Original Article
Protective effect of Xiaoyao-jieyu-san on oxidative damage induced by hydrogen peroxide in PC12 cells

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Abstract: This study aimed to evaluate the protective effect of Xiaoyao-jieyu-san (XYJY) on oxidative damage induced by H2O2 in PC12 cells. Oxidatively damaged PC12 cells were induced by H2O2 (100 μmol/l). CCK-8 assays were carried out to determine the effects of XYJY on cell viability of H2O2 induced PC12 cells. Subsequently, flow cytometry analysis was used to evaluate apoptosis and ROS levels of H2O2 induced PC12 cells after treatment with XYJY. Furthermore, malondialdehyde (MDA), reduced glutathione (GSH), and superoxide dismutase (SOD) were determined by using commercial kits, and expression of caspase-3, AKT, p-AKT, eNOS, and p-eNOS were evaluated by using Western blotting. The results reveal that XYJY increased cell viability of H2O2 induced PC12 cells during 50 to 800 μg/ml, and XYJY dose-dependently reduced apoptotic cells and ROS levels in H2O2 induced PC12 cells. Furthermore, XYJY treatments (100, 200, and 400 μg/ml) significantly reduced the levels of MDA whereas they increased the levels of SOD and GSH-PX in H2O2 induced PC12 cells. In addition, XYJY treatments (200 and 400 μg/ml) significantly up-regulated the expression of p-AKT and p-eNOS in H2O2 induced PC12 cells, whereas they down-regulated caspase-3. In conclusion, the results presented here suggest that XYJY has a protective effect against oxidative damage in PC12 cells mediated by regulation of AKT/eNOS signaling pathway. The present study could be beneficial for interpretation of the possible molecular mechanisms corresponding to the curative effects of XYJY against stroke and its related diseases.

Keywords: Xiaoyao-jieyu-san, oxidative damage, PC12 cells, stroke, AKT/eNOS

Introduction

Stroke is a worldwide epidemic severe life-threatening disease with high fatality and disability rate [1], among which ischemic stroke patients constitute most of the cases of stroke patients [2]. Furthermore, due to lack of blood flow in the brain after ischemic stroke and the following ischemia-reperfusion, it commonly leads to damage of neurons and the related connected neural circuits and even unexpected behavioral impairments, resulting in heavy burden to stroke patients and their families [3, 4]. Consequently, it is important and necessary to protect the neurons in ischemic stroke.

Increasing evidence has suggested that herbal medicines are natural precious resource for finding potential drugs for human being and traditional Chinese medicines (TCMs) have been used for curing various diseases for thousands years [5, 6]. Xiaoyao-jieyu-san (XYJY) is an empirical TCM prescription for treating stroke and its related diseases for decades [7, 8]. The XYJY is derived from the famous Chinese medicinal formulae, and consists of 11 herbal medicines, including whole plant of Bupleurum chinense (15 g), rhizome of Cyperus rotundus (12 g), radix of Curcuma rcenyujin (12 g), radix of Angelica sinensis (15 g), radix of Cynanchum otophyllum (30 g), fructus of Citrus aurantium (15 g), cortex of Albizia julibrissin (12 g), radix of Angelica sinensis (15 g), radix of Cynanchum otophyllum (30 g), fructus of Citrus aurantium (15 g), cortex of Albizia julibrissin (12 g), rhizome of Acorus tatarinowii (12 g), caulis of Fallopia multiflora (30 g), semen of Ziziphus jujuba Mill. var. spinosa (15 g), and rhizome of Atractylodis macrocephalae (15 g). In prior work, XYJY was found to possess significant curative effects on post-stroke depression via regulation of brain derived neurotrophic factor (BDNF), and the constituents of XYJY mainly
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includes paeoniflorin, imperatorin, naringin, arnesene, 2,3,5,4'-tetrahydroxy- diphenylethylene-2-O-glucoside, kaempferol-3-O-rutinoside, quercetin, hesperidin, cycloastragenol, and atracylenolide III [7-9]. In the present study, the protective effect of Xiaoyao-jieyu-san (XYJY) on the oxidative damage induced by hydrogen peroxide (H$_2$O$_2$) was evaluated in rat pheochromocytoma derived cell line (PC12) cells. This work could be beneficial for interpretation of the molecular mechanisms corresponding to the curative effects of XYJY against stroke.

