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Protective Effect of Puerarin Against Oxidative Stress Injury of Neural Cells and Related Mechanisms

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Background: Parkinson's disease (PD) is manifested as degeneration of dopaminergic neurons in substantia nigra compacta. The mitochondrial dysfunction induced by oxidative stress is believed to be a major cause of PD. Puerarin has been widely applied due to its estrogen nature and anti-oxidative function. This study thus investigated the protective role of puerarin against oxidative stress injury on PC12 neural cells, in addition to related mechanisms.

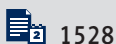
Material/Methods: PC12 cells were pre-treated with gradient concentrations of puerarin, followed by the induction of 0.5 mM H₂O₂. MTT assay was used to detect cell viability. Enzyme-linked immunosorbent assay (ELISA) was employed to detect intracellular level of superoxide dismutase (SOD), malondialdehyde (MDA), and glutathione (GSH). Cell apoptosis was determined by Annexin-V/7-AAD double labelling. Reactive oxidative species (ROS) and lactate dehydrogenase (LDH) activities were then measured. Cellular levels of caspase-3 and caspase-9 were also determined.

Results: The pre-treatment using puerarin significantly reversed H₂O₂-induced oxidative stress injury, as it can increase proliferation, SOD and GSH activities, decrease MDA activity, suppress apoptosis of PC12 cells, and decrease ROS and LDH production (p<0.05 in all cases). Further assays showed depressed up-regulation of caspase-3 and caspase-9 after puerarin pretreatment.

Conclusions: Puerarin pretreatment can decrease activity of caspase-3 and caspase-9 activity in PC12 cells, thus protecting cells from oxidative injury.

MeSH Keywords: **Brachial Plexus Neuritis • Pueraria • Respiratory Burst**

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Background

Parkinson disease (PD) and Alzheimer disease (AD) are two common neurodegenerative diseases in people over 60 years old. The major pathogenesis mechanism of PD has been recognized as the degeneration of dopaminergic neurons in substantia nigra compacta [1–4]. The mitochondrial dysfunction induced by oxidative stress is believed to be a major cause of PD [5]. The incidence rate of AD is as high as 5% in people over 60 years old, and it imposes heavy burdens on affected families and society. AD's pathogenesis has also been recognized as being related with oxidative stress, which may cause cytotoxicity as a consequence of imbalance between production and clearance of the body's oxygen free radicals. In addition to PD and AD, oxidative stress is also related with the occurrence of other neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS) [6].

Puerarin is a type of isoflavone compound extracted from the Chinese herb Gegen [7,8]. Due to its pluripotent biological activities, especially estrogen-like activity and anti-oxidative function, puerarin has been developed as an alternative medicine in prevention and treatment of cardiovascular disease, diabetes mellitus, cancer, and osteoporosis [9–12]. Puerarin can suppress the function of intercellular adhesion molecule-1 (ICAM-1) to alleviate the infiltration of neutrophils in cerebral tissues in an ischemia-reperfusion rat model. Puerarin can decrease the expression of inflammatory cytokines, including tumor necrosis factor α (TNF- α) and interleukin 1 β (IL-1 β) in injured brain tissues [13]. In a diabetic model, puerarin inhibited the apoptosis of Schwann cells by its anti-oxidative function, thus exerting its protective function [14–16]. It has been found that oxidative stress can decrease transmembrane potential of mitochondria ($\Delta\psi_m$) and facilitate cell apoptosis via activating caspase-3 and caspase-9 [17].

Currently, there were few studies on the protective role of puerarin against oxidative stress injury in neural cells. We proposed that the pre-treatment using puerarin might exert a protective function against H₂O₂-induced oxidative stress injury in cultured neural cells. We thus established an *in vitro* model of oxidative stress injury on neural cells, in which the protective effect of puerarin pre-treatment was observed, along with explorations of related mechanisms.

Material and Methods

Cell culture and MTT assay

PC12 cells (Hongshun Bio, China) were kept in RPMI-1640 medium containing 10% fetal bovine serum (FBS). Log-phased cells were seeded into a 96-well plate containing 20 μ L MTT

solutions in each well. After 4-h incubation, supernatants were replaced by 0.15 mL DMSO, followed by quantification of optical density (OD) values at 490 nm.

