Original Article

# Effects of *Salvia miltiorrhiza* injection on apoptosis of Schwann cells induced by hydrogen peroxide

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**Background:** *Salvia miltiorrhiza* is a traditional Chinese medicine with remarkable antioxidant, antibacterial, and anticoagulant properties. In the present study, we investigated the effects of *Salvia miltiorrhiza* injection in protecting Schwann cells (SCs) from hydrogen peroxide ($H_2O_2$)-induced cell apoptosis.

**Methods:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and immunofluorescence staining were used to detect the establishment of the SC apoptosis model induced by $H_2O_2$. The effect of *Salvia miltiorrhiza* injection on injured cell morphology was observed, and the effect on cell apoptosis was determined by Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. Western blotting analysis was used to detect the effect of *Salvia miltiorrhiza* injection on apoptosis-related protein expression.

**Results:** The results of the MTT assay showed that cell activity significantly decreased after treatment with 1 mM $H_2O_2$, but different concentrations of *Salvia miltiorrhiza* injection could improve cell activity at different degrees. The number of cells increased significantly after treatment with *Salvia miltiorrhiza* injection. Annexin V-FITC/PI double staining and TUNEL results revealed that *Salvia miltiorrhiza* injection could significantly reduce apoptosis induced by $H_2O_2$. Western blotting analysis showed that the expression of Bcl-2 was significantly upregulated, while the expression level of Bax was significantly downregulated.

**Conclusions:** *Salvia miltiorrhiza* injection can protect SCs from $H_2O_2$-induced cell apoptosis, and has potential therapeutic effects in neurological disease.

**Keywords:** *Salvia miltiorrhiza* injection; Schwann cells (SCs); cell apoptosis; hydrogen peroxide ($H_2O_2$)

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## Introduction

With the development of tissue engineering and molecular biology technology, research on peripheral nerve injury is now extensive (1-3). After peripheral nerve injury, the preservation of nerve cells and prevention of apoptosis are important for nerve regeneration. The gradual restoration of its regenerative ability depends on the microenvironment provided by the relevant cytokines. As an important glial cell in the peripheral nervous system, Schwann cell (SCs) play a key role in the process of nerve injury, regeneration, and repair (4,5). Damage to SCs likely induces cell apoptosis, which will cause neurodegenerative diseases and restrict the functional recovery of peripheral nerves (6-8).

Various reactive oxygen species, such as superoxide anion, hydrogen peroxide ($H_2O_2$), and nitric oxide, are

all associated with cell apoptosis (9,10). If the body overproduces these reactants, it will accelerate cell damage and tissue dysfunction. As a permeable membrane oxidant, $H_2O_2$ can freely diffuse into various cells and organelles, and induce oxidative stress effects, which may lead to mitochondrial dysfunction and ultimately destroy cell function integrity (11,12). Many studies have found that the SC apoptosis induced by $H_2O_2$ can affect nerve regeneration (13,14).

The advantages of traditional Chinese medicine are mainly reflected in the definite clinical efficacy and relatively safe medication, which has generated interest among researchers. In recent years, many studies have reported the protective effect of *Salvia miltiorrhiza* on myocardial ischemia-reperfusion injury, which fully shows that *Salvia miltiorrhiza* is widely used in the treatment of cardiovascular diseases and its anti-inflammatory and anti-apoptotic effects (15-17). There are also studies to observe the protective effect of *Salvia miltiorrhiza* after exposing cardiomyocytes to $H_2O_2$, but there are few studies on the protective effect of *Salvia miltiorrhiza* on SCs with oxidative damage (18-20).

In the present study, we used an $H_2O_2$-induced SCs apoptosis model to investigate the potential protective role of different concentrations of *Salvia miltiorrhiza* injection. The findings indicated that *Salvia miltiorrhiza* injection can protect SCs from apoptosis caused by $H_2O_2$. We hope these findings could provide an experimental basis for the clinical application of *Salvia miltiorrhiza*.

We present the following article in accordance with the MDAR checklist (available at http://dx.doi.org/10.21037/apm-20-2580).

## Methods

### *Ethics Statement*

Experiments were performed under a project license (No.: 20190303-15) granted by Laboratory Animal Ethics Committee of Nantong University, in compliance with Nantong University institutional guidelines for the care and use of animals.

