SOD activity was tested with the xanthine oxidase method as previously described (28). Briefly, rBMSCs were pretreated with BBR for 2 h and incubated with 600 μM H_2O_2 for 24 h. Then, the cells were harvested and sonicated with cold 0.9% sodium chloride to obtain cell homogenates. Following centrifugation at 3,500 x *g* at 4˚C for 5 min, the supernatants were obtained and then used for detecting intracellular SOD. The level of SOD was calculated by measuring the absorbance at 570 nm according to the instructions of a Superoxide Dismutase Assay kit (cat. no. A001-2; Jiancheng Biological Engineering Research Institute). The basal contents of SOD in untreated control cells were taken as 100%.

Western blotting analysis. Western blotting analysis was performed as previously described (29). Briefly, at the end of the treatment period, cells were collected and lysed with cold lysis buffer. Total protein concentration was determined using a BCA assay kit (cat. no. KGET13; Keygen, Nanjing, China). Protein samples (30 μg/kg) were separated by 10% SDS-PAGE and transferred onto polyvinylidenedifluoride membranes. After being blocked with 5% non-fat dry milk in Tris-buffered saline and Tween-20 buffer, the membranes were incubated overnight at 4˚C with the following the primary antibodies: Rabbit polyclonal anti-phosphorylated (p)-Akt (cat. no. 5012S), anti-B-cell lymphoma 2 (Bcl-2; cat. no. 3498) and anti-caspase-3 (Cat. no. 9661) (all purchased from Cell Signaling Technology, Inc., Danvers, MA, USA; 1:1,000), rabbit polyclone anti-Bcl-2-associated X protein (cat. no. sc-526; Santa Cruz Biotechnology, Inc., Dallas, TX, USA; 1:500) and mouse polyclonal anti-β-actin (cat. no. A1978; Sigma-Aldrich; Merck Millipore1 1:10,000) at 4˚C overnight. The next day, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-rabbit secondary antibody (cat. no. sc-3836; Santa Cruz Biotechnology, Inc.; 1:5,000) and anti-mouse secondary antibody (cat. no. W4021; Promega Corporation, Madison, WI, USA; 1:10,000). The bands were detected with enhanced chemiluminescence (GE Healthcare Life Sciences, Chalfont, UK). The blots were quantified using Quantity One software (version 4.62; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. All quantified data represent an average of at least three samples, and the results are presented as the mean ± standard deviation. Differences among groups were tested by one-way analysis of variance (ANOVA). Statistical analyses between two groups were performed by unpaired Student's t-test. Following ANOVA analyses, the Tukey's test was used. $P<0.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed using GraphPad version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Morphology and cell surface phenotype detection of BMSCs. Fifteen days after being obtained from rat femur, cultured rBMSCs were adherent to the dish and showed an elongated, spindle-shaped and fibroblast-like morphology (Fig. 1A). Furthermore, the phenotype of BMSC-related cell surface markers CD90, CD34 and CD11b were detected. Results of flow cytometry demonstrated that 89.4% of the cells expressed CD90, 4.8% of the cells expressed CD11b and 0.2% of the cells expressed CD34 (Fig. 1B).

Effect of BBR and H_2O_2 on rBMSC cell viability. CCK-8 assay results demonstrated that BBR (Fig. 2A) did not cause cell death at a concentration of 30 μM, but significantly reduced cell viability at 30 μM compared with the control ($P<0.01$; Fig. 2B). To determine the optimum concentration of H_2O_2 for next experiment, cells treated with 500, 600, 700, 800, 900 and 1000 μM for 24 h in the complete medium were examined. H_2O_2 exhibited cytotoxicity dose-dependently, and 600 μM H_2O_2 significantly reduced cell viability by 48.6% compared with the control ($P<0.01$; Fig. 2C). This concentration was used for the following experiments.

BBR inhibited H_2O_2-induced cell inhibition in rBMSCs. To examine whether BBR protects rBMSCs from H_2O_2-induced cell death, cells were pretreated with 1, 3 and 10 μM BBR for 2 h, then incubated with H_2O_2 (600 μM) for 24 h. The results demonstrated that the cell viability of BMSCs in the H_2O_2 group decreased dramatically, while BBR dose-dependently increases the cell viability. BBR at 3 μM showed the best effect against H_2O_2 ($P<0.01$ vs. the H_2O_2 group; Fig. 2D).

