A combination of four effective components derived from Sheng-mai san attenuates hydrogen peroxide-induced injury in PC12 cells through inhibiting Akt and MAPK signaling pathways

CAO Guo-Sheng, LI Shao-Xia, WANG Yan, XU Ying-Qiong, LV Yan-Ni, KOU Jun-Ping *, YU Bo-Yang

Jiangsu Key Laboratory of TCM Evaluation and Translational Research, Department of Complex Prescription of TCM, China Pharmaceutical University, Nanjing, 211198, China

Available online 20 Jul., 2016

[ABSTRACT] The present study was designed to investigate whether a combination of four effective components derived from Sheng-mai san (SMXZF; ginsenoside Rb1: ginsenoside Rg1: DT-13: Schizandrol A as 6 : 9 : 4 : 5) could attenuate hydrogen peroxide (H₂O₂)-induced injury in PC12 cells, focusing on the Akt and MAPK pathways. The PC12 cells were exposed to H₂O₂ (400 μmol·L⁻¹) for 1 h in the presence or absence of SMXZF pre-treatment for 24 h. Cell viability was measured by MTT assay. The efflux of lactate dehydrogenase (LDH), the intracellular content of malondialdehyde (MDA), the activities of superoxide dismutase (SOD), and caspase-3 were also determined. Cell apoptosis was measured by Hoechst 33342 staining and Annexin V-FITC/PI staining method. The expression of Bcl-2, Bax, cleaved caspase-3, Akt, and MAPKs were detected by Western blotting analyses. SMXZF pretreatment significantly increased the cell viability and SOD activity and improved the cell morphological changes, while reduced the levels of LDH and MDA at the concentrations of 0.1, 1 and 10 μg·mL⁻¹. SMXZF also inhibited H₂O₂-induced apoptosis in PC12 cells. Moreover, SMXZF reduced the activity of caspase-3, up-regulated the protein ratio of Bcl-2 and Bax and inhibited the expression of cleaved caspase-3, p-Akt, p-p38, p-JNK and p-ERK1/2 in H₂O₂-induced PC12 cells. Co-incubation of Akt inhibitor or p38 inhibitor partly attenuated the protection of SMXZF against H₂O₂-injured PC12 cells. In conclusion, our findings suggested that SMXZF attenuated H₂O₂-induced injury in PC12 cells by inhibiting Akt and MAPKs signaling pathways, which might shed insights on its neuroprotective mechanism.

[KEY WORDS] Combination of effective TCM components; Sheng-mai san; SMXZF; PC12 cells; Hydrogen peroxide; Akt; MAPKs

[CLC Number] R965

Introduction

Most neurodegenerative diseases, such as Alzheimer’s disease [1], Parkinson’s disease (PD) [2-3], and stroke [4-5], are characterized by oxidative stress-induced cell damage, which can lead to a progressive loss of cognitive function, mitochondrial dysfunction, and apoptosis in neuronal cells [6-7]. Previous studies have indicated that increasing levels of reactive oxygen species (ROS) and hydrogen peroxide (H₂O₂) through oxidative metabolism or decreasing clearance of H₂O₂ induced by oxidative stress and subsequent cell death is owing to anti-oxidant deficiency in the cell [8-9]. Enzymes and antioxidant nutrients, which have several natural defense mechanisms and capture the chain reaction of ROS initiation and production, cannot prevent the damage completely [10-11]. And the brain is vulnerable to oxidative stress damage due to its high energy use and metabolic demands, high cellular content of lipids and proteins, extensive axonal and dendritic networks, and low levels of endogenous scavengers [12-14]. A number of signaling pathways are involved in protecting cells against H₂O₂-induced neuronal...
damages, such as anti-inflammation, anti-oxidation, and anti-apoptosis pathways. Cell apoptosis is one of the major factors responsible for H₂O₂-induced damages \[15-17\]. In the process of cell apoptosis, caspase-3 is the most important terminal enzyme; Bax and its related protein Bcl-2 act as regulators of apoptosis at the mitochondrial level. Overexpression of Bax protein following oxidation stress plays a key role in DNA fragmentation and neuronal death and induces the release of apoptogenic factors by heterodimerized at the Bel-2-interacting domain of the mitochondrial membrane. On the contrary, the anti-apoptotic protein Bcl-2 neutralizes Bax by interacting with it and inhibiting activation of the apoptosis signaling cascade \[18\]. In addition, two important signaling pathways, including Akt and MAPKs (p38, JNK, and ERK1/2) pathways, majorly regulate the caspase-3 activity and the ratio of Bcl-2/Bax proteins level in the process of cell apoptosis \[19-21\].