### *Cell culture*

SCs were cultured from the sciatic nerves of 1-day-old Sprague-Dawley rats, and digested by collagenase and trypsin, as previously described (21). The 1-day-old rats were acquired from the Experimental Animal Center of Nantong University [license No. SCXK (Su) 2014-0001 and SYXK (Su) 2012-0031, No. 20190225-004]. Briefly, the SCs were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) containing Recombinant Human NRG1-β1/HRG1-β1 (HRG) (R&D System, Minneapolis, MN, USA) and forskolin (Sigma, St. Louis, MO, USA). Then anti-Thy1.1 antibody (Sigma, St. Louis, MO, USA) and rabbit complement (Invitrogen, Carlsbad, CA, USA) were further added to SCs isolated, and the purified SCs was observed and photographed.

### *Immunofluorescence staining*

Cells were fixed in 4% formaldehyde for 15 min at room temperature. After washing with phosphate-buffered saline (PBS) twice; blocking solution was added at room temperature for 90 min. After incubation with anti-S100 (1:500; Abcam) antibody at 4 ℃ overnight, the secondary antibody (donkey anti-mouse IgG-Alex-488, 1:200; Invitrogen) was incubated for 1 h at room temperature under dark conditions. After staining the nucleus with Hoechst 33258, the cells were observed under a fluorescence microscope (AxioImager M2; Zeiss).

### *Cell treatment*

Prior to each experiment, $H_2O_2$ was freshly diluted to a 1 mM final concentration with DMEM. *Salvia miltiorrhiza* injection (Zhengda Qingchunbao, China) was diluted into different concentrations (1/160, 1/80, 1/40) with DMEM, according to the concentration of the stock solution (1–1.5 g/mL). To determine the effect of *Salvia miltiorrhiza* injection, SCs were pretreated with *Salvia miltiorrhiza* injection for 24 h, followed by co-treatment with 1 mM $H_2O_2$ for 30 min. In a single experiment, each treatment was performed in triplicate.

### *Cell viability assay*

The cells were inoculated onto 96-well plates, and each group was subjected to corresponding treatment. A total of 20 μL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well and cultured at 37 ℃ for 4 h. The supernatant was discarded, and 150 μL dimethyl sulfoxide (DMSO) was added to each well and gently shaken. The light absorption value of each group was

 

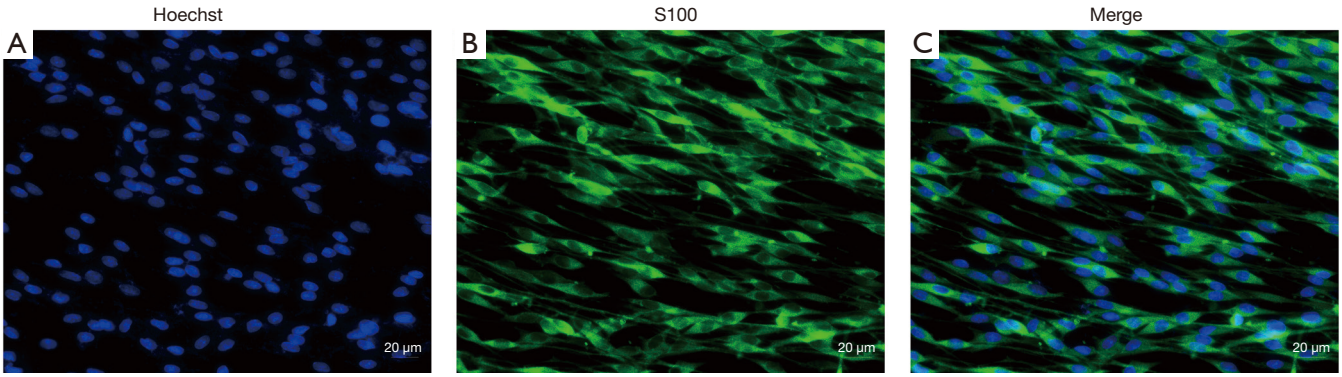


**Figure 1** Immunocytochemistry identification of Schwann cells (SCs) in primary culture. (A) Hoechst 33342 staining; (B) S100 immunocytochemical staining; (C) Hoechst 33342 staining (blue) is combined with S100 (green) immunocytochemical staining. Scale bar =20 μm.

determined at a wavelength of 490 nm with a microplate analyzer.