Enzyme-linked immunosorbent assay (ELISA)

The activity of superoxide dismutase (SOD), malondialdehyde (MDA), and glutathione (GSH) inside cells was determined by ELISA using relevant test kits (Beyotime, China) following the manual instructions. In brief, test samples and standard samples were added into a 96-well plate (0.1 mL each). The plate was incubated at 37°C for 90 min, followed by the addition of 0.25 mL washing buffer. Biotin-labelled antibody working solution was then added for 1-h incubation at 37°C. The plate was developed in the dark for 20 min, and was quenched by use of stopping buffer. OD values were measured.

Cell apoptosis assay

Using APC-Annexin VI/7-AAD double labelling kit (Biolegend, USA), the apoptosis of PC12 cells was quantified. In brief, cells were first washed in PBA twice, and were re-suspended in Annexin-V binding buffer at 1×10^7 /mL. Cell suspension was then mixed with 5% Annexin-V/7-AAD staining buffer. After dark incubation for 15 min, Annexin-V binding solution was then added for subsequent flow cytometry assay. Early apoptotic cells were identified as being positive for Annexin VI, while double-positive cells were identified as late-stage apoptotic cells.

Reactive oxidative species (ROS) and lactate dehydrogenase (LDH) assay

Treated cells were re-suspended in PBS, with addition of 1 μ L DCFH2DA probe (Jiancheng, China). After dark incubation for 30 min at 37°C, cells were measured for fluorescent intensity by flow cytometry.

LDH release was tested by kit (Promega, USA) following the manual instructions. In brief, supernatants of culture medium were added into an enzyme-linked plate along with 60 μ L LDH test reagent. After dark incubation for 30 min, OD values at 490 nm were measured. LDH level (in percentage) was calculated as: $(A_{\text{Sample}} - A_{\text{Blank}}) / (A_{\text{Control}} - A_{\text{Blank}}) \times 100\%$.

Caspase activity assay

PC12 cells were digested by trypsin, and were washed with PBS. Lysis buffer was then added to extract supernatants following iced incubation and centrifugation. Activities of caspase-3 and caspase-9 were determined by measuring absorbance values as U/mgprot.