Sheng-mai san, which is composed of Panax ginseng, Ophiopogon japonicas, and Schisandra chinensis, is one of the famous complex prescriptions in traditional Chinese medicine with the effects of nourishing the Qi, tonifying the Yin, restoring pulse, and treating collapse \[22-23\]. It has been mostly used for cardiovascular and cerebrovascular diseases in clinic with significant therapeutic effects \[24-25\], which is partly ascribed to its anti-oxidative activities \[26-27\]. The proper proportion of effective constituents of SMXZF (the proportion of ginsenoside Rb1 : ginsenoside Rg1 : DT-13 : Schizandrol A as 6 : 9 : 4 : 5, their chemical structures were shown in Fig. 1) is derived from Sheng-mai san, a complex prescription for prevention and treatment of cardiovascular and cerebrovascular diseases. Recent studies have shown that SMXZF exerts significant protection against cerebral ischemia-reperfusion injury in a mouse model of stroke in vivo \[28\] and inhibits H₂O₂-induced PC12 cell apoptosis linked with caspase-3/ROCK1/MLC pathway in vitro \[29\]. However, the other possible underlying mechanisms of SMXZF on PC12 cells induced by H₂O₂ remain unclear. To provide further evidence for its potential use for most neurodegenerative diseases, we evaluated its neuroprotective activities and potential cellular mechanisms in the present study.

![Fig. 1 Chemical structures of four components in SMXZF. A: Ginsenoside Rb1. B: Ginsenoside Rg1. C: DT-13. D: Schizandrin A](image)

**Materials and Methods**

**Test compounds, chemicals, and reagents**

The effective constituents of SMXZF (ginsenoside Rb1, ginsenoside Rg1, and schizandrin) were purchased from Nanjing Zelang Medical Technology Co., Ltd. (Nanjing, China), and DT-13 with the purity greater than 95% was kindly provided by Dr. Qi Jin at the Department of Complex Prescription of TCM, China Pharmaceutical University (Nanjing, China). They were dissolved in Dulbecco’s modified Eagle’s medium (DMEM), which was purchased from Gibco (New York, USA). Assay kits for lactate dehydrogenase (LDH), malondialdehyde (MDA), and superoxide dismutase (SOD) were purchased from Nanjing Jiancheng Biological Engineering Institute (Nanjing, China). N-acetylcysteine (NAC, as an anti-oxidant) was purchased from Sigma (St. Louis, MO, USA). Hoechst 33342 (bissbenzimide), enhanced chemiluminescence (ECL) reagent, wortmannin (Akt inhibitor) and SB203580 (p38MAPK inhibitor) were obtained from Beyotime (Haimen, Jiangsu, China). Polyvinylidene fluoride (PVDF) membranes were purchased from Millipore (Bedford, MA, USA). 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Ameresco (Ameresco, OH, USA).
Rabbit anti-Bcl-2, anti-Bax, anti-cleaved caspase-3, anti-pro-caspase-3, anti-Akt, anti-phospho-Akt (Thr308), anti-p38, anti-phospho-p38(Thr180/Tyr182), anti-JNK, anti-phosphor-JNK (Thr183/Tyr185), anti-ERK1/2, and anti-phosphor-ERK1/2 (Thr202/Tyr204) anti-bodies were purchased from Cell Signaling Technology (Boston, MA, USA). Glyceraldehyde phosphate dehydrogenase (GAPDH) antibody was obtained from Kangchen Bio-Tech (Shanghai, China). Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG and horseradish peroxidase (HRP)-conjugated goat anti-rabbit and anti-mouse IgGs were purchased from Boster (Wuhan, China).

