Protective effect of gigantol against hydrogen peroxide-induced apoptosis in rat bone marrow mesenchymal stem cells through the PI3K/Akt pathway

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Received July 5, 2017; Accepted October 24, 2017

DOI: 10.3892/mmr.2017.8242

Abstract. Bone marrow mesenchymal stem cell (BMSC) transplants are promising for the treatment of certain central nervous system diseases. However, oxidative stress is one of the major factors that may limit the survival of the transplanted BMSCs. The present study investigated the effect of pretreatment with gigantol on hydrogen peroxide (H_2O_2)-induced apoptosis in rat BMSCs (rBMSCs) and the potential underlying mechanisms. The results demonstrated that gigantol pretreatment significantly inhibited H_2O_2-induced apoptosis of rBMSCs. rBMSCs were incubated with 600 μM H_2O_2 in the absence or presence of different doses of gigantol (1-100 μM). Cell viability and apoptosis ratios were assessed by MTT assays and flow cytometry, respectively. Morphological alterations and reactive oxygen species were measured by the fluorescent-based methods of Hoechst staining and dichlorodihydrofluorescein diacetate, respectively. Furthermore, the protein levels of phosphorylated-protein kinase B (Akt), B-cell lymphoma-2 (Bcl-2), Bcl-2-associated X (Bax), caspase-3 and caspase-9 were investigated by western blotting. Following incubation with H_2O_2 for 2 h, gigantol significantly inhibited the H_2O_2-induced reductions in the cell viability of rBMSCs in a dose-dependent manner. Furthermore, gigantol upregulated Akt phosphorylation and Bcl-2 expression, downregulated Bax expression, and reduced the expression of caspase-3 and caspase-9 in H_2O_2-treated rBMSCs, whereas an opposite effect was detected when LY294002, an inhibitor of phosphatidylinositol 3-kinase, was administered in combination with gigantol. These results indicate that gigantol may be developed as a promising neuroprotective agent for successful MSC transplantation in ischemic diseases.

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Abbreviations: rBMSCs, rat bone marrow mesenchymal stem cells; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X; H_2O_2, hydrogen peroxide; PI3K, phosphatidylinositol 3-kinase; Akt, protein kinase B; ROS, reactive oxygen species

Key words: gigantol, mesenchymal stem cells, oxidative stress, apoptosis, phosphatidylinositol 3-kinase/Akt pathway

Introduction

Diseases associated with cerebral ischemia are a major cause of mortality in developing countries. Ischemic stroke is associated with the acute loss of neurons, astroglia and oligodendroglia, in addition to disruption to synaptic architecture, as a result of cerebral artery occlusion (1). Certain studies have focused on the potential use of mesenchymal stem cell (MSC) transplantation in the treatment of central nervous system (CNS) diseases and injures, such as cerebral ischemia (2,3). MSC therapy is considered a novel and promising strategy for the treatment of ischemic stroke, and may exert neuroprotective effects and promote the repair of neurons by secreting various neural trophic factors and replacing damaged neurons (4). However, the ischemic microenvironment negatively influences the survival rate of transplanted MSCs in injured CNS conditions due to oxidative stress (5,6). Thus, improving the survival of MSCs during oxidative stress may improve the efficacy of MSC-based therapies.

Gigantol is a biphenolic compound that is primarily extracted from the stem of *Dendrobium aurantiacum* (7). Phenols derived from natural plants contain numerous antioxidants and therefore are typically used to study antioxidative activities (8-11). Furthermore, gigantol is reported to exhibit numerous biological functions, including anti-osmosis effects (12), antitumor effects in human liver (13) and lung (14) cancer, antimutagenic effects (15) and immunomodulatory activities (16). Additionally, gigantol

was reported to be a potent compound for restoring sight in diabetics with cataracts (17). However, to the best of our knowledge, no previous studies have investigated the protective effect of gigantol on hydrogen peroxide (H_2O_2)-induced oxidative stress in rat bone marrow MSCs (rBMSCs). Therefore, the present study investigated whether gigantol protects against H_2O_2-induced oxidative stress in rBMSCs and whether the antioxidant mechanism of gigantol involves the phosphatidylinositol 3-kinase (PI3K)-protein kinase B (Akt) pathway.