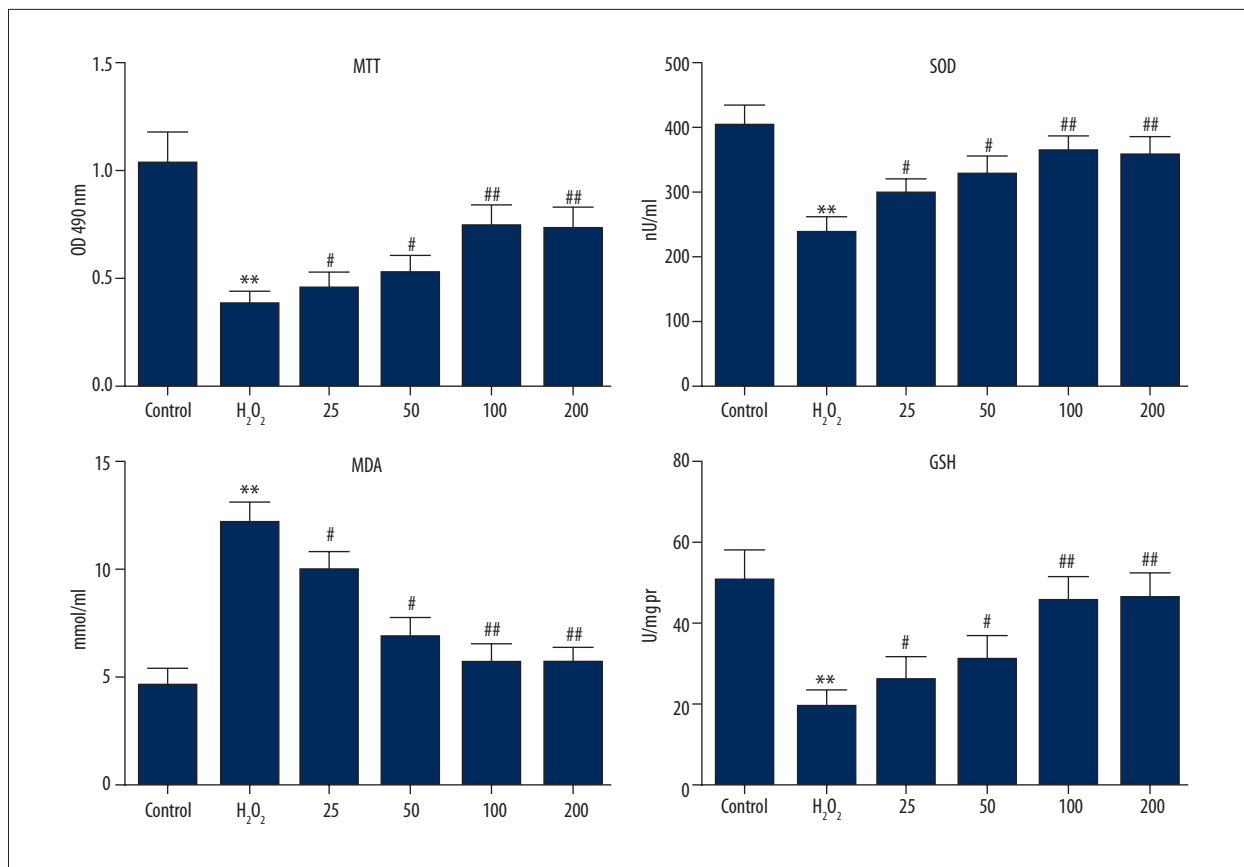


Figure 1. Effects of puerarin on cell proliferation and activity of SOD, MDA, and GSH. ** $p < 0.01$ compared to control group; # $p < 0.05$, ## $p < 0.01$ compared to H₂O₂ group.

Statistical analysis

SPSS 16 software was used to process all collected data, which were analyzed by analysis of variance (ANOVA). A statistical significance was defined when $p < 0.05$.

Results

Cell proliferation and activity of SOD, MDA, and GSH

As shown in Figure 1, MTT assay showed the significant inhibition of PC12 proliferation ability by H₂O₂ ($p < 0.01$). The pre-treatment using gradient concentrations of puerarin (25, 50, 100, and 200 mg/L) restored proliferation of PC12 cells in a dose-dependent manner, with the peak level at 100 mg/L. The activity of SOD and GSH was significantly depressed, but MDA activity was elevated after H₂O₂ induction ($p < 0.05$, Figure 1). Such alternations can be partially reversed by puerarin pre-treatment. Based on the dose-dependent curves, 100 mg/L was chosen as the optimal dosage for consequent experiments.

Neural cell apoptosis

As shown in Figure 2, apoptotic (both early- and late-stage) level of PC12 cells in the control group was $(8.84 \pm 2.64\%)$. The induction by H₂O₂ elevated the apoptotic cell ratio to $(16.10 \pm 3.31\%)$. The pre-treatment using 100 mg/L puerarin depressed the apoptotic level to $(10.90 \pm 2.88\%)$, which was significantly lower than in the H₂O₂ group ($p < 0.01$).

ROS level and LDH production

Figure 3 shows the significantly elevated ROS and LDH production in PC12 cells after H₂O₂ induction ($p < 0.01$). The pre-treatment using 100 mg/L puerarin, however, significantly depressed ROS and LDH levels ($p < 0.01$).

Caspase-3 and caspase-9 activity

As shown in Figure 4, H₂O₂ induction significantly increased cellular activity of caspase-3 and caspase-9 in PC12 cells ($p < 0.01$). The pre-treatment using 100 mg/L puerarin significantly depressed activities of both apoptotic proteins ($p < 0.01$).