Cell culture and drug treatment

Differentiated rat pheochromocytoma PC12 cells purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences, were grown in tissue culture flasks in DMEM supplemented with 10% new-born calf serum (NCS), and 100 U·mL−1 of penicillin and 100 U·mL−1 of streptomycin. The cultures were maintained at 37 ºC in a humidified atmosphere containing 5% CO2. Prior to exposure to 400 μmol·L−1 of SMXZF or NAC (500 μmol·L−1) and inhibitor (10 μmol·L−1) of SMXZF or NAC (500 μmol·L−1) for 24 h prior to exposure to 400 μmol·L−1 H2O2 for 1 h. For pharmacological evaluations, the PC12 cells were treated with SMXZF at the final concentrations of 0.1–10 μmol·L−1 of SMXZF or NAC (500 μmol·L−1) for 24 h.

Cell morphology evaluation

Cell morphology was analyzed under an inverted microscope as described previously [29]. Briefly, the cells were synchronized with serum-free high glucose DMEM medium and cultured for 1 h before the experiment. After synchronization, the cells were treated with SMXZF at the final concentrations of 0.1–10 μmol·L−1, 500 μmol·L−1 NAC, or the medium for 24 h. Then, 400 μmol·L−1 H2O2 was added and incubated for another 1 h. The cells were washed twice with ice-cold PBS and then observed under an inverted microscope for morphological changes.

Cell viability and LDH release assays

The cell viability assay was performed as described previously [29]. Briefly, 10 μL of 0.5 mg mL−1 tetrazoline in phosphate buffer saline was added to the media. The plates were cultured for 3 h at 37 ºC in a humidified atmosphere of 5% CO2, followed by the addition of 150 μL of dimethyl sulfoxide. The 96-well plates were shaken for 10 min and the OD values at 570 nm were detected using a microplate reader, with a reference wavelength at 650 nm. Cell viability was expressed as a percentage of the control cells treated with vehicle (100%). The culture supernatant was used to measure the levels of LDH, according to the manufacturer’s instructions (Nanjing Jiancheng Co.).

Detection of apoptotic cells

Hoechst 33342 staining and Annexin V-FITC/propidium iodide (PI) staining were used for evaluation of cell apoptosis, according to the methods described previously [30-31]. After drug treatment, the PC12 cells were washed with PBS and then fixed with 0.2 mL of cell staining buffer. The cells were incubated in 1 mL of Hoechst 33342 at 4 ºC for 30 min in the dark, and the fluorescence images were obtained under an inverted fluorescence microscope.

In addition, the cells in each well were incubated at room temperature for 10 min in the dark with 5 μL of Annexin V-FITC and 5 μL of PI. The cells were analyzed on a flow cytometer (Becton–Dickinson, San Jose, CA, USA) using FL1 channel for fluorescein detection and FL2 channel for PI detection. In the final analysis of results, the total cells and damaged cells were counted and the percentage of surviving cells was calculated.

Measurement of caspase-3 Activity

The caspase-3 activity was determined using a caspase-3 assay kit (Beyotime, Nanjing, China) [19]. The cells were cultured in 6-well plates, and after drug treatment, the medium was discarded and the cells were washed twice with cold PBS; After 0.125% trypsin digestion about 50 s, the cells were harvested and centrifuged at 2 000 r·min−1 for 5 min at 4 ºC. The supernatant was removed and the cells were washed with PBS once. Two million cells were added to 100 μL of lysis buffer, and the resuspended precipitate was centrifuged at 12 000 r·min−1 for 15 min at 4 ºC. The supernatant was transferred to a pre-chilled centrifuge tube, and caspase-3 activity was measured, according to kit instructions.