**Materials and methods**

**Chemicals and reagents**

Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco. Ltd. Co. (USA); Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Biochem (Shanghai, China). Trypsinase, acrylamide, sodium lauryl sulfate (SDS), penicillin, streptomycin and phosphate buffered saline (PBS) were purchased from JRDun Biotech. (Shanghai, China). Malondialdehyde (MDA), reduced glutathione (GSH) and superoxide dismutase (SOD) assay kits were supplied by Nanjing Jiangcheng Bioengineering Institute (Nanjing, China). Annexin V/FITC kit and reactive oxygen species (ROS) testing kit were purchased from BD Biosciences (Shanghai, China). Anaxxin V/FITC kit and reactive oxygen species (ROS) testing kit were purchased from BD Biosciences (Shanghai, China). Caspase-3, AKT, p-AKT, eNOS, p-eNOS, and GAPDH antibodies were purchased from Abcam Biotech. (Shanghai, China). BCA protein assay kit, goat-anti-rabbit/rat horseradish-peroxidase-conjugated secondary antibodies were obtained from Beyotime Biotechnology (Shanghai, China).

**Cell culture**

Rat pheochromocytoma derived cell line (PC12) was purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China). PC12 Cells were cultured in DMEM medium containing 10% heat-inactivated FBS, 100 U/mL penicillin and 100 mg/mL streptomycin at 37°C in 5% CO$_2$ atmosphere.

**Preparation of XYJY extracts**

The XYJY is supplied by the pharmacy department of our hospital. In brief, all the 11 herbal medicines were decocted with 8 times water (v/w) for 1.5 hours. The filtrates concentrated by using rotary evaporators under 60°C, and the fluid extracts of XYJY were afforded (yields of XYJY extracts were calculated as approximately 7.81%) [9].

**CCK-8 assay**

Cells (5 x 10$^4$/100 μl) were plated in the 96-well plates for growth of 24 hours. Then, XYJY at the finally concentrations of 50, 100, 200, 400 and 800 μg/ml was added and cultured for 0, 24, 48, and 72 hours at 37°C with the presence of H$_2$O$_2$ (100 μmol/l). Consequently, 100 μl serum free DMEM containing 10% CCK-8 reagents (v/v) were consequently added in each well, then cells were cultured for 1 hour at 37°C. Finally, optical density values (OD) were read under 450 nm by micro-plate reader. The experiment was repeated three times.

**Apoptosis and ROS assays by flow cytometry analysis**

Cells were plated in the 24-well plates for growth of 24 hours. Then, XYJY at the finally concentrations of 100, 200, and 400 μg/ml was added and cultured for 24 hours at 37°C with the presence of H$_2$O$_2$ (100 μmol/l). Then, cells were harvested, and then cells (5 x 10$^4$) were washed using PBS and stained by the Annexin V/FITC kit, and cell apoptosis was detected by the flow cytometry (FCM) assay on a FACS Calibur flow cytometer (BD Bioscience, USA). In addition, DCFH-DA ROS kit was used to determine the intracellular ROS level by the flow cytometry (FCM) assay. The experiment was repeated three times.

**Antioxidant indexes analysis**

Cells were plated in the 24-well plates for growth of 24 hours. Then, XYJY at the finally concentrations of 100, 200, and 400 μg/ml was added and cultured for 24 hours at 37°C with the presence of H$_2$O$_2$ (100 μmol/l). Then, cells were harvested and MDA, GSH, and SOD were determined by commercial kits following the manufacture’s instruction.