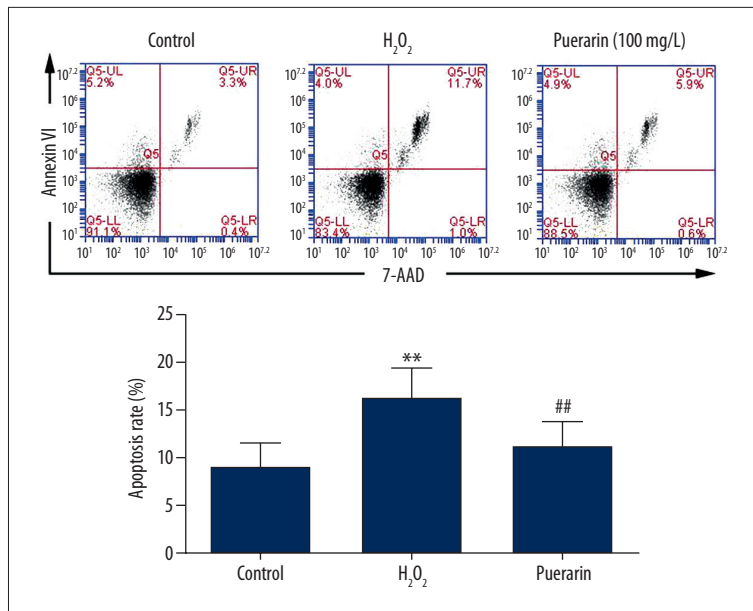


Figure 2. Apoptosis of PC12 cells. ** p<0.01; ## p<0.01 compared to H₂O₂ group.

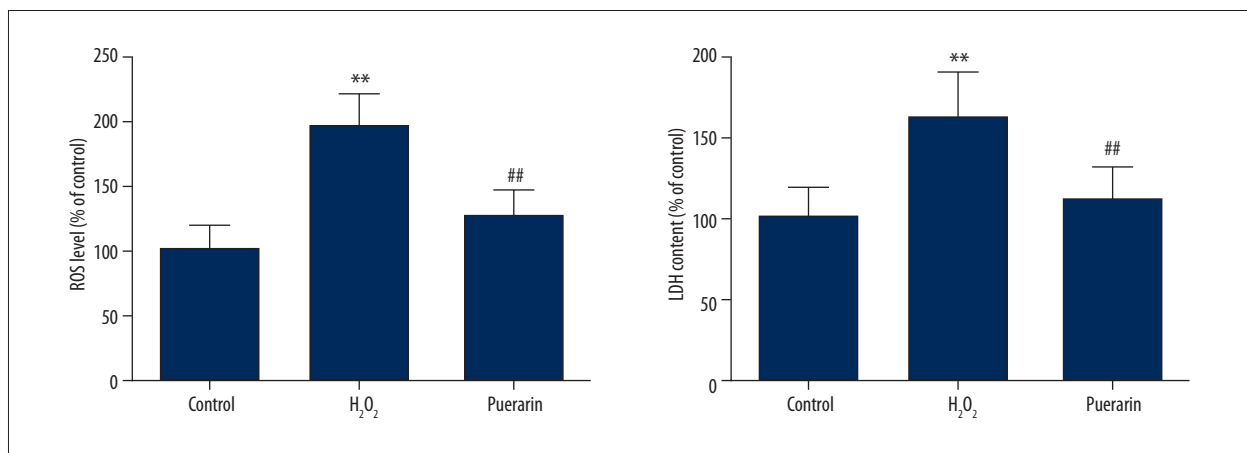


Figure 3. ROS level and LDH production in PC 12 cells. ** p<0.01 compared to control group; ## p<0.01 compared to H₂O₂ group.