Measurement of MDA and SOD Levels

In brief, the cells were harvested and centrifuged at 2 000 r·min−1 for 5 min at 4 ºC after homogenized in ice-cold PBS. The supernatant was used to measure the levels of SOD and MDA, according to the manufacturer’s instructions.

Western blotting analysis

For Western blotting, the whole cell lysates were centrifuged at 12 000 r·min−1 for 10 min at 4 ºC. Equal amounts of proteins (30 μg) were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes. Immunodetection was performed after blocking nonspecific binding sites on the membrane with 5% bovine serum albumin (BSA) for 1.5 h. The membranes were incubated overnight at 4 ºC with the appropriate primary antibodies: Bcl-2 (1 : 1 000), Bax (1 : 1 000), cleaved caspase-3 (1 : 1 000), pro-caspase-3(1 : 1 000), Akt (1 : 1 000), phospho-Akt (1 : 1 000), p38 (1 : 1 000), phospho-p38 (1 : 1 000), JNK (1 : 1 000), phosphor-JNK (1 : 1 000), ERK1/2 (1 : 1 000), phosphor-ERK1/2 (1 : 1 000), and GAPDH (1 : 8 000; Kang Cheng, Shanghai, China). After washing, the membranes were incubated with the goat anti-rabbit or anti-mouse antibody conjugated with horseradish peroxidase as the secondary antibody for 2 h. The antigen-antibody complexes were then detected with ECL reagent and visualized by Quantity One.
software 4.6.2 (Bio-Rad).

**Statistical analysis**

All the data analyses were performed with GraphPad Prism Version 5.01 (GraphPad Software Inc., La Jolla, CA, USA). The data were expressed as mean ± standard deviation (SD) of at least three independent experiments. Differences among different groups were analyzed with one-way analysis of variance (ANOVA) with subsequent post hoc analysis using Dunnett’s test when the data involved three or more groups. \( P < 0.05 \) was considered statistically significant.

**Results**

**Effects of SMXZF on cell morphology, viability, and LDH release in PC12 cells treated with \( \text{H}_2\text{O}_2 \)**

As shown in Fig. 2A, compared with the control group, exposure to \( \text{H}_2\text{O}_2 \) (400 \( \mu \text{mol·L}^{-1} \)) for 1 h resulted in the edge of cell body shrinkage and shortened neurite fracture, while the morphological changes induced by \( \text{H}_2\text{O}_2 \) was significantly attenuated after pre-incubation with SMXZF (0.1, 1, and 10 \( \mu \text{g·mL}^{-1} \)) for 24 h, and a positive control, NAC at 500 \( \mu \text{mol·L}^{-1} \) also protected cell morphology against \( \text{H}_2\text{O}_2 \) injury. Fig. 2B shows that exposure to \( \text{H}_2\text{O}_2 \) (400 \( \mu \text{mol·L}^{-1} \)) for 1 h resulted in a 30.77% of cell loss, which was significantly attenuated by the pretreatment with SMXZF (0.1, 1 and 10 \( \mu \text{g·mL}^{-1} \)) or NAC (500 \( \mu \text{mol·L}^{-1} \)) (\( P < 0.01 \)). LDH, as a biomarker for injured cells, is a cytosolic enzyme and leaks out of cells when the membrane is damaged \[^{52}\]. As shown in Fig. 2C, the level of LDH leakage was inhibited significantly in PC12 cells by SMXZF at concentrations of 0.1–10 \( \mu \text{g·mL}^{-1} \) for 24 h, the LDH leakage rate dropped to 29.41%, 28.59% and 26.02%, significantly lower than that of the model group (41.07%). All those data indicated that 0.1–10 \( \mu \text{g·mL}^{-1} \) SMXZF reduced the \( \text{H}_2\text{O}_2 \)-induced membrane damage in PC12 cells.