### *Morphological observation of cells*

SCs were inoculated onto a 24-well plate at a density of $5\times10^4$/mL. After the treatment of cells in each group, morphological changes of cells were observed under an inverted microscope. Six fields were randomly selected for each group, and the experiment was repeated 3 times.

### *Flow cytometry*

To detect the apoptosis rate of SCs, flow cytometry apoptosis was detected in each group. Cells were resuspended in binding buffer and stained with 5 μL Annexin V-FITC and 10 μL propidium iodide (PI) at room temperature for 15 min under dark conditions. Apoptotic cells were analyzed by flow cytometry (BD Biosciences, San Jose, CA, USA), and the number of early apoptotic cells were calculated.

### *Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay*

Cells were seeded at a density of $2\times10^5$ cells/mL with different treatments. TUNEL assay (Promega, Madison, WI, USA) was used to detect apoptotic cells. Hoechst 33342 was added for observation. The apoptotic rate was observed by calculating the number of TUNEL-positive cells in each field.

### *Western blotting analysis*

After the cell model was established, the proteins of each group were extracted; 30 μg cell proteins were separated on 10% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). After blocking for 2 h with 5% skim milk, anti-Bcl-2 (1:1,000; Abcam), anti-Bax (1:1,000; Abcam), and anti-GAPDH (1:5,000; Abcam) antibodies were added and incubated overnight at 4 ℃. The secondary antibody was incubated at room temperature for 1 h, and protein data were analyzed.

### *Statistical analysis*

All data are presented as mean ± standard error. Statistical significance was conducted by one-way analysis of variance. Differences between groups were compared by *t*-test, and $P<0.05$ was considered to be statistically significant.

## Results

### *Establishment of SC apoptosis model induced by $H_2O_2$*

The purity of primary cultured SCs was determined by immunochemistry with anti-S100 staining (*Figure 1*). After adding 1 mM $H_2O_2$ to the SCs for 15, 30, 60, and 120 min, the cell activity decreased significantly. As shown in *Figure 2A*, an obvious decrease of viability was observed at 30 min with 1 mM $H_2O_2$ exposure (51.3%±1.01%, compared with the control). Treatment with 1 mM $H_2O_2$