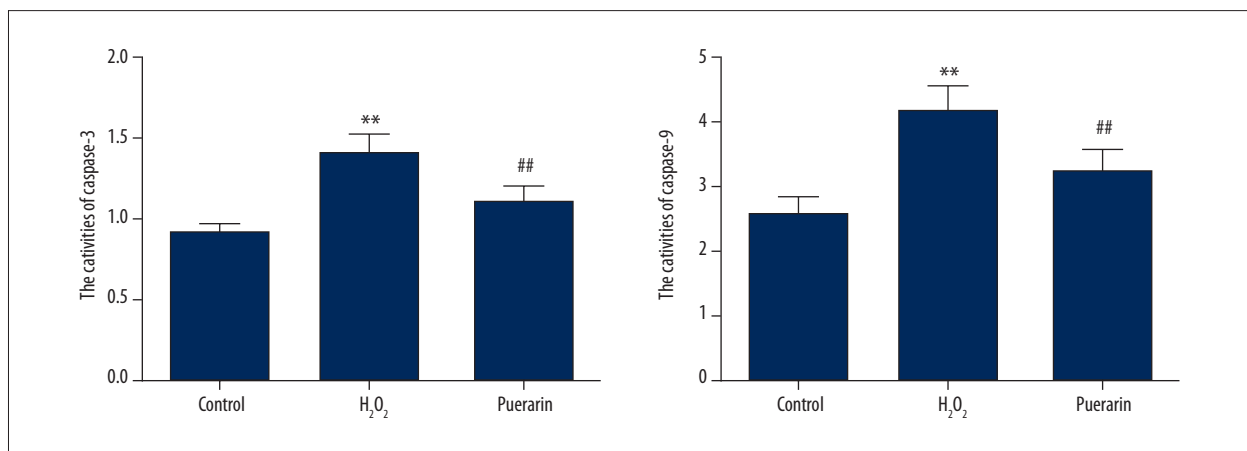


Figure 4. Caspase-3 and caspase-9 activities. ** p<0.01 compared to control group; ## p<0.01 compared to H₂O₂ group.

Discussion

More than 2% of people over 65 years old in China now have PD, accounting for over 40% of patients worldwide [18–22]. PD's occurrence is believed to be related with oxidative stress-induced neural cell damage. Therefore, the development of anti-oxidative stress injury drug candidates is a research hotspot in this field. Our study generated an *in vitro* oxidative stress injury model using H₂O₂ induction on PC12 cell line, which is widely applied across different research groups [23,24]. Our study showed significantly depressed proliferative ability and elevated apoptosis after H₂O₂ induction. H₂O₂ induction can decrease intracellular activity of SOD and elevate MDA activity, as well as leading to higher ROS level and LDH release. As a key enzyme in the body's anti-oxidation system, SOD plays a crucial role in clearing oxygen free radicals [25]. GSH is a tri-peptide formed by glutamate, cysteine, and glycine with a sulfhydryl group, presenting anti-oxidation and detoxification functions [26]. The activity of SOD and GSH may reflect the body's ability to clear free radicals. As one of the most important by-products of membrane lipid peroxidation, MDA can reflect the degree of peroxidation [27] in addition to the ability to clear free radicals. Our results regarding cell proliferation, apoptosis, SOD, GSH, MDA, ROS, and LDH levels all suggest the successful generation of an oxidative stress injury model.

Based on previous reports, effective drugs for PD include garlicin, vitexin B-1, and propofol. Garlicin may exert its protective function against 6-hydroxyl dopamine-induced oxidative stress of PC12 cells via mediating dynamics of mitochondria [28]. Vitexin B-1 can protect PC12 cells from oxidative stress injury by inhibiting NADPH oxidase [29]. Propofol, however, can exert its protective function on PC12 cells against oxidative stress injury by inhibiting Ca²⁺-dependent NAPDH oxidase [30]. An isoflavone compound extracted from the Chinese herb

Gegen, puerarin has been widely used due to its estrogenic nature and anti-oxidative function. We thus hypothesized a protective function of puerarin against H₂O₂-induced oxidative stress in cultured PC12 cells. Our results showed significantly elevated cell proliferation ability ($p < 0.01$) compared to the H₂O₂-induced group, along with higher levels of SOD and GSH, and depression of MDA level. Pre-treatment using puerarin can decrease apoptosis level of PC12, whose intracellular ROS and LDH release were depressed. These results suggest the existence of a protective function by puerarin pre-treatment against oxidative stress injury.

Previous studies have established the inhibition of the apoptosis of Schwann cells by its anti-oxidative function, in a diabetic model [14–16]. Actually, puerarin can decrease transmembrane potential of mitochondria ($\Delta\psi_m$) and facilitate cell apoptosis via activating caspase-3 and caspase-9 [17]. Currently, there were few reports on the role of caspase-3 and caspase-9. This study mainly focused on the protective function against oxidative stress injury by puerarin. We thus further explored if expression levels of caspase-3 and caspase-9 was affected by puerarin, for exerting protective roles in H₂O₂-induced oxidative stress injury. Our results showed significantly elevated caspase-3 and caspase-9 in the model group. The pre-treatment significantly reversed this elevation ($p < 0.01$). These results show the activity of caspase-3 and caspase-9, whose activity can be used to protect neural cells by H₂O₂-induced oxidative stress injury.

Conclusions

This study suggests the protective function of puerarin against H₂O₂-induced oxidative stress injury, possibly via modulating caspase-3 and caspase-9 activity.

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