![Fig. 2](image_url)

**Fig. 2** Effects of SMXZF on the oxidative injury in PC12 cells induced by \( \text{H}_2\text{O}_2 \). The PC12 cells were pretreated with SMXZF (0.1–10 \( \mu \text{g·mL}^{-1} \)) for 24 h and then exposed to 400 \( \mu \text{mol·L}^{-1} \) \( \text{H}_2\text{O}_2 \) for 1 h. (A) The cell morphology was detected by an inverted microscope. (a) Control; (b) 400 \( \mu \text{mol·L}^{-1} \) \( \text{H}_2\text{O}_2 \); (c) 0.1 \( \mu \text{g·mL}^{-1} \) SMXZF + 400 \( \mu \text{mol·L}^{-1} \) \( \text{H}_2\text{O}_2 \); (d) 1 \( \mu \text{g·mL}^{-1} \) SMXZF + 400 \( \mu \text{mol·L}^{-1} \) \( \text{H}_2\text{O}_2 \); (e) 10 \( \mu \text{g·mL}^{-1} \) SMXZF + 400 \( \mu \text{mol·L}^{-1} \) \( \text{H}_2\text{O}_2 \); (f) 10 \( \mu \text{g·mL}^{-1} \) SMXZF + 400 \( \mu \text{mol·L}^{-1} \) \( \text{H}_2\text{O}_2 \); (g) 500 \( \mu \text{mol·L}^{-1} \) NAC + 400 \( \mu \text{mol·L}^{-1} \) \( \text{H}_2\text{O}_2 \); (B) The cell viability was detected using MTT assay described in methods. (C) The activity of LDH was detected using the CytoTox 96 nonradioactive cytotoxicity assay. Results were obtained from three independent experiments and were expressed as mean ± SD. \(^{\#\#} P < 0.01 \) vs Control, \(^* P < 0.05 \), \(^{\#\#} P < 0.01 \) vs Model

**Effects of SMXZF on the cell apoptosis induced by \( \text{H}_2\text{O}_2 \) in PC12 cells**

As shown in Fig. 3A, of the apoptotic cells (light blue) in the \( \text{H}_2\text{O}_2 \) injury group increased significantly, compared with that of the control group, while SMXZF group reduced apoptosis significantly. As shown in Fig. 3B, the apoptotic
cells in the model group were increased to 39.81% (7.30% in normal group), and SMXZF (0.1, 1, 10 μg·mL⁻¹) decreased the apoptosis to 31.78%, 25.81% and 19.13%, respectively, significantly lower than that in the model group(all *P* < 0.05). These data indicated that SMXZF could significantly inhibit H₂O₂-induced apoptosis in the PC12 cells.

Effects of SMXZF on the activity and expression of caspase-3 in H₂O₂-treated PC12 cells

As shown in Figs. 4A and 4B, the activity of caspase-3 and expression of cleaved caspase-3 were significantly increased by 400 μmol·L⁻¹ H₂O₂ treatment in the PC12 cells, which was remarkably reversed with SMXZF incubation for 24 h at concentrations of 0.1–10 μg·mL⁻¹, without affecting pro-caspase-3 expression. These data indicated that SMXZF could inhibit cell apoptosis by regulating caspase-3 activation.

Effects of SMXZF on the ratio of Bcl-2/Bax in H₂O₂-treated PC12 cells

As shown in Fig. 4C, the Bcl-2/Bax ratio was significantly decreased by 400 μmol·L⁻¹ H₂O₂ treatment in PC12 cells, which was reversed by SMXZF pretreatment for 24 h at concentrations of 0.1–10 μg·mL⁻¹. SMXZF at 1 μg·mL⁻¹ significantly increased the Bcl-2/Bax ratio, indicating that SMXZF had a regulatory role in mitochondrial pathway of apoptosis.