Materials and methods

Chemicals and materials. Male 4-week-old Sprague-Dawley rats (n=10) weighing 80-100 g were used in the present study and were obtained from Guangzhou Laboratory Animal Center, Guangzhou University of Chinese Medicine (Guangzhou, China). Low glucose Dulbecco's modified Eagle's medium (DMEM) and PBS were acquired from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). H_2O_2 was purchased from Guangzhou Chemical Reagent Factory (Guangzhou, China). Basal medium of Sprague-Dawley rat MSCs, fetal bovine serum (FBS), glutamine, penicillin-streptomycin and trypsin were purchased from Cyagen Biosciences, Inc. (Guangzhou, China). MTT and dimethyl sulfoxide were acquired from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Gigantol was purchased from the National Institute for Food and Drug Control (cat. no. 111875; Beijing, China). The chemical structure of gigantol is presented in Fig. 1A. Annexin V-fluorescein isothiocyanate (FITC) apoptosis, Hoechst 33258 and reactive oxygen species (ROS) assay kits were provided by Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China). The PI3K/Akt inhibitor LY294002 was purchased from Selleck Chemicals (Houston, TX, USA). All other chemicals were of analytical grade.

Isolation and culture of rBMSCs. MSCs were immediately isolated from the Sprague-Dawley rats as previously described, with minor modifications (18). Briefly, Sprague-Dawley rats were sacrificed by cervical dislocation. The experimental procedures were approved by the Laboratory Animal Committee of Guangdong Province (Guangzhou, China). All treatments on animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals (19). The femurs and tibias of rats were carefully cleaned of adherent soft tissue, the marrow was harvested and flushed with serum-free DMEM with 1% penicillin-streptomycin until the bone washed pale. Cells were resuspended in DMEM medium with 10% FBS and 1% penicillin -streptomycin of Sprague-Dawley rBMSCs at 37˚C with 5% CO_2 After being allowed to attach for 24 h, hematopoietic and non-adherent cells were removed by changing the medium. Subsequently, rBMSCs were harvested for the experiments described below between the second and third passage. Cells were pretreated with gigantol for 12 h followed by treatment with H_2O_2 for 2 h, both at room temperature. To determine the effect of LY294002, cells were pretreated with LY294002 (25 μmol/l) for 1 h at room temperature, followed by the treatments with gigantol and H_2O_2.

Cell viability assay. Cells were seeded in 96-well plates ($1x10^5$ cells/ml) for 24 h at room temperature. To determine the effects of gigantol and H_2O_2 on rBMSC viability, cells were treated with 1, 10, 40, 80 and 100 μM gigantol for 12 h, or 400, 500, 600, 700, 800 and 900 μM H_2O_2 for 2 h, respectively. As a control, cells were treated with DMEM medium only. Furthermore, in another cell viability assay, cells were pretreated with different concentrations of gigantol (1, 10, 40, 80 and 100 μM) for 12 h followed by treatment with 600 μM H_2O_2 for 2 h, both at room temperature. Subsequently, 20 μl MTT was added to each well and incubated at 37˚C for 4 h prior to removal and addition of 100 μl dimethyl sulfoxide. The absorbance value was measured in a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 490 nm. Statistical analysis was performed on absorbance value readings.

Assessment of morphological changes. Cells were cultured in 24-well plates ($5x10^5$ cells/well) and treated with 80 μM gigantol for 12 h followed by the addition of 600 μM H_2O_2 for 2 h. Cells in the H_2O_2 group were treated with 600 μM H_2O_2 only. Cells were fixed with 4% paraformaldehyde for 10 min and washed with PBS twice prior to staining with Hoechst 33258 for 5 min at 4˚C in the dark. Condensed nuclei and cell shrinkage were observed using an inverted and fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany). A bright blue stain indicated apoptotic cell nuclei.

Measurement of ROS. Cells were cultured in 6-well plates ($1x10^6$ cells/well) and treated with 80 μM gigantol for 12 h followed by the addition of 600 μM H_2O_2 for 2 h, both at room temperature. Cells in the H_2O_2 group were treated with 600 μM H_2O_2 only. Cells were stained with 10 μM 2'7'-dichlorofluorescin diacetate (DCFH-DA) diluted with serum-free medium at 37˚C for 20 min and later washed with serum-free medium three times. Fluorescence intensity was analyzed using a microplate reader (Bio-Rad Laboratories, Inc.) at excitation and emission wavelengths of 488 and 525 nm, respectively. Images were captured using a fluorescence microscope (Leica Microsystems GmbH). The absorbance values were obtained for statistical analysis.

Flow cytometric analysis of cell apoptosis. Cells were seeded in 6-well plates ($1x10^6$ cells/well) for 24 h and and treated with 80 μM gigantol for 12 h followed by the addition of 600 μM H_2O_2 for 2 h. Cells in the H_2O_2 group were treated with 600 μM H_2O_2 only. Subsequently, cells were harvested and washed twice using PBS, and were resuspended in 500 μl binding buffer. Annexin V-FITC stock (5 μl) and propidium iodide solution (5 μl) was added to the cells and incubated for 10 min at room temperature in the dark, and immediately analyzed using flow cytometer (BD FACSCanto II). The percentage of apoptotic cells was obtained for statistical analysis.