BBR reduced H_2O_2-induced apoptosis-like cell death in rBMSCs. Hoechst 33258 staining was applied and observed by fluorescence microscope to detect changes in cell nuclei. After pretreatment with BBR, the number of cells with bright blue fluorescence were reduced notably, indicating that BBR can markedly decrease the number of apoptotic cells and nuclear condensation (Fig. 3A) caused by H_2O_2. The results of Annexin V-FITC/PI staining detected by flow cytometery revealed (Fig. 3B and C) that the H_2O_2 treatment group had a significantly higher rate of apoptosis (36±3.39%) compared with the control group (0.14± 0.02%) ($P<0.01$). However, the addition of BBR was able to protect the cells against H_2O_2-induced apoptosis and significantly reduce the rate of apoptotic cells ($P<0.01$; 22± 4.21%).

BBR suppressed H_2O_2-induced intracellular ROS formation. To elucidate the potential mechanisms underlying the protection of BBR following H_2O_2 treatment, H2DCF-DA staining, a ROS probe, was used to measure cellular oxidative stress by DCF fluorescence. Treatment with 600 μM

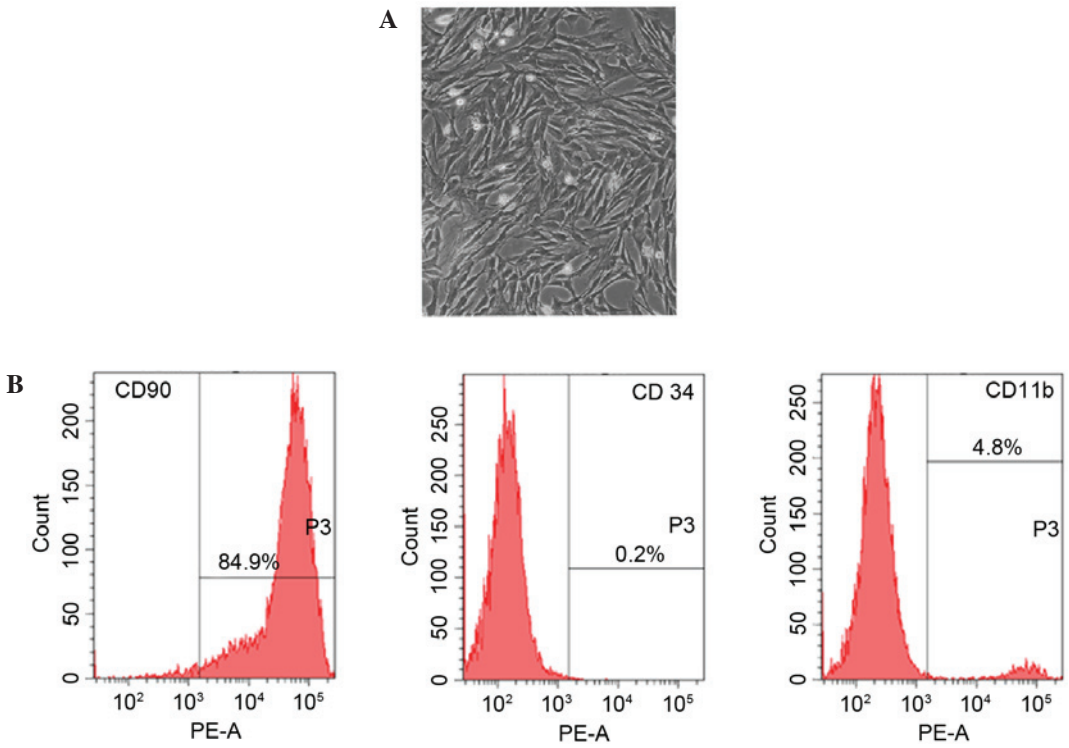

Figure 1. Characterization of cell morphology and surface phenotype of rBMSCs at passage 3. (A) Representative phase-contrast microscopy image of rBMSCs with typical spindle-shaped and fibroblast-like morphology. Magnification, x100. (B) Flow cytometry analysis showing that rBMSCs express CD90, CD34 and CD11b. CD, cluster of differentiation; PE-A, phycoerythrin absorbance; rBMSC, rat bone marrow-derived mesenchymal stem cell.