**Western blot assay**

Total proteins of the PC12 cells were extracted. Subsequently, 35 μg total proteins were separated by sodium dodecylsulfate-polyacrylamide
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Effects of XYJY on H$_2$O$_2$ induced injury in PC12 cells

As can be seen from the Figure 1, cell viability was evaluated by CCK-8 assay. The results show that the cell viability was significantly reduced by treatment with H$_2$O$_2$ at the time points designed, including 24, 48, and 72 hours ($p<0.01$), compared to the normal cells. Furthermore, the results also indicate that XYJY can increase the cell viability of H$_2$O$_2$ induced PC12 cells from the concentration of 50 μg/ml compared to the control cells ($p<0.01$), and show a significant dose-dependent manner during the concentration of 200 μg/ml to 400 μg/ml at the time points designed, including 24, 48, and 72 hours ($p<0.01$). Consequently, the results indicate that XYJY has a potential protective effect against the H$_2$O$_2$ induced injury in PC12 cells.

Effects of XYJY on H$_2$O$_2$ induced apoptosis of PC12 cells

As shown in Figure 2, flow cytometry assay with Annexin V-FITC/PI staining was carried out to evaluate the effects of XYJY on H$_2$O$_2$ induced apoptosis of PC12 cells. After exposure to H$_2$O$_2$, the percentage of apoptotic cells sharply increased compared to the normal PC12 cells ($p<0.01$). However, our results also reveal that XYJY (100, 200 and 400 μg/ml) could dose-dependently reduce the percentage of apoptotic cells in H$_2$O$_2$ induced PC12 cells ($p<0.01$), compared to the control PC12 cells.

Effects of XYJY on ROS levels of H$_2$O$_2$ induced PC12 cells

As shown in Figure 3, flow cytometry assay with DCFH-DA staining was carried out to evaluate the effects of XYJY on ROS levels of H$_2$O$_2$ induced PC12 cells. After exposure to H$_2$O$_2$, the ROS levels of PC12 cells sharply increased compared to the normal PC12 cells ($p<0.01$). However, the present results also suggest that XYJY (100, 200 and 400 μg/ml) could dose-dependently reduce the ROS levels of PC12 cells induced by H$_2$O$_2$ ($p<0.01$), compared to the control PC12 cells.

Effects of XYJY on MDA, GSH and SOD of H$_2$O$_2$ induced PC12 cells

Furthermore, our present study also investigated the effects of XYJY on levels of MDA, GSH and SOD in H$_2$O$_2$ induced PC12 cells (Figure 4). After exposure to H$_2$O$_2$, the levels of GSH-PX, and SOD were significantly decreased ($p<0.01$) whereas the MDA level was increased significantly ($p<0.01$), compared to the normal PC12 cells. However, the results show that XYJY treatments (100, 200 and 400 μg/ml) could signifi-

Statistical analysis

Data are presented as mean ± standard deviations (SD). Statistical comparisons were made by one-way analysis of variance (ANOVA) using SPSS software (version 18.0, USA), followed by Dunnet $t$ multiple comparison test. $P<0.05$ was set as the significance level.

Results

Effects of XYJY on the cell viability of H$_2$O$_2$ induced PC12 cells
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cantly reduce the levels of MDA (p<0.01) whereas increase the levels of SOD and GSH-PX in H$_2$O$_2$ induced PC12 cells (p<0.01), compared to the control PC12 cells.

Effects of XYJY on caspase-3, P-AKT and P-eNOS in H$_2$O$_2$ induced PC12 cells

To explore the further molecular mechanisms for the activities of XYJY, the protein expression of caspase-3, AKT, eNOS, p-AKT and p-eNOS in H$_2$O$_2$ induced PC12 cells were carried out (Figures 5 and 6). As shown in Figure 5, after treatment with H$_2$O$_2$, caspase-3 expression was significantly up-regulated compared to the normal group (p<0.01), and the XYJY treatments (200 and 400 μg/ml) significantly decreased caspase-3 expression in H$_2$O$_2$ induced PC12 cells (p<0.01), compared to normal cells.
In addition, the results in Figure 6 also indicate that after treatment with $\text{H}_2\text{O}_2$, no obvious difference could be found in the expression of AKT, eNOS, p-AKT and p-eNOS of PC12 cells ($p>0.05$), compared to the normal cells. However, XYJY treatments (200 and 400 μg/ml) could significantly up-regulate expression of p-AKT ($p<0.01$) and p-eNOS ($p<0.01$) in $\text{H}_2\text{O}_2$ induced PC12 cells, compared to the control cells.