Protein extraction and western blot analysis. Cells were seeded in 6-well plates ($1x10^6$ cells/well) for 24 h and treated with 80 μM gigantol for 12 h followed by the addition of 600 μM H_2O_2 for 2 h, both at room temperature. Cells in the H_2O_2 group were treated with 600 μM H_2O_2 only. Cells in the gigantol + H_2O_2 + LY294002 group were pretreated

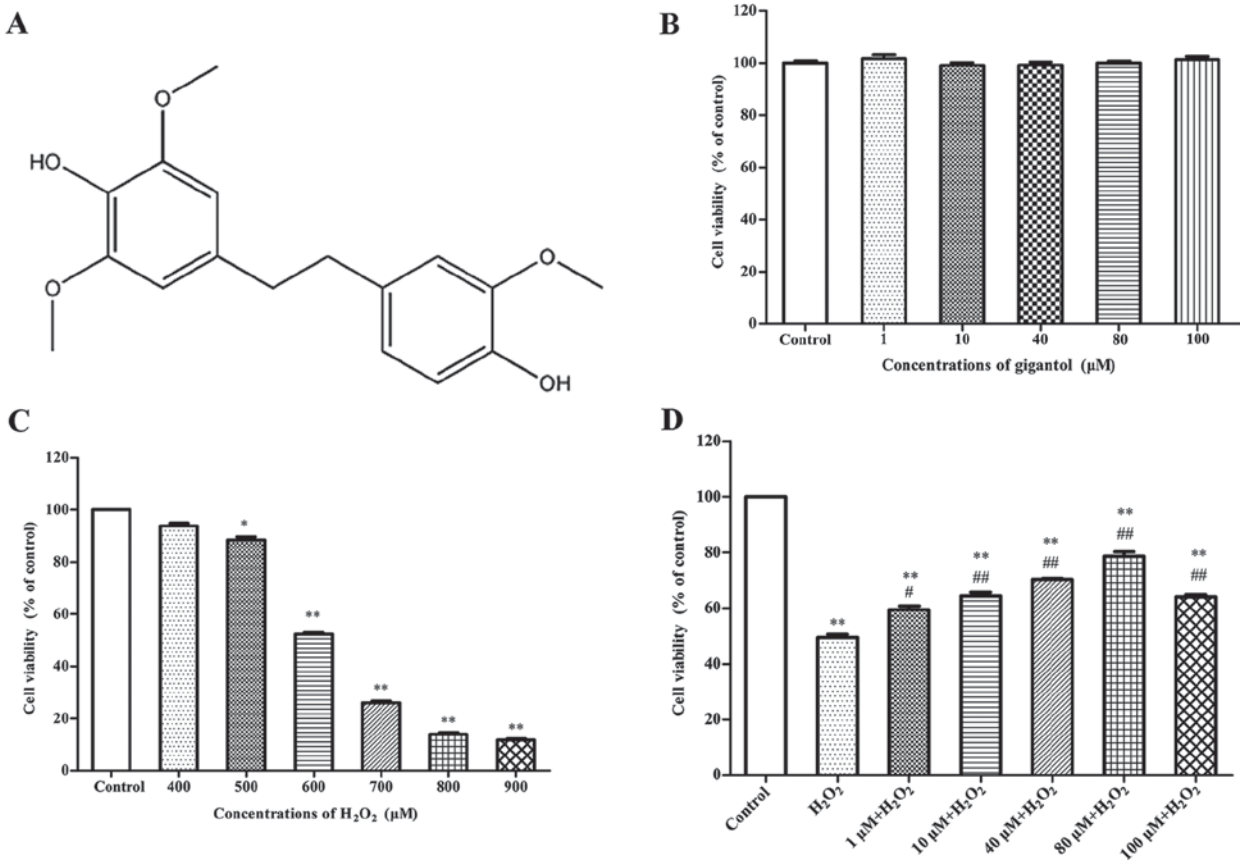

Figure 1. Effect of gigantol on the cell viability of rat bone marrow mesenchymal stem cells, as determined by MTT assays. (A) Chemical structure of gigantol. (B) Cells were cultured with various concentrations of gigantol for 12 h. (C) Cell viability was reduced dose-dependently following treatment of cells with 400, 500, 600, 700, 800 and 900 μM H_2O_2. (D) Cell viability was increased dose-dependently in cells that were pretreated with various concentrations of gigantol followed by 600 μM H_2O_2 treatment. *P<0.05 and **P<0.01 vs. control group. $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. H_2O_2 only group. H_2O_2, hydrogen peroxide.