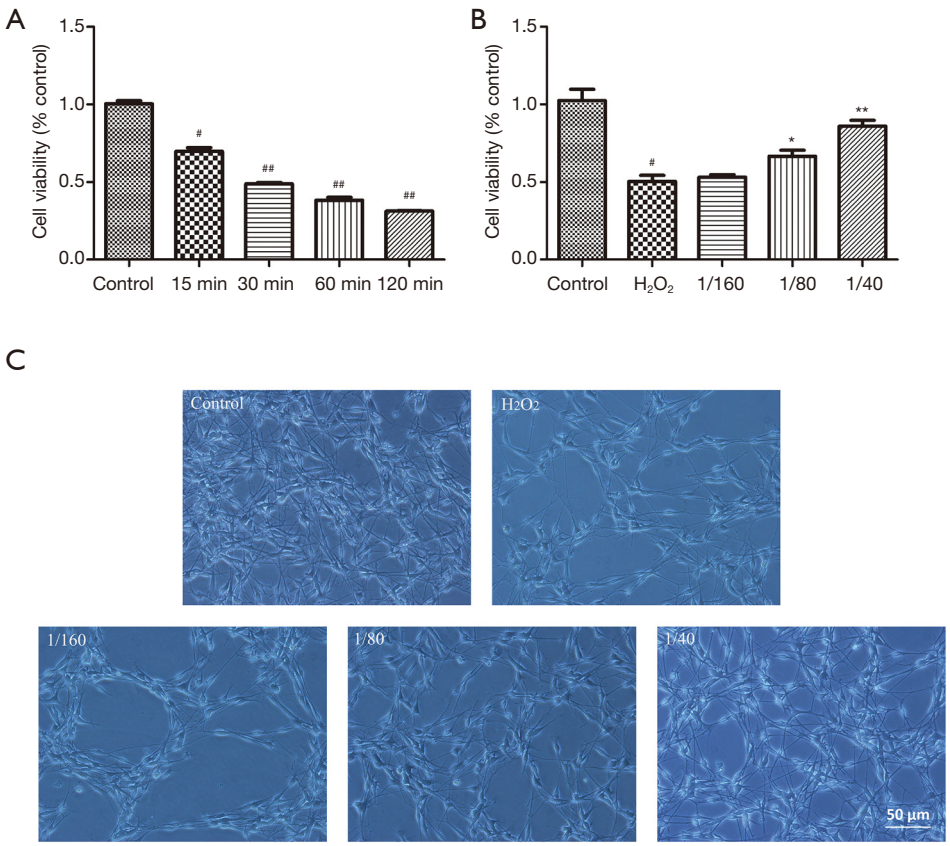


**Figure 2** Effects of *Salvia miltiorrhiza* injection on viability in hydrogen peroxide ($H_2O_2$)-treated Schwann cells (SCs). (A) SCs were exposed to 1 mM $H_2O_2$ for the indicated times (15, 30, 60, 120 min). (B) SCs were incubated with *Salvia miltiorrhiza* injection (1/160, 1/80, 1/40) for 24 h, and 1 mM $H_2O_2$ was added for 30 min. (C) Changes in SC morphology. $^{\#}P<0.05$, $^{\#\#}P<0.01$ *vs.* control cells. *P<0.05, **P<0.01 *vs.* cells exposed to $H_2O_2$ alone. Scale bar =50 μm.

for 30 min was used for subsequent experiments.

### *Effect of Salvia miltiorrhiza injection on $H_2O_2$-induced cytotoxicity of SCs*

To evaluate the potential cytoprotective effect of *Salvia miltiorrhiza* injection against $H_2O_2$-induced SC death, SCs were pretreated with *Salvia miltiorrhiza* injection at various concentrations (1/160, 1/80, 1/40) for 24 h, followed by exposure to $H_2O_2$ at a final concentration of 1 mM for 30 min. As shown in *Figure 2B*, a low concentration of *Salvia miltiorrhiza* injection had no significant effect on the viability of apoptotic SCs, while moderate and high concentrations of *Salvia miltiorrhiza* injection increased the survival to 71.8%±1.6% and 85.4%±1.2%, respectively (P<0.05). These data suggest that *Salvia miltiorrhiza* injection at these concentrations was not significantly cytotoxic.

### *Effect of Salvia miltiorrhiza injection on $H_2O_2$-induced morphological changes of SCs*

After treatment with different concentrations of *Salvia miltiorrhiza* injection, we observed the morphology of SCs in each group. As shown in *Figure 2C*, cells in the control group had good morphology. The number of cells in the $H_2O_2$ group was significantly reduced, and shrinking and rounding of cell bodies and decreased connections between cells were observed. There was little change in cell number and morphology in the low concentration group. The number of cells in the moderate concentration group increased significantly. Compared with the moderate concentration group, the number of cells in the high concentration group increased more and the cells are better

