Original Article

Ginsenoside Rd pretreatment attenuates apoptosis of PC12 cells induced by oxygen-glucose deprivation

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Abstract: The current study explored the inhibitory mechanisms of ginsenoside Rd on apoptosis of PC12 cells induced by oxygen-glucose deprivation (hypoxia). An in vitro cell model was established. Cellular activity and apoptosis rates were measured using CCK8 assays and Annexin V-FITC staining. Expression levels of Fas, Fas ligand (FasL), caspase-8, caspase-9 and Fas-associated via death domain (FADD) were determined using quantitative real-time PCR and Western blotting. Changes in the mitochondrial membrane potential and release of cytochrome C were measured using appropriate kits. Ginsenoside Rd inhibited apoptosis in PC12 cells induced by hypoxia. It also decreased mRNA expression levels of caspase-8 and caspase-9 but did not significantly affect expression of Fas or FasL. At the protein level, ginsenoside Rd decreased expression of caspase-8, caspase-9, and FADD. It also attenuated mitochondrial membrane potential reduction induced by hypoxia and decreased the release of cytochrome C. Ginsenoside Rd protects PC12 cells against hypoxia, likely through downregulating caspase-8 expression and inhibiting activation of mitochondrial pathways.

Keywords: Ginsenoside Rd, PC12 cell, apoptosis, neuroprotective effect, signal pathway

Introduction

Spinal cord ischemia-reperfusion injury (SCI) is a secondary injury of spinal cord ischemia. It leads to severe loss of sensorimotor function and even death. SCI is a common complication of spinal injuries and thoracoabdominal aortic aneurysm surgeries. No effective treatment methods have been established. The loss of labor ability, demand of long-term nursing, and high treatment expenses of SCI patients place a heavy burden on society. SCI pathogenesis has not been fully studied. However, it has been associated with several factors, including oxidative stress, inflammatory responses, apoptosis, and autophagy [1]. Apoptosis is the main cause of neuronal death in SCI [2, 3]. Thus, inhibition of apoptosis is a potential new treatment strategy.

PC12 rat pheochromocytoma is a single cell type that undergoes stable passaging. It has consistent characteristics. Extensive nerve growth factor (NGF) receptors are present on PC12 cell surfaces. Thus, these cells can be induced by NGF to differentiate into sympathetic-like cells, which resemble neurons in their physiology, morphology, function, and signal transduction. Therefore, they provide an important model for in vitro experiments [4, 5].

A traditional medicine, ginseng has been used for over 2,000 years in Asia. Ginsenosides are the major active components of ginseng. Several different types have been isolated and identified. Some of their products have been marketed. Ginsenoside Rd is a ginsenoside with relatively high activity. It has been predicted to be a strong voltage-independent Ca\(^{2+}\) channel blocker [6], with protective roles in ischemic strokes and cardiovascular diseases [7, 8]. Previous studies have demonstrated the neuroprotective activity of ginsenoside Rd in SCI model rats, resulting in improvements to Basso, Beattie, and Bresnahan (BBB) scores. This activity has been related to inhibition of apoptotic protein caspase-3 [9].
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In the present study, PC12 cells under oxygen-glucose deprivation (OGD) were used as SCI cell models. Effects of ginsenoside Rd on Fas pathways and mitochondrial pathways in apoptosis were observed, providing a theoretical basis for SCI treatment.

Materials and methods

Induced differentiation of PC12 cells

PC12 cells were recovered and cultured in DMEM medium supplemented with 5% fetal bovine serum and 5% HS. When the cells reached 50% confluence, the medium was replaced with a differentiation medium (DMEM medium supplemented with 50 ng/mL NGF and 2% HS). The cells were induced for 48-72 hours.

In preparing hypoxic cell models, differentiated PC12 cells were cultured in serum- and glucose-free MEM medium. Sodium thiosulfate (Na₂S₂O₃) was added to a final concentration of 5 mmol/L, removing dissolved oxygen. The cells were cultured in a hypoxia incubator chamber with 5% CO2 and 95% N2 for 6 hours. They were then cultured again in a high-glucose DMEM medium with serum for 24 hours.

Cell activity measurement using CCK8 assays

Differentiated PC12 cells were digested by trypsin, re-suspended, and inoculated into 96-well plates. They were divided into five groups, including the control group, hypoxia model group, 0.1 µmol/L Rd group, 1.0 µmol/L Rd group, and 10.0 µmol/L Rd group. Each group contained five replicates. After treatment with ginsenoside Rd for 1 hour, the cells were cultured using serum- and glucose-free MEM medium in a hypoxia incubator chamber at 37°C with 5% CO₂ and 95% N₂ for 6 hours. They were then cultured again in a high-glucose DMEM medium with serum for 24 hours.