Effects of SMXZF on the levels of SOD and MDA in H₂O₂-treated PC12 cells

MDA, the degradation product of the oxygen-derived free radicals and lipid oxidation, reflects the damage caused by ROS [8]. And the intracellular enzyme, SOD, with antioxidant activity, is involved in the elimination of ROS [33-34]. As shown in Figs. 5A and 5B, the activity of MDA was decreased and the level of SOD was increased significantly in PC12 cells treated with H₂O₂, which were reversed by pre-incubation with the SMXZF at concentrations of 0.1–10 μg·mL⁻¹ for 24 h. These findings suggested SMXZF could reduce oxidative injury induced by H₂O₂ in the PC12 cells.
Fig. 4 Effects of SMXZF on caspase-3 activity, the ratios of Bcl-2/Bax induced by H$_2$O$_2$ in PC12 cells. The PC12 cells were pretreated with SMXZF (0.1–10 $\mu$g·mL$^{-1}$) for 24 h and then exposed to 400 $\mu$mol·L$^{-1}$ H$_2$O$_2$ for 1 h. (A) Caspase-3 activity was tested by test kit described in the method. Protein expressions were tested by western blot. Protein expressions of (B) Cleaved caspase-3 and Pro-caspase-3, (C) Bcl-2 and Bax in Control, H$_2$O$_2$ and SMXZF treatments and Graphic representations of the ratios of cleaved caspase-3/pro-caspase-3 and Bcl-2/Bax. The band intensities were assessed by scanning densitometry. The results were obtained from three independent experiments and were expressed as mean ± SD. **$P < 0.01$ vs Control, *$P < 0.05$, **$P < 0.01$ vs Model

Fig. 5 Effects of SMXZF on the activity of MDA and SOD content in PC12 cells treated with H$_2$O$_2$. The PC12 cells were pretreated with SMXZF (0.1–10 $\mu$g·mL$^{-1}$) for 24 h and then exposed to 400 $\mu$mol·L$^{-1}$ of H$_2$O$_2$ for 1 h. (A) MDA contents and (B) SOD activities were tested by SOD and MDA test kits. Results were obtained from three independent experiments and were expressed as mean ± SD. **$P < 0.01$ vs Control, *$P < 0.05$, **$P < 0.01$ vs Model

JNK (Thr183/Tyr185) and ERK1/2 (Thr202/Tyr204) without affecting on Akt, p38, JNK and ERK1/2 expression, which had the similar effects as NAC (500 $\mu$mol·L$^{-1}$), indicating that SMXZF could inhibit the activation of Akt and MAPKs induced by H$_2$O$_2$ in PC12 cells. Furthermore, combined treatment of SMXZF with wortmannin (Akt inhibitor) or SB203580 (p38MAPK inhibitor) could partly reverse neuroprotective efficacy of SMXZF (Fig. 6E). These data suggested the involvement of Akt and MAPKs signaling pathways in the potential mechanisms of SMXZF on H$_2$O$_2$-induced cell apoptosis in PC12 cells.

Discussion

Increasing evidence suggests that oxidative stress is a key modulator of the biochemical changes that lead to activation of the apoptotic process and neuronal cell death in neurodegenerative diseases [8, 10, 35-36]. H$_2$O$_2$ is one of the main ingredients in reactive oxygen species that can diffuse into the cell membrane, and also is a relatively common cellular oxidative stress inducer, which is widely used to induce cellular oxidative stress model [6, 37]. And cultures of PC12 cells are useful model system for studying the neuronal cell death, which occurs after H$_2$O$_2$ generation [38]. However, different signaling pathways might be involved owing to differences in the stimulation time and dosage of H$_2$O$_2$. Previous studies have indicated that the caspase-3/ROCK1/MLC signaling pathway is induced by H$_2$O$_2$. 