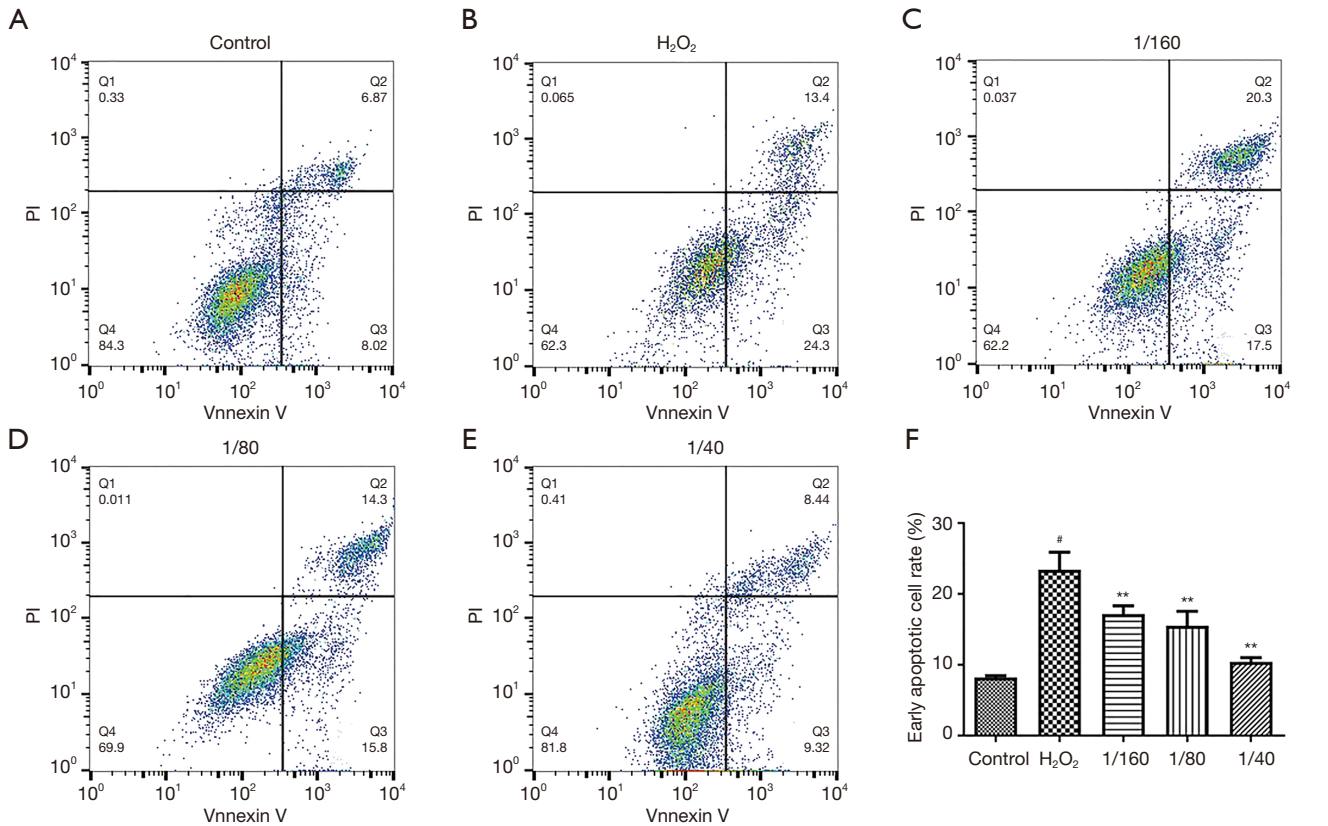


**Figure 3** Effects of *Salvia miltiorrhiza* injection on hydrogen peroxide ($H_2O_2$)-induced cell apoptosis measured by flow cytometry. (A) Schwann cells (SCs) were cultured with normal medium. (B) SCs were exposed to 1 mM $H_2O_2$ for 30 min. (C) SCs were incubated with *Salvia miltiorrhiza* injection at a concentration of 1/160 for 24 h, followed by co-treatment with 1 mM $H_2O_2$ for 30 min. (D) SCs were incubated with *Salvia miltiorrhiza* injection at a concentration of 1/80 for 24 h, followed by co-treatment with 1 mM $H_2O_2$ for 30 min. (E) SCs were incubated with *Salvia miltiorrhiza* injection at a concentration of 1/40 for 24 h, followed by co-treatment with 1 mM $H_2O_2$ for 30 min. (F) The percentages of the rate of early apoptosis are shown by the histogram. Results are expressed as the mean ± standard error of mean, n=5. $^{\#}P<0.05$, *vs.* control cells. $^{**}P<0.01$ *vs.* cells exposed to $H_2O_2$ alone.

connected to each other.

### *Salvia miltiorrhiza injection protects SCs against $H_2O_2$-induced cell apoptosis*

Flow cytometry results showed that the apoptosis of SCs increased rapidly in the $H_2O_2$ group. The number of apoptotic cells decreased significantly after treatment with different concentrations of *Salvia miltiorrhiza* injection, and the protective effect became more obvious with the drug concentration increase. Compared with the control group, the difference was statistically significant (P<0.01) (*Figure 3*). TUNEL-positive cells significantly increased from 3.78%±1.05% (control) to 13.88%±1.89% after exposure to $H_2O_2$ alone (P<0.01). After treatment with different concentrations of *Salvia miltiorrhiza* injection, the number of TUNEL-positive cells reduced to 9.03%±1.92%, 8.46%±1.23%, and 4.88%±2.24%, respectively (P<0.01) (*Figure 4*).