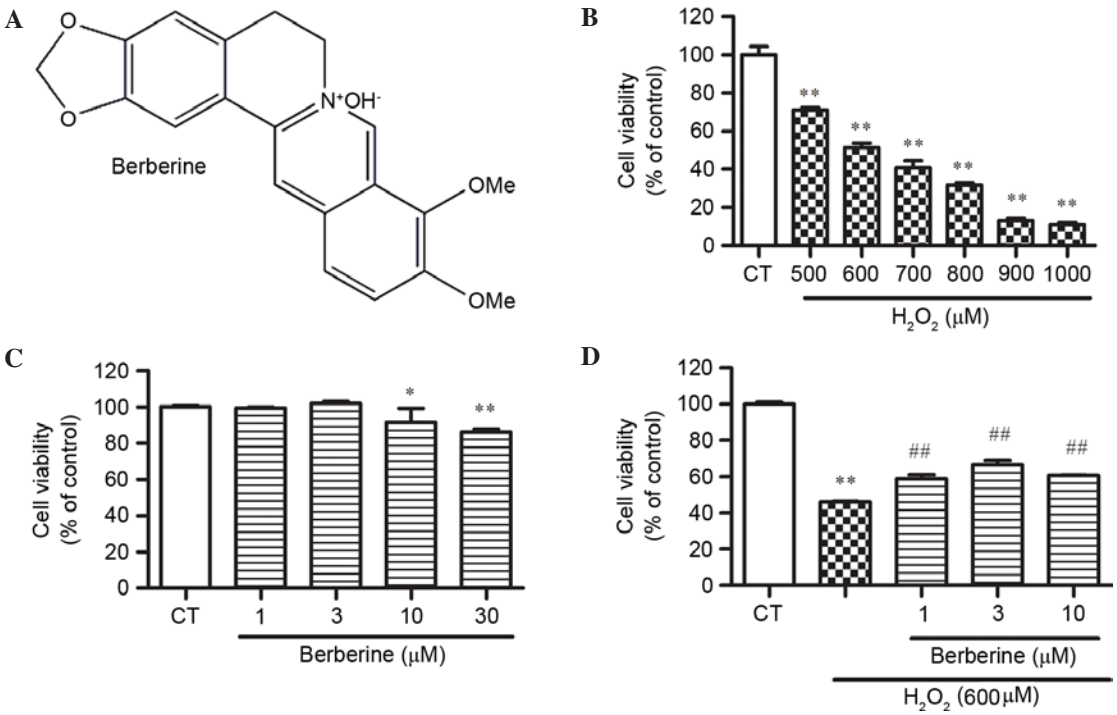

Figure 2. Effect of BBR and H_2O_2 on cell viability detected by CCK-8 assay. (A) Structure of BBR. (B) Cells were treated with different concentrations of BBR for 24 h. (C) The viability of cells treated with 500-1000 μM H_2O_2 decreased dose-dependently. (D) rBMSCs were pretreated with 1, 3 and 10 μM BBR for 2 h, then incubated with H_2O_2 (600 μM) for 24 h. *P<0.05, **P<0.01 vs. the control group; $^{\#\#}$P<0.01 vs. the H_2O_2-treated group. Data are presented as mean plus standard deviation. The bar graphs represent three independent experiments. BBR, berberine; CCK-8, cell counting kit-8; CT, control; rBMSCs, rat bone marrow-derived mesenchymal cells.

H_2O_2 for 24 h significantly resulted in the increase of DCF fluorescence compared with the control group (P<0.01), which was significantly reversed by BBR (P<0.01; Fig. 4A and B).