As shown in Figs. 6A-D, 400 $\mu$mol·L$^{-1}$ of H$_2$O$_2$ significantly increased the phosphorylation of Akt and MAPKs (p38, JNK and ERK1/2) in the PC12 cells, while SMXZF at concentration of 10 $\mu$g·mL$^{-1}$ markedly inhibited the phosphorylation of Akt (Thr308), p38 (Thr180/Tyr182), JNK (Thr183/Tyr185) and ERK1/2 (Thr202/Tyr204) without affecting on Akt, p38, JNK and ERK1/2 expression, which had the similar effects as NAC (500 $\mu$mol·L$^{-1}$), indicating that SMXZF could inhibit the activation of Akt and MAPKs induced by H$_2$O$_2$ in PC12 cells. Furthermore, combined treatment of SMXZF with wortmannin (Akt inhibitor) or SB203580 (p38MAPK inhibitor) could partly reverse neuroprotective efficacy of SMXZF (Fig. 6E). These data suggested the involvement of Akt and MAPKs signaling pathways in the potential mechanisms of SMXZF on H$_2$O$_2$-induced cell apoptosis in PC12 cells.
Fig. 6  Effects of SMXZF on Akt and MAPKs signaling pathways induced by H₂O₂ in PC12 cells. Protein expressions of (A) Akt and p-Akt, (B) p38 and p-p38, (C) JNK and p-JNK, (D) ERK1/2 and p-ERK1/2 in Control, H₂O₂, SMXZF (10 μg·mL⁻¹) and NAC (50 μmol·L⁻¹) treatments and graphic representations of the ratios of p-Akt/Akt, p-p38/p38, p-JNK/JNK and p-ERK1/2 and ERK1/2. PC12 cells were pretreated with SMXZF (10 μg·mL⁻¹), NAC (500 μmol·L⁻¹) for 24 h and then exposed to 400 μmol·L⁻¹ H₂O₂ for 1 h. The band intensities were assessed by scanning densitometry. The results were obtained from three independent experiments and were expressed as mean ± SD. *P < 0.05, **P < 0.01 vs Control group, *P < 0.05, **P < 0.01 vs Model group. (E) The cell viability was detected using MTT assay. PC12 cells were treated with SMXZF at 10 μg·mL⁻¹ with Akt inhibitor (Wortmannin, 10 μmol·L⁻¹) or p38MAPK inhibitor (SB20358, 10 μmol·L⁻¹) for 24 h. W: Wortmannin, S: SB20358. Results were obtained from six independent experiments and were expressed as mean ± SD. *P < 0.05, **P < 0.01 vs Control, *P < 0.05, **P < 0.01 vs Model, §§P < 0.01 vs group treated with SMXZF and H₂O₂.
H$_2$O$_2$ superoxide anion to H$_2$O$_2$, and the formed H$_2$O$_2$ is and increased the Bcl-2/Bax ratio (Figs. 4A-C). While SMXZF significantly inhibited caspase-3 activation impairment (MCI) and AD, which can be a marker for lipid shown in the serum of patients with mild cognitive MDA and SOD. An increased production of MDA has been induce cell morphological changes, cell loss, and cell apoptosis in PC12 cells, which is probably associated with the inhibition of anti-oxidants [29, 34, 39]. Our results indicated that SMXZF pretreatment at concentrations of 0.1, 1 and 10 μg·mL$^{-1}$ significantly increased the cell viability, reversed the cell morphological changes, and reduced the levels of LDH and apoptosis in the H$_2$O$_2$ treated PC12 cells (Fig. 2). Moreover, the results of Hoechst 33342 staining and Annexin V/PI analysis confirmed that SMXZF inhibited the H$_2$O$_2$-induced apoptosis in the PC12 cells (Fig. 3), which was consistent with the previous study [30]. Therefore, we could conclude that SMXZF protected the PC12 cells from H$_2$O$_2$-induced injury through the inhibition of cell apoptosis.