### *Salvia miltiorrhiza injection inhibits apoptosis, as measured by Bax and Bcl-2 in SCs*

To confirm the protection of *Salvia miltiorrhiza* injection against $H_2O_2$-induced apoptosis, apoptosis-associated protein levels were measured. Treatment with different concentrations of *Salvia miltiorrhiza* injection significantly increased the expression of Bcl-2 and significantly decreased

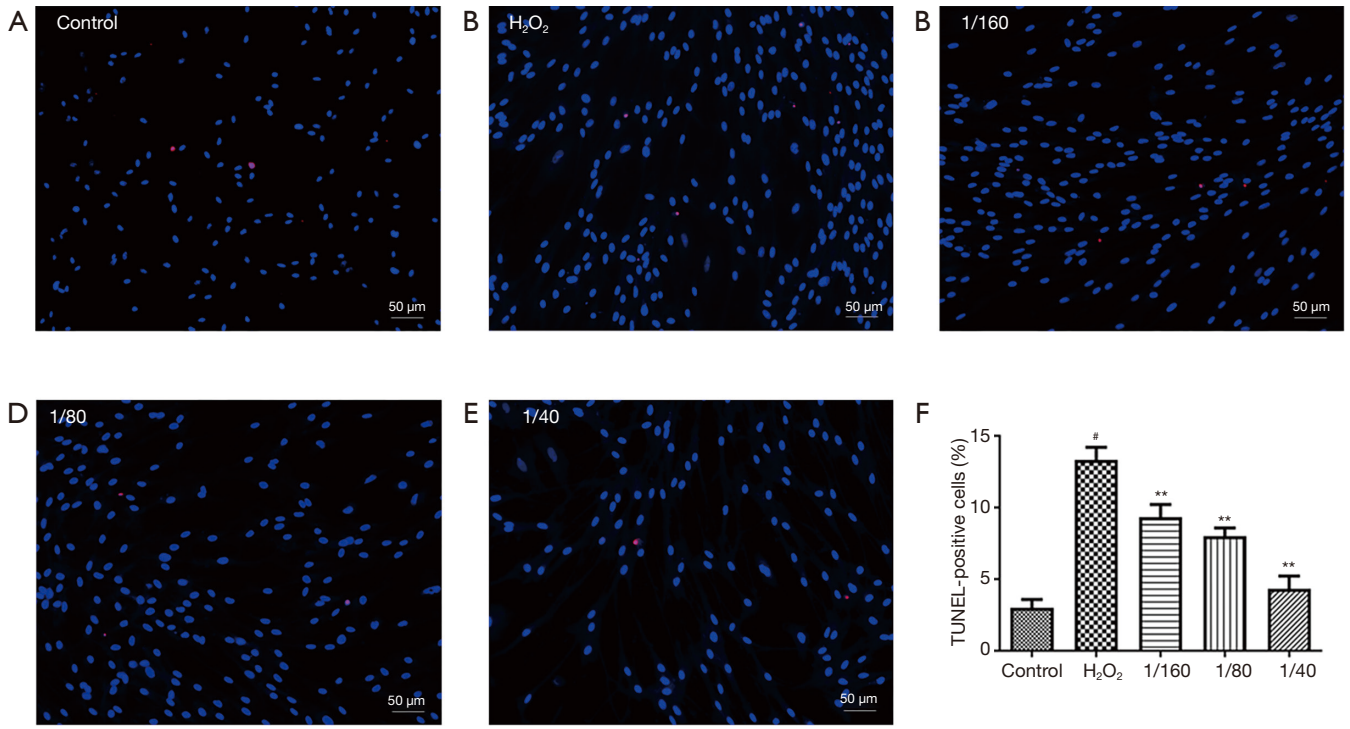


**Figure 4** Effects of *Salvia miltiorrhiza* injection on hydrogen peroxide ($H_2O_2$)-induced cell apoptosis detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. (A) Schwann cells (SCs) were cultured with normal medium. (B) SCs were exposed to 1 mM $H_2O_2$ for 30 min. (C) SCs were incubated with *Salvia miltiorrhiza* injection at a concentration of 1/160 for 24 h, followed by co-treatment with 1 mM $H_2O_2$ for 30 min. (D) SCs were incubated with *Salvia miltiorrhiza* injection at a concentration of 1/80 for 24 h, followed by co-treatment with 1 mM $H_2O_2$ for 30 min. (E) SCs were incubated with *Salvia miltiorrhiza* injection at a concentration of 1/40 for 24 h, followed by co-treatment with 1 mM $H_2O_2$ for 30 min. (F) The percentages of TUNEL-positive cells are shown by the histogram. TUNEL-positive cells (red) under fluorescence microscopy; number of positive cells was counted. Results are expressed as the mean ± standard error of mean, n=5. $^{\#}P<0.05$, *vs.* control cells. $^{**}P<0.01$ *vs.* cells exposed to $H_2O_2$ alone. Scale bar =50 μm.

the expression of Bax compared with that of $H_2O_2$ treatment alone (*Figure 5*).