**Discussion**

The present study shows that *Xiaoyao-jieyu-san* (XYJY), an empirical TCM prescription for treatment of stroke and its related diseases in our hospital, possesses promising protective effect on the oxidative damage induced by hydrogen peroxide ($\text{H}_2\text{O}_2$) in PC12 cells and its potential pharmacological mechanisms.
Oxidative stress induced damage is one of the important molecular possible mechanisms for the tissue injury after ischemic stroke [10, 11]. In addition, reactive oxygen species (ROS) could result in oxidative damage of the bio-macromolecules and tissues of body, and subsequently lead to the lipid peroxidation, breakage of DNA and polypeptide, etc, and eventually leading to neuronal degeneration and necrosis [12, 13]. Hydrogen peroxide ($H_2O_2$) is a commonly used reagent for preparation of oxidative damaged cells due to the $H_2O_2$ could easily go through the cytoplasmic membrane and form the powerful radicals (such as hydroxyl radical and singlet oxygen) via Fenton reaction with transition metal [14]. In addition, $H_2O_2$ could also induce the release of cytochrome C from the mitochondria, and subsequently result in the activation of caspase-3 which is a crucial activated death protease and bio-marker for cells undergoing apoptosis, leading to the apoptosis of cells [13, 15]. In the present study, $H_2O_2$ induced oxida-

Figure 4. Effects of XYJY on MDA, GSH, and SOD of $H_2O_2$ induced PC12 cells. Data are expressed as mean ± SD (n=3), **$p<0.01$, vs. normal; ***$p<0.01$, vs. control.

Figure 5. Effects of XYJY on protein expression of caspase-3 in $H_2O_2$ induced PC12 cells. Data are expressed as mean ± SD (n=3), **$p<0.01$, vs. normal; ***$p<0.01$, vs. control.
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Figure 6. Effects of XYJY on protein expression of p-AKT and p-eNOS in H$_2$O$_2$ induced PC12 cells. Data are expressed as mean ± SD (n=3); **p<0.01, vs. normal; ***p<0.01, vs. control.

tive damaged PC12 cells were successfully prepared to investigate the related activities of XYJY, and the results showed that XYJY could significantly alleviate the cell viability inhibition and apoptosis induced by H$_2$O$_2$ in PC12 cells. Furthermore, intracellular MDA, SOD, and GSH are important biomarkers to evaluate the oxidative stress. MDA is the production of peroxidation of membrane lipids induced by ROS, which could result in membrane damage and destruction; while SOD and GSH are important antioxidant enzymes in mammalian cells [16, 17]. In the present study, XYJY could significantly reduce MDA whereas it could increase SOD and GSH in H$_2$O$_2$ induced PC12 cells. In addition, the results of the flow cytometry assay with DCFH-DA staining also indicated the XYJY could obviously decrease the ROS levels in H$_2$O$_2$ induced PC12 cells. All these results suggest that XYJY could significantly reduce the oxidative damage of H$_2$O$_2$ induced PC12 cells. AKT plays important role in the growth, proliferation, migration of nerve cells, and previous researches have revealed that activation (phosphorylation) of the AKT could protect the nerve cells from cell apoptosis induced by oxidative toxins, consequently it has been reported that activation of AKT pathways might potentially be a new target for treating ischemic stroke [18, 19]. Furthermore, NO mediated by eNOS could protect the nerve cell and tissues, and up-regulation of eNOS would be beneficial for treatment of ischemic stroke [20, 21]. In summary, these data show that XYJY treatment could significantly up-regulation of P-AKT and P-eNOS in H$_2$O$_2$ induced PC12 cells.

Conclusion

In conclusion, the results presented here suggest that XYJY has protective effect against oxidative damage in PC12 cells mediated by regulation of AKT/eNOS signaling pathway. The present study would be beneficial for interpretation of the possible molecular mechanisms corresponding to the curative effects of XYJY against stroke and its related diseases.

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Disclosure of conflict of interest

None.
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