with LY294002 (25 μmol/l) for 1 h prior to gigantol with H_2O_2 treatment. Subsequently, cells were washed with PBS and lysed in cold radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) with protein inhibitor. Cellular proteins were collected and their concentrations were determined using a Bradford assay. Equal amounts of protein (40 μg/lane) were separated on 15% SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes via electrophoresis. After blocking with tris-buffered saline (TBS) containing 5% skimmed milk and 0.05% Tween-20 for 1 h at room temperature, the membranes were incubated with the following primary antibodies: p-Akt (ser 473; cat. no. Sc7985r; 1:100; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), Akt (ser 473; cat. no. Sc8312; 1:200; Santa Cruz Biotechnology, Inc.), B-cell lymphoma-2 (Bcl-2)-associated X (Bax; cat. no. 2772; 1:1,000; CST Biological Reagents Co., Ltd., Shanghai, China), Bcl-2 (cat. no. 2872; 1:1,000; CST Biological Reagents Co., Ltd.), Caspase-3 (cat. no. 9662; 1:1,000; CST Biological Reagents Co., Ltd.), Caspase-9 (cat. no. 9504; 1;1,000; CST Biological Reagents Co., Ltd.) and β-actin (cat. no. sc58673; Santa Cruz Biotechnology, Inc.) at 4˚C overnight. After washing with TBS three times, the membranes were incubated with goat anti-rabbit immunoglobulin G antibodies conjugated with horseradish peroxidase (cat. no. 111-035-003; 1:1,000; Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA) for 1 h at room temperature. Following three washes with TBS-Tween-20, the intensity of bands was visualized using an enhanced chemiluminescence western blotting kit (Merck KGaA) and quantified by densitometric analysis with ImageJ software (version 3.0; National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. All experiments were conducted at least three times. Data are presented as the mean + standard error of the mean. Differences among groups were analyzed by one-way analysis of variance, followed by Dunnett's post-hoc test, using SPSS version 20 (IBM Corp., Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Gigantol inhibits H_2O_2-induced inhibition of cell viability in rBMSCs. To determine an appropriate concentration of gigantol, cells were treated with gigantol (1, 10, 40, 80 and 100 μM), and the results indicated that none of these concentrations exhibited a damaging effect on cell viability (Fig. 1B). Cell viability was reduced in a dose-dependent manner when treated with 400, 500, 600, 700 and 800 μM H_2O_2 for 2 h, compared with the control group. H_2O_2 at the concentration of 600 μM significantly reduced cell viability compared with the control by 51.6±3.2% (Fig. 1C). In addition, results in Fig. 1D demonstrated that gigantol significantly increased the cell