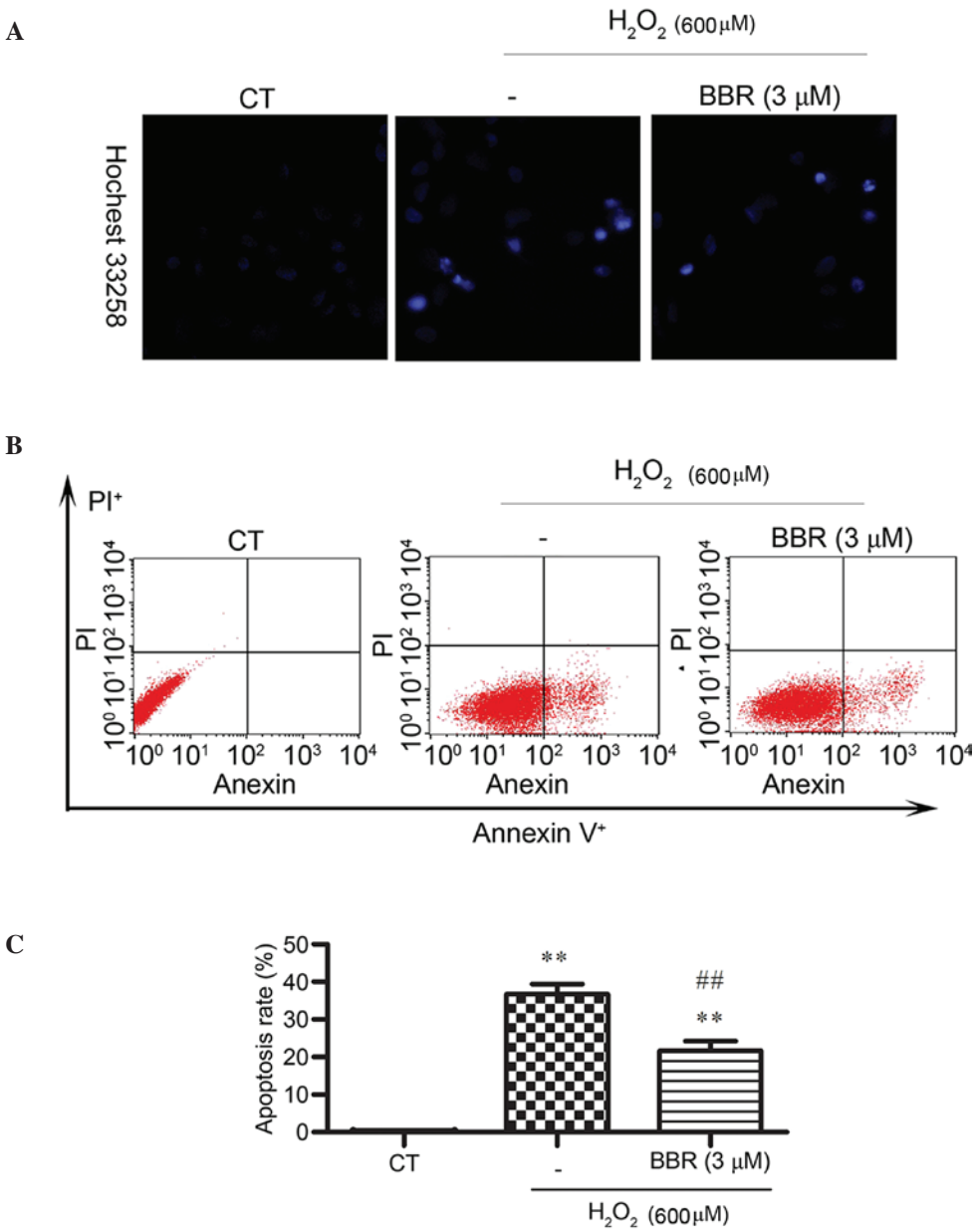

Figure 3. BBR significantly lowered H_2O_2-induced apoptosis in rBMSCs. Cells were pretreated with or without 3 μM BBR for 2 h prior to exposure to H_2O_2 (600 μM). (A) Nuclear condensation was assessed using Hoechst 33258. Magnification, x200. (B) Apoptosis was assessed using Annexin V-FITC/PI staining. (C) Quantitative analysis of apoptotic cells. **P<0.01 vs. the control group; $^{\#\#}$P<0.01 vs. the H_2O_2 group. Results are shown as the mean plus standard deviation. BBR, berberine; CT, control; FITC, fluorescein; PI, propidium iodide; rBMSCs, rat bone marrow-derived mesenchymal stem cells.