## Discussion

*Salvia miltiorrhiza* is a well-known traditional Chinese medicine with many functions and effects, including enhancing myocardial contractility, regulating heart function and lowering blood lipids (22,23). In the present study, we found that *Salvia miltiorrhiza* injection could protect SCs from $H_2O_2$-induced damage. To the best of our knowledge, our study is the first to report on the protective effect of *Salvia miltiorrhiza* injection on SCs during oxidative stress.

In the process of nerve regeneration, SCs can secrete a large number of growth factors to promote axon growth and myelin formation, which play a key role in peripheral nerve regeneration (24,25). Oxidative stress can affect DNA synthesis and mitochondrial function, and completely destroy cell integrity. Therefore, apoptosis induced by oxidative stress is an important pathogenic factor in many neurodegenerative diseases (26).

In the present study, we constructed a $H_2O_2$ injury model in primary SCs to explore the protective effects of different concentrations of *Salvia miltiorrhiza* injection on SC apoptosis induced by $H_2O_2$. We found that *Salvia miltiorrhiza* injection can significantly improve the morphological changes and the viability of SCs exposed to $H_2O_2$. Annexin V-FITC/PI flow cytometry and TUNEL test results also showed that *Salvia miltiorrhiza* injection can inhibit the apoptosis of SCs induced by $H_2O_2$ in a dose-dependent manner. In addition, it was also found that the expression of Bax and Bcl-2 both changed significantly after adding different concentrations of *Salvia miltiorrhiza*

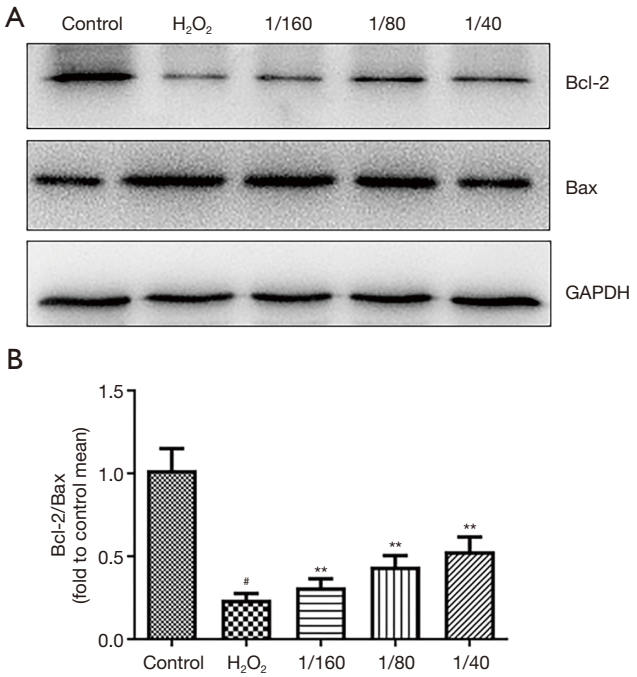


**Figure 5** *Salvia miltiorrhiza* injection inhibited apoptosis measured by the protein levels in Schwann cells (SCs) exposed to hydrogen peroxide ($H_2O_2$). (A) The level of Bax and Bcl-2 were measured by western blot. (B) Statistic analysis of (A). GAPDH was used as a loading control. Results are expressed as the mean ± standard error of mean, n=5. #P<0.05, *vs.* control cells. **P<0.01 *vs.* cells exposed to $H_2O_2$ alone.

injection.

The findings of the present study indicate that different concentrations of *Salvia miltiorrhiza* injection can protect SCs from apoptosis caused by $H_2O_2$. The findings provide a basis for the application of *Salvia miltiorrhiza* in peripheral nerve injury repair, but further research on its molecular mechanism warrants further investigation.

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## Footnote

*Reporting Checklist:* The authors have completed the MDAR reporting checklist. Available at http://dx.doi.org/10.21037/apm-20-2580

*Data Sharing Statement:* Available at http://dx.doi.org/10.21037/apm-20-2580

*Conflicts of Interest:* All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/apm-20-2580). The authors have no conflicts of interest to declare.

*Ethics Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Experiments were performed under a project license (No.: 20190303-15) granted by Laboratory Animal Ethics Committee of Nantong University, in compliance with Nantong University institutional guidelines for the care and use of animals.



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