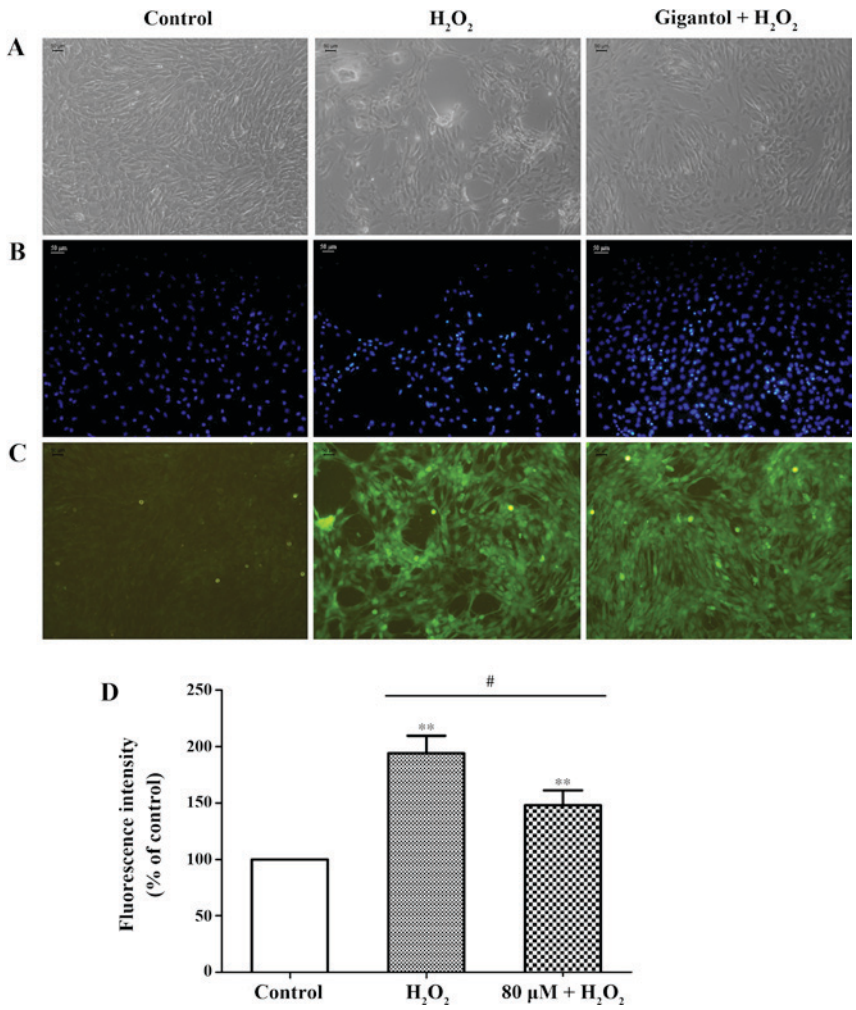

Figure 2. Effect of gigantol on apoptosis-associated morphology in rBMSCs. (A) Gigantol markedly decreased the amount of rBMSCs with apoptosis-associated morphologies induced by H_2O_2, as observed using an inverted microscope. (B) Gigantol protected rBMSCs from H_2O_2-induced morphological alterations, as observed by fluorescence microscopy using Hoechst 33258 staining. (C) Gigantol decreased the ROS levels in rBMSCs treated with H_2O_2, as determined by observing dichlorofluorescein fluorescence using fluorescence microscopy. (D) ROS levels were quantified in rBMSCs treated with H_2O_2 with or without gigantol by analyzing the fluorescence intensity with a microplate reader. Magnification, 640x. **P<0.01 vs. control group. $^{\#}$P<0.05, as indicated. rBMSCs, rat bone marrow mesenchymal stem cells; H_2O_2, hydrogen peroxide; ROS, reactive oxygen species.

viability of rBMSCs in a dose-dependent manner compared with cells treated with H_2O_2 only. Furthermore, pretreatment with 80 μM gigantol significantly enhanced cell viability compared with the H_2O_2 only group (Fig. 1D). Concentrations of gigantol >80 μM reduced the stimulatory effect. Therefore, 600 μM H_2O_2 and 80 μM gigantol were selected for the following experiments.

Assessment of morphological changes. Following treatment with H_2O_2, apoptosis-associated morphology was observed in rBMSCs, including detachment, irregular shape and nuclear shrinkage. However, the number of apoptosis-like cells decreased in the group pretreated with gigantol, which indicated a potential protective effect of gigantol from apoptosis induction (Fig. 2A and B).

Detection of ROS. Cellular oxidative stress was examined by a DCFH-DA assay. The results demonstrated that, in the H_2O_2-treated group, a significant increase in 2',7'-dichlorofluorescein fluorescence was observed (Fig. 2C and D). However, pretreatment with gigantol significantly reduced the intracellular production of ROS compared with the H_2O_2-treated group (Fig. 2C and D).

Analysis of cell apoptosis. Cell apoptosis was analyzed using an Annexin V and propidium iodide double-staining assay by flow cytometry. The percentage of apoptotic cells in Q2 and Q4 increased from 0.5±0.45% in the control group to 49.5±3.30% in the H_2O_2 group, while apoptosis was significantly reduced to 23.4±2.06% in the gigantol + H_2O_2 group, compared with the H_2O_2 only group (Fig. 3).

Gigantol activates the PI3K/Akt pathway. The results of western blot analysis demonstrated that H_2O_2 treatment reduced the protein levels of phosphorylated (p)-Akt and the antiapoptotic protein Bcl-2 (Fig. 4A and B), and increased the levels of the proapoptotic proteins Bax, caspase-3 and caspase-9 (Fig. 4B-D). However, gigantol pretreatment lowered the caspase-3, caspase-9 and Bax levels, and increased the levels of p-Akt and Bcl-2, compared with the H_2O_2 only group (Fig. 4). Furthermore, LY294002 (a PI3K inhibitor) significantly inhibited the protective effect of gigantol against