BBR alleviated H_2O_2-induced decrease of the activity of intracellular SOD. To further study whether BBR could alleviate H_2O_2-induced oxidative stress, the activity of SOD in rBMSCs was assessed. As shown in Fig. 4C, the activity of SOD was significantly decreased in the H_2O_2-treated group compared with that of the control group (P<0.01), while pretreatment of BBR significantly restored this decrease in SOD activity (P<0.05).

BBR protected rBMSCs from H_2O_2-induced apoptosis via regulation of the expression of apoptosis-related proteins p-Akt, Bcl-2, Bax and caspase-3. To further explore the protective mechanisms of BBR, the expression of anti-apoptotic protein Bcl-2, and the pro-apoptotic proteins Bax and caspase-3, were investigated. As shown in Fig. 5A-D, following treatment with H_2O_2 (600 μM) for 12 h, it was observed that the expression of Bcl-2 significantly decreased (P<0.05), and the expression of Bax and caspase-3 significantly increased (P<0.01), compared with the control group. Pretreatment with BBR (3 μM) for 2 h significantly inhibited the downregulation of anti-apoptotic proteins (P<0.01) and significantly reduced the upregulation of pro-apoptotic proteins (P<0.01). The phosphorylation of Akt had been reported to be important in cell survival and apoptosis (30). In the current study (Fig. 5E), treatment with H_2O_2 (600 μM) significantly decreased the expression level of pSer473-Akt compared with the control group (P<0.01), and this was significantly reversed with pretreatment with BBR (3 μM; P<0.01). These results suggest that BBR can protect rBMSCs from H_2O_2-induced apoptosis by increasing the level of p-Akt.

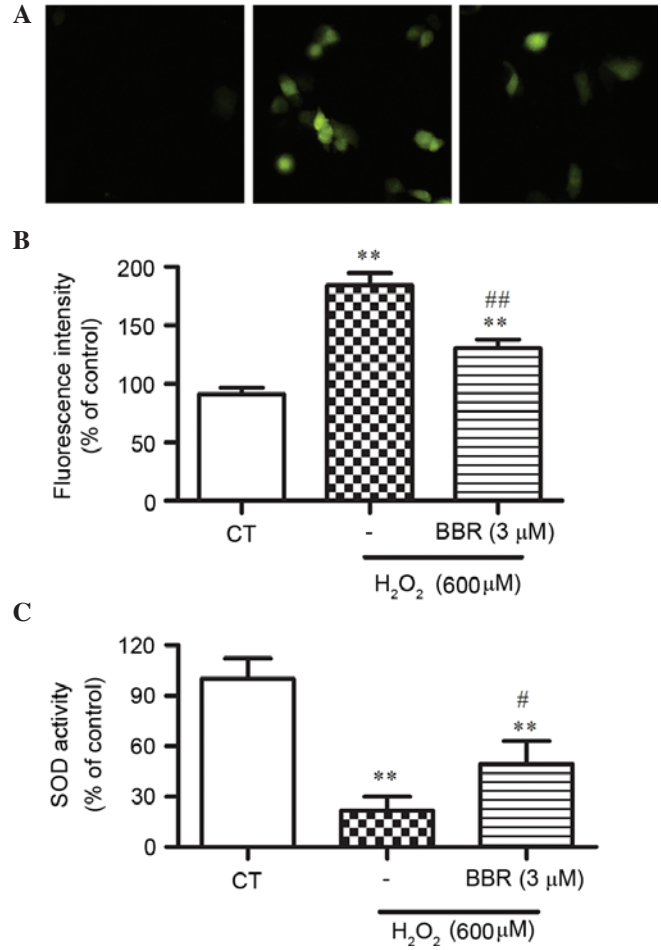

Figure 4. BBR scavenges intracellular ROS produced by H_2O_2 in rBMSCs. (A) ROS production induced by H_2O_2 detected by H_2DCFH-DA assay. The higher the ROS level, the lighter the fluorescence in the typical photos acquired by a fluorescence microscope. Magnification, x200. (B) Quantitative analysis of DCF fluorescent intensity. (C) The activity of intracellular SOD. **P<0.01 vs. the control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. the H_2O_2 group. Results are shown as the mean plus standard deviation. n=3 in each group. BBR,berberine; DCF, 2'7'-dichlorofluorescein; rBMSCs, rat bone marrow-derived mesenchymal stem cells; SOD, superoxide dismutase.