Experimental design and grouping

In this study, four groups of PC12 cells were established. The hypoxia group contained cells cultured in a glucose-deficient medium containing 5 mM Na₂S₂O₃ in a hypoxia incubator chamber for 6 hours. They were then cultured in a differentiation medium for 24 hours. The Rd group contained differentiated PC12 cells treated with 1.0 µmol/L ginsenoside Rd for 24 hours. The Rd pretreatment combining hypoxia group containing cells pretreated with 1.0 µmol/L ginsenoside for 1 hour. They were treated with hypoxia for 6 hours, then cultured in a medium containing ginsenoside Rd for 24 hours. The control group contained cells cultured in a medium for 24 hours.

Cell apoptosis detection

PC12 cells were counted and centrifuged after grouping. Annexin V-FITC solution and propidium iodide solution were then added, according to manufacturer instructions of the Annexin V-FITC Apoptosis Detection Kit (C1062, Beyotime). Confocal microscopy was used to observe five random fields of view. The apoptotic index (apoptotic cell number/total cell number * 100%) was calculated.

Measurement of mRNA expression levels

For total RNA extraction, PC12 cells, after grouping, were transferred into 35 mm diameter culture plates. They were lysed with 1 mL of TRizol Reagent, incubated at room temperature for 5 minutes, and transferred into 1.5 mL RNase-free EP tubes. A total of 200 µl of chloroform was added to each tube. The contents were then centrifuged at 10,000 rpm and 4°C for 10 minutes. The upper water phase was transferred into another tube. Isopropanol with equal volume was added and mixed 15 minutes. Centrifugation at 10,000 rpm and 4°C for 10 more minutes was conducted. The supernatant was discarded and 75% ethanol was added from 1 mL of DEPC water, washing away residual isopropanol. They were then centrifuged at 7,500 rpm and 4°C for 5 minutes. The supernatant was again discarded. The RNA pellet was air-dried on a clean bench for 10 minutes, then dissolved in 25 µl DEPC water.

Reverse transcription was conducted using the Transcriptor First Strand cDNA Synthesis Kit.
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Table 1. Sequences of PCR primers

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequences</th>
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<tbody>
<tr>
<td>Rat-Fas-F</td>
<td>5'-AAGATGCAGCTGACAGAAA-3'</td>
</tr>
<tr>
<td>Rat-Fas-R</td>
<td>5'-GGATTAAGCTTGCACAGCA-3'</td>
</tr>
<tr>
<td>Rat-FasL-F</td>
<td>5'-CACAGCTCCACAGGTCAAG-3'</td>
</tr>
<tr>
<td>Rat-FasL-R</td>
<td>5'-TTCCTTCTTGGCCTGATTTG-3'</td>
</tr>
<tr>
<td>Caspase-8-F</td>
<td>5'-CCCCACCCTCAGTTCTGT-3'</td>
</tr>
<tr>
<td>Caspase-8-R</td>
<td>5'-CGAGAGACCCATCTGCGA-3'</td>
</tr>
<tr>
<td>Caspase-9-F</td>
<td>5'-ACACCTGGCTCAGTGTGACC-3'</td>
</tr>
<tr>
<td>Caspase-9-R</td>
<td>5'-AGGTCATCCCAGAAGCTC-3'</td>
</tr>
<tr>
<td>GAPDH-F</td>
<td>5'-AGATGGCAGCCACCTTTG-3'</td>
</tr>
<tr>
<td>GAPDH-R</td>
<td>5'-AAGGTCATCCCAGAAGCTC-3'</td>
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(Roche) in a 20 µl reaction. According to manufacturer instructions, the system was set up and placed in a PCR machine with the following conditions: 50°C for 1 hour, then 85°C for 5 minutes. Products were stored at -20°C.

Quantitative real-time PCR was conducted using the FastStart Essential DNA Green Master mix (Roche) and LC96 PCR machine. The amplification program was as follows: 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 seconds, 55°C for 10 seconds, and 72°C for 15 seconds, with a final elongation at 72°C for 10 minutes. GAPDH was used as an internal standard. Sequences of primers used for PCR amplification are shown in Table 1.