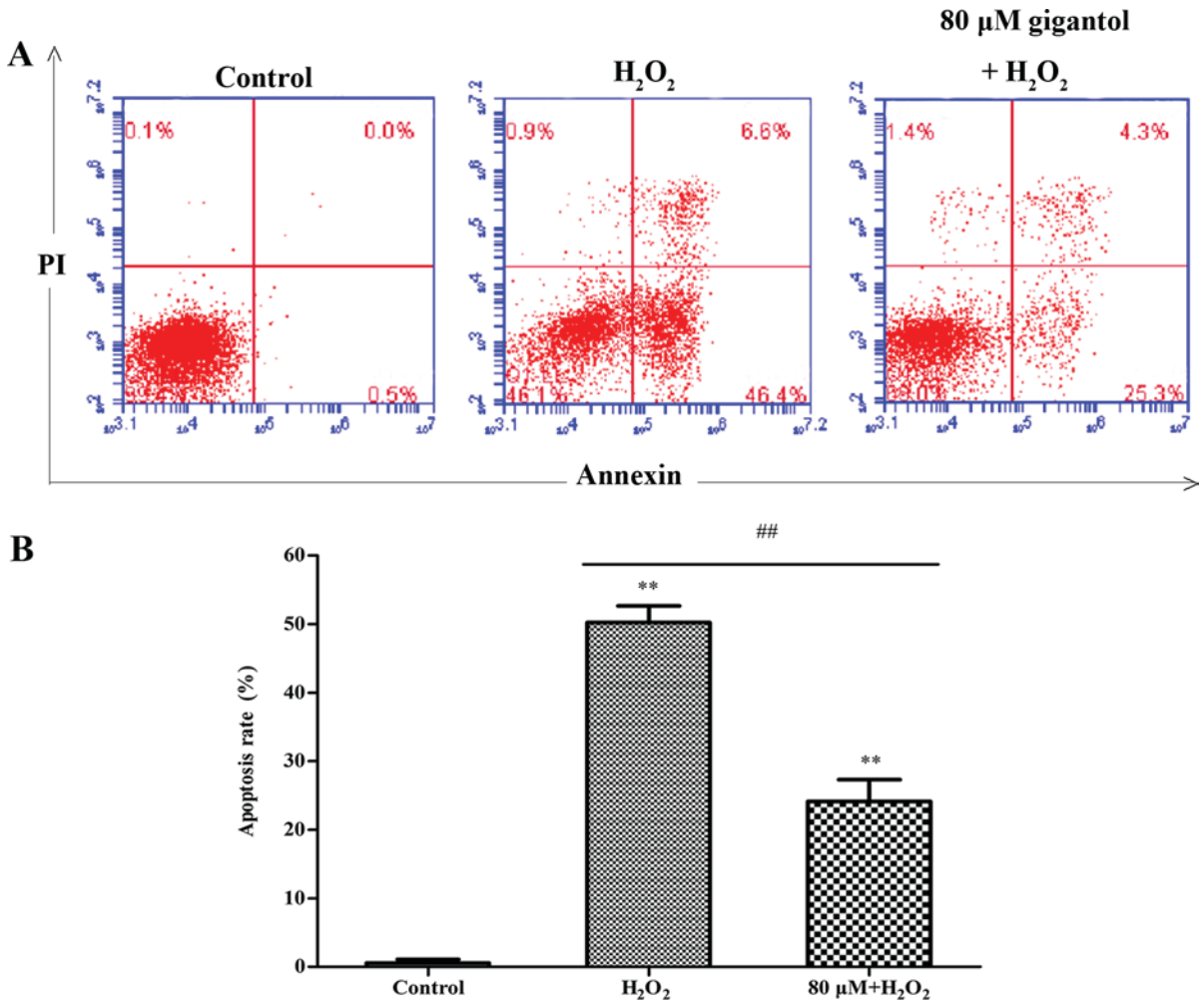

Figure 3. The effect of gigantol on rat bone marrow mesenchymal stem cell apoptosis was determined by Annexin V/PI staining. (A) Representative flow cytometry plots following Annexin V/PI staining in control, H_2O_2 and gigantol + H_2O_2 groups. (B) Apoptosis rates were statistically analyzed. The percentage of apoptotic cells in the H_2O_2 only group was 49.5±3.30%, while gigantol significantly reduced the percentage to 23.4±2.06% in Q2 and Q4. **P<0.01 vs. control group. $^{\#\#}$P<0.01, as indicated. PI, propidium iodide; H_2O_2, hydrogen peroxide.

H_2O_2-induced apoptosis by increasing the levels of caspase-3, caspase-9 and the ratio of Bax/Bcl-2, and decreasing the ratio of p-Akt/Akt (Fig. 4).