Discussion

Treatment of delayed healing, nonunion or a persistent bone defect represents a major challenge for traumatology department (31). Owing to the low immunogenicity and transplantability (32), BMSCs are an effective resource for transplantation in clinical application, with the establishment of cell banks for bone regenerative medicine (2,33). Previously, much attention has been directed towards the poor survival of transplantation in MSC-based therapy (5,9,34,35). For basic and clinician scientists, it is necessary to utilize strategies to improve low cell survival rates.

H_2O_2 has been extensively used to imitate the microenvironment surrounding transplanted cells in injured tissue *in vitro* (36). When exposed to H_2O_2, cells are faced with high concentration of ROS, which consequently damages the balance of oxidants and antioxidants, resulting in apoptosis and eventually necrosis. In the experiments in the current study, BBR significantly reduced the level of apoptosis in H_2O_2-treated cells. In addition, the results demonstrated that BBR exposure inhibited the overproduction of intracellular ROS induced by exogenous H_2O_2, and significantly enhanced the activity of antioxidant enzyme SOD, which promotes a balance between the ROS and anti-oxidative system in targeting oxidative stress.

Caspase-3 is the most crucial downstream apoptosis protease in the caspase cascade, which executes apoptosis through DNA degradation, chromatin condensation and nuclear fragmentation (37). In the present study, treatment of rBMSCs with 600 μM H_2O_2 induced marked nuclear condensation and apoptotic death, and increased the expression of cleaved caspase-3, indicating that the apoptosis of the caspase cascade may be activated by H_2O_2. However, following pretreatment with BBR (3 μM), the expression of cleaved caspase-3 was significantly reduced, indicating the BBR effectively attenuates oxidative injury by suppressing H_2O_2-induced caspase activation.

To further explore the molecular mechanisms underlying the moderation of apoptosis by BBR, the expression level of vital apoptotic proteins was assessed. The Bcl-2 family of proteins comprise the anti-apoptotic proteins and pro-apoptotic proteins, of which the relative proportions control the fine balance between cell survival and cell death via the intrinsic apoptotic pathway (38). In the present study, western blot indicated that BBR could reverse the reduction of the anti-apoptotic protein Bcl-2, and could increase the level of pro-apoptotic protein Bax, following treatment with H_2O_2. In addition, pretreatment with BBR prior to incubation with H_2O_2 significantly increased the ratio of Bcl-2/Bax compared with cells treated with H_2O_2 alone, showing that the Bcl-2 family has a close association with the protective effects of BBR in rBMSCs.

As a survival pathway, the Akt signaling pathway mediating the anti-apoptosis mechanism is well understood (30). Exogenous H_2O_2 can influence Akt activation, which promotes cell survival (39). The present study showed that BBR pretreatment prevented the downregulation of p-Akt induced by H_2O_2. This suggests that BBR caused an increase of Akt phosphorylation in parallel with an increase of protective effects. However, whether BBR can protect rBMSCs from oxidative stress-induced apoptosis via the Akt pathway requires further investigation.

In conclusion, the results of the present study provide powerful evidence that pretreatment with BBR can alleviate H_2O_2-induced apoptosis and enhance the viability of rBMSCs via improvement of antioxidant activities and regulation of apoptosis and the anti-apoptotic pathway. This may be a useful strategy to improve low cell survival rates in treatment of delayed union and nonunion with or without defects.