Depending on the extent of oxidative stress, it can induce proliferation, growth arrest, senescence and apoptosis. Many genes have been reported to be linked with apoptosis under physiological and pathological conditions, in which Bcl-2 and Bax genes play a major role in determining cell’s survival or death after exposure to apoptotic stimuli [30-42]. Thus, the effects of SMXZF on the protein levels of Bcl-2 and Bax were detected by Western blot in the present study. The results of the study showed that SMXZF treatment up-regulated the expression of anti-apoptotic protein, Bcl-2, and down-regulated the expression of pro-apoptotic protein Bax. Based on these results, it could be reasonably speculated that SMXZF might attenuate apoptosis. To further support our finding, the caspase-3 activity and expression of cleaved-caspase-3 were determined. The results indicated that induction of apoptosis with H$_2$O$_2$ was associated with the activation of caspase-3 and decrease of Bcl-2/Bax ratio in H$_2$O$_2$-treated alone group, while SMXZF significantly inhibited caspase-3 activation and increased the Bcl-2/Bax ratio (Figs. 4A-C).

In addition, there are some antioxidants in cells, such as MDA and SOD. An increased production of MDA has been shown in the serum of patients with mild cognitive impairment (MCI) and AD, which can be a marker for lipid peroxidation [43]. SOD is able to transform intracellular superoxide anion to H$_2$O$_2$, and the formed H$_2$O$_2$ is subsequently scavenged by catalase through enzymatic reactions [53]. In the present study, H$_2$O$_2$ significantly inhibited SOD and increased MDA levels in the PC12 cells (Figs. 5A and 5B), which was reversed by SMXZF-pretreatment, suggesting that the neuroprotective effects of SMXZF were related to its anti-oxidant ability.

Previous studies have shown that Akt and MAPKs are two important signaling pathways involved in oxidative stress-induced cells injury [44-46]. Our findings demonstrated that SMXZF inhibited phosphorylation of Akt and MAPKs in response to H$_2$O$_2$-induced oxidative stress (Figs. 6A-D). In addition, we also verified that combined treatment of SMXZF with Akt inhibitor (Wortmannin) or p38MAPK inhibitor (SB203580) could partly reverse SMXZF’s neuroprotective activity (Fig. 6E). These results implicated that SMXZF increased the cell viability and inhibited cell apoptosis partly through Akt and MAPKs signaling pathways. In previous reports, ginsenoside Rg1, a tetracyclic triterpenoids saponin from ginseng, is of interest because of its prominent neuroprotective and anti-oxidant activities [21, 47-49]. Moreover, Rg1 promotes nonamyloidogenic cleavage of APP via estrogen receptor signaling to MAPK/ERK and PI3K/Akt [50] and down-regulates the shear stress induced MCP-1 expression by inhibiting MAPKs signaling pathway [51]. On the other hand, DT-13, a saponin compound, could inhibit MDA-MB-435 cell adhesion and invasion by inhibiting MMP-2/9 via the p38MAPK pathway [52]. Rb1 also could protect H$_2$O$_2$-induced PC12 cells from caspase-3-dependent apoptosis through MAPKs pathway [53]. Whether single compound or their combination contributes to such activity of SMXZF needs to be explored in the future. And whether other signaling pathways are involved in this process and there is synergy among the four active components need further investigation.

In conclusion, pretreatment with SMXZF effectively alleviated oxidative stress through restoring SOD activities, decreasing MDA level, elevating the ratio of Bcl-2/Bax and inhibiting caspase-3 activity in the PC12 cells caused by H$_2$O$_2$. Our findings also suggested that Akt and MAPKs signaling pathways play key roles in anti-apoptosis activity of SMXZF in reducing oxidative damage after exposure to H$_2$O$_2$ in the PC12 cells, which provide some explanation on its neuroprotective mechanism.

References