Discussion

Previous studies have reported that the transplantation of human or rat MSCs led to a substantial functional improvement in stroke treatment (20-22). However, the low survival rate of MSCs that are transplanted for the treatment of an ischemic myocardium indicates that the hypoxic microenvironment may impair the survival of MSCs. H_2O_2 has successfully been used to induce oxidative stress, which led to cell apoptosis and mimicked the hypoxic microenvironment of the ischemic brain (23-25). In addition, Sun *et al* (26) employed a H_2O_2-induced cytotoxicity model of BMSCs to investigate damage induced by oxidative stress.

We previously reported that gigantol is abundant in *Dendrobium aurantiacum* among the herbal medicines grouped as Huangcao Shihu, which includes *Dendrobium nobile*, *Dendrobium fimbriatum* and *Dendrobium aurantiacum* (27). It is uncommon for such high concentrations of active compounds to occur naturally within plants; therefore, gigantol may be of clinical value if beneficial effects are observed. The present study, to the best of our knowledge, is the first to indicate that gigantol may have protective activities against ischemic diseases, as MTT and flow cytometry results demonstrated that gigantol inhibited H_2O_2-induced cell apoptosis in rBMSCs. Furthermore, gigantol reduced the generation of ROS in H_2O_2-treated rBMSCs, which indicates that gigantol may exhibit beneficial antiapoptotic activities through inhibition of ROS generation.

A previous report demonstrated that extracellular H_2O_2 enhanced intracellular concentrations of ROS, which subsequently inactivated p-Akt (28). In the present study, treatment of MSCs with H_2O_2 led to decreased levels of p-Akt, indicating that the PI3K/Akt signaling pathway may be inhibited in MSCs following exposure to H_2O_2. In the present study, treatment with gigantol activated the expression of p-Akt in H_2O_2-induced rBMSCs. Previous studies have demonstrated that the PI3K/Akt pathway is involved in various biological processes, including cell growth, survival and apoptosis, and also has roles in cell metabolism, proliferation and migration (29-31).

PI3K/Akt is reported to prevent cell apoptosis by reducing the expression of various proapoptotic proteins, including