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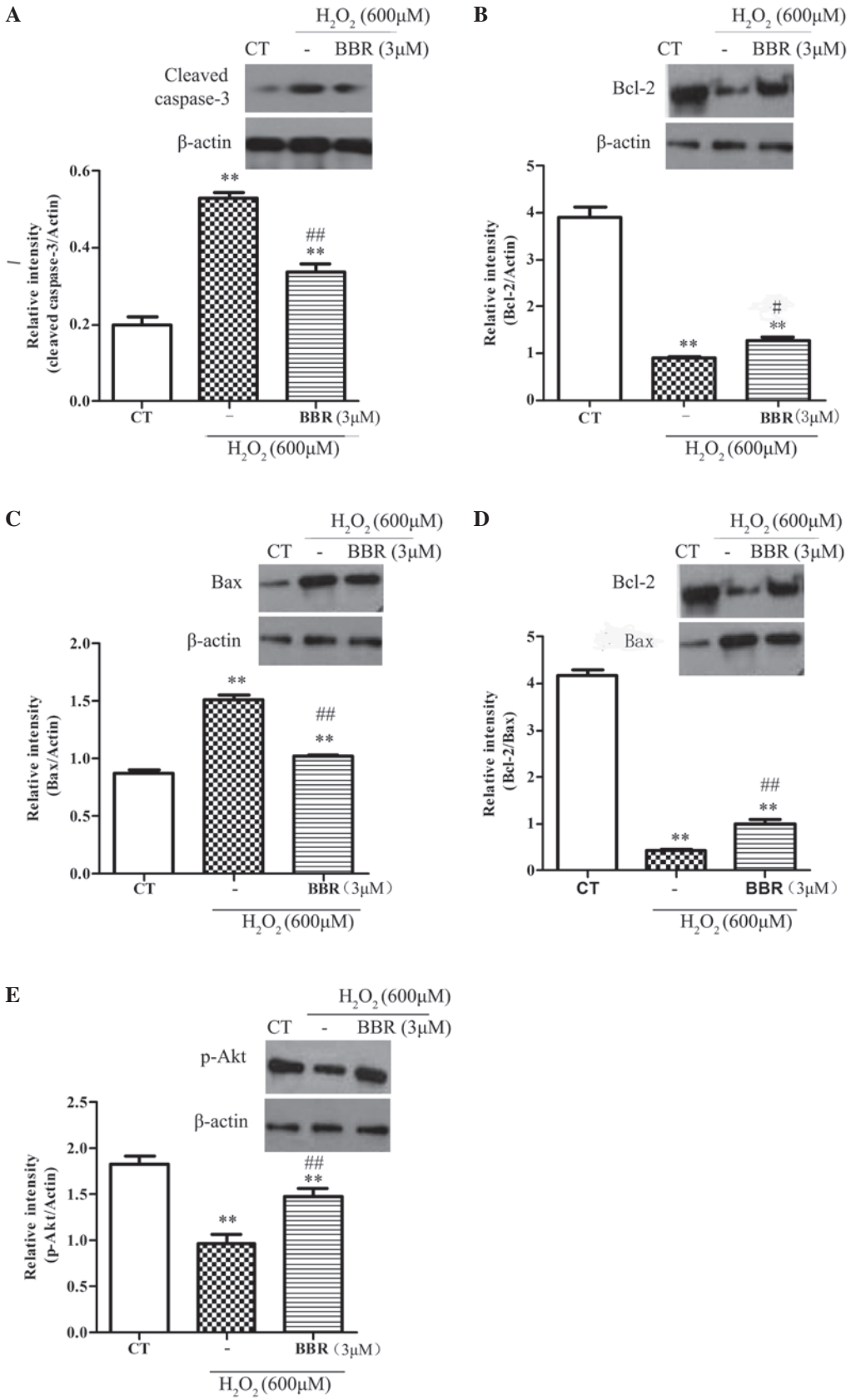

Figure 5. Representative western blots showing the effects of BBR on Bcl-2, Bax, caspase-3 and p-Akt expression in rBMSCs. β-actin was used as a loading control. Cells were pretreated with BBR for 2 h followed by treatment with H_2O_2 (600 μM) for 12 h. (A) Following pretreatment with BBR, the expression of caspase-3 significantly reduced compared with the H_2O_2 group. (B) BBR inhibited the H_2O_2-induced downregulation of Bcl-2. (C) BBR suppressed the H_2O_2-induced upregulation of Bax. (D) Increased ratio of Bcl-2/Bax was observed when the cells were pretreated with BBR prior to incubation with H_2O_2. (E) Treatment with H_2O_2 significantly decreased the level of p-Akt, which was blocked following pretreatment with BBR (3 μM). **P<0.01 vs. the control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. the H_2O_2 group. All data are presented as the mean plus standard deviation of three independent experiments. BBR, berberine; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; CT, control; p-Akt, phosphorylated Akt; rBMSCs, rat bone marrow-derived mesenchymal stem cells.

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