Western blotting analysis

Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Grouped PC12 cells were digested and centrifuged at 1,000 rpm for 5 minutes. They were then lysed using 200 µl lysate and centrifuged at 12,000 × g for 10 minutes. Supernatants were kept. After protein quantitation (according to manufacturer instructions, P0010s, Beyotime), the loading buffer and 2-mercaptoethanol were added. The samples were then boiled, incubated on ice, and loaded onto gels. After separation, proteins were transferred to polyvinylidene fluoride membranes. Membranes were blocked in 5% skimmed milk powder at room temperature for 1 hour, then incubated in diluted primary antibodies at 4°C overnight. Primary antibodies included Caspase 9 (Sigma, USA), Caspase 8 (SAB, USA), Cytochrome C (Abcam, USA), and FADD (Abcam, USA). Membranes were washed the next day and incubated with a secondary antibody at room temperature for 1 hour. Secondary antibodies included Goat anti-Mouse IgG (H+L), HRP and Goat anti-Rabbit IgG (H+L), HRP. Membranes were then treated with the ECL chemiluminescent substrate and exposed. Protein bands were analyzed using the gel imaging system. Proteins were quantified relative to GAPDH levels.

Mitochondrial membrane potential tests

Mitochondrial membrane potential reduction is a specific event that occurs during the early stages of apoptosis. When the membrane potential is high, the fluorescent probe JC-1 accumulates in the mitochondrial matrix and emits red fluorescence, measured at an excitation wavelength of 564 nm. When the membrane potential is low, JC-1 does not accumulate. Thus, the JC-1 monomer emits green fluorescence, measured at an excitation wavelength of 488 nm. Present experiments were conducted according to manufacturer instructions of the mitochondrial membrane potential assay kit with tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) (C2006, Beyotime). A total of 20 mL of washing solution was made by mixing 4 mL of JC-1 staining buffer and 16 mL of distilled water. Next, 1 mL of JC-1 staining working solution was added to grouped PC12 cells and mixed thoroughly. Cells were then incubated for 20 minutes and washed twice with the washing solution. After adding 2 mL of medium, the cells were observed and pictured under a confocal microscope.

Cytochrome C detection

Cytoplasm was isolated, according to the manufacturer instructions of the cell mitochondria isolation kit (C3601, Beyotime). PC12 cells were cultured in 50 mL culture flasks, re-suspended in 1 mL of cold phosphate-buffered saline after grouping, centrifuged at 600 × g for 5 minutes at 4°C, and re-suspended in 4 mL of mitochondria isolation solution containing 1 mM of phenylmethanesulfonyl fluoride. They were then incubated on ice for 15 minutes, transferred into a 2 mL glass homogenizer, and homogenized 15 times. Homogenates were transferred into EP tubes and centrifuged at 600 × g at 4°C for 10 minutes. The supernatant was transferred into another tube and centrifuged at 11,000 × g at 4°C for 10 minutes. Supernatants containing cytoplasm proteins were collected in another tube and stored at -80°C.

Cytoplasm and mitochondrial proteins were quantified using the BCA protein quantification
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kit. They were then used for Western blotting analysis. Cytochrome C expression levels were analyzed using GAPDH as an internal standard.

Statistical analysis

Data are expressed as mean ± SD. Statistical analysis was conducted using SPSS Statistics software 17.0. Comparisons between groups were conducted using one-way analysis of variance, with the significance level set at $P<0.05$.

Results

PC12 cell differentiation

After 50 ng/mL NGF induction, PC12 cells were grown with neurites. They were observed to connect in a net (Figure 1).

PC12 cell activity

PC12 cell activities were shown to be altered following ginsenoside Rd treatment. Interestingly, high ginsenoside Rd concentrations did not result in high PC12 cell activities. Compared with the control group, ODs of the hypoxia group were significantly decreased at 0.57±0.02 ($P<0.05$). Values of the hypoxia+Rd 0.1 µM and hypoxia+Rd 10 µM groups were 0.61±0.01 and 0.58±0.02, respectively. These values were not significantly different from the hypoxia group ($P>0.05$). However, the OD value of the hypoxia+Rd 1.0 µM group was 0.68±0.03, significantly higher than that of the hypoxia group ($P<0.05$). Thus, the optimal pretreatment concentration of ginsenoside Rd under OGD conditions was 1.0 µmol/L (Figure 2).

Cell apoptosis

Neuronal apoptosis plays an important role in SCI. Little PC12 cell apoptosis was detected in control and Rd groups, with rates of 5.60±1.60 and 4.68±2.24, respectively. The apoptosis rate of the hypoxia group was 24.01±6.90, significantly higher than that of the control group ($P<0.05$). After ginsenoside Rd treatment, the apoptosis rate of the hypoxia+Rd group was 9.83±2.60, significantly lower than that of the HYPOXIA group ($P<0.05$) (Figure 3).

Results of mRNA expression measurement

Fas, Fasl, caspase-8, and caspase-9 are key proteins in intrinsic and extrinsic apoptotic pathways. As shown in Figure