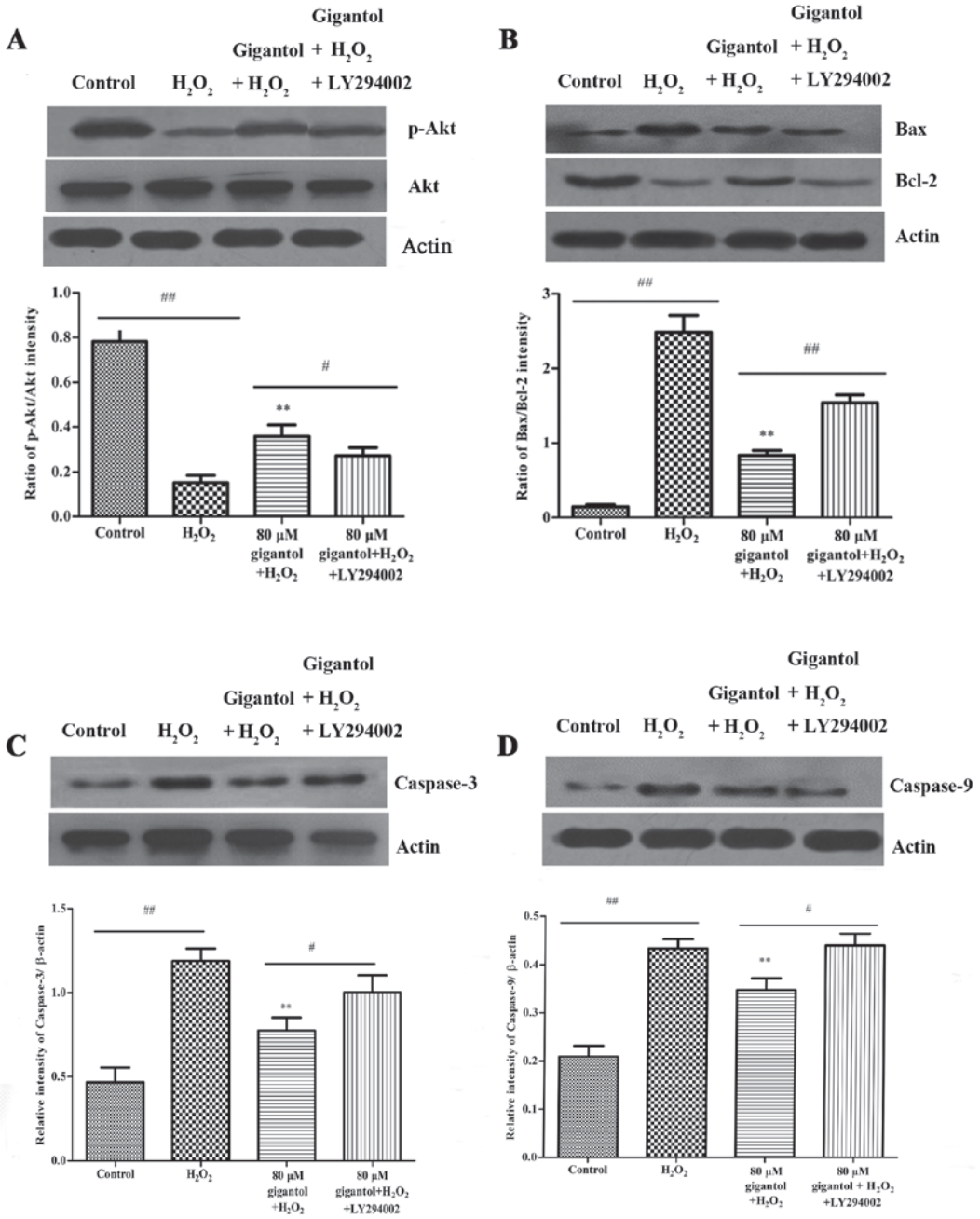

Figure 4. The effect of gigantol and LY294002 on the expression of apoptosis-associated proteins was investigated by western blot analysis. (A) Exposure of rBMSCs to 80 μM gigantol significantly increased the expression of p-Akt compared with the H_2O_2 only group, while LY294002 reduced the increase in p-Akt. Akt was used as an internal control and densitometric analysis indicates the p-Akt/Akt ratio. (B) Gigantol pretreatment attenuated H_2O_2-induced upregulation of Bax and downregulation of Bcl-2, and these effects were reversed by the application of LY294002. β-actin was used as an internal control and densitometric analysis indicates the Bax/Bcl-2 ratio. (C) Gigantol inhibited the protein expression of caspase-3 in H_2O_2-induced rBMSCs and LY294002 reversed this inhibition partially. β-actin was used as an internal control. (D) Gigantol suppressed the expression of caspase-9 in H_2O_2-induced rBMSCs, while LY294002 reversed the inhibition partially. β-actin was used as an internal control. **P<0.01 vs. H_2O_2 group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01, as indicated. rBMSCs, rat bone marrow mesenchymal stem cells; p-, phosphorylated-; Akt, protein kinase B; H_2O_2, hydrogen peroxide; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X; actin, β-actin.

caspase-3, caspase-9 and Bax, and by elevating the levels of the antiapoptotic protein Bcl-2 (32). These results are consistent with those of an earlier report, which indicated that PI3K-Akt signaling increased the intracellular levels of ROS and activated the proapoptotic proteins caspase-3, caspase-9 and Bax, and inhibited the expression of Bcl-2 (33). The results of the current study demonstrated that H_2O_2 treatment increased the Bax/Bcl-2 ratio, and caspase-3 and caspase-9 protein expression, in rBMSCs. However, pretreatment with gigantol suppressed the Bax/Bcl-2 ratio, and caspase-3 and caspase-9 levels, which indicates that gigantol may protect against rBMSC apoptosis via the PI3K/Akt signaling pathway. Furthermore, LY294002, a specific PI3K/Akt inhibitor, blocked the protective effects of gigantol. These results confirmed that PI3K/Akt may be activated by gigantol to protect rBMSCs from H_2O_2-induced apoptosis.

In conclusion, the present study demonstrated that gigantol significantly inhibited H_2O_2-induced apoptosis in rBMSCs.

The protective effect of gigantol was accompanied by reductions in intracellular ROS generation, the expression ratio of Bax/Bcl-2, and caspase-3 and caspase-9 protein expression, in addition to increases in the ratio of p-Akt/Akt and Bcl-2 expression. Therefore, gigantol may have the potential to be developed as a protective agent for the clinical treatment of patients with ischemic diseases. Regarding the utilization of gigantol in ischemic stroke, however, further *in vitro* and *in vivo* experiments are required to investigate the effect of gigantol on transport and differentiation in rBMSCs.

Acknowledgements

The present study was financially supported by the Special Foundation of 2015 High Level University Construction (grant no. 2050205), the Special Foundation of High Level University Construction of Guang Zhou University of Chinese Medicine (grant no. A1-AFD018171Z11024) and the Science and Technology Planning Project of Guangdong Province (grant no. 2013B060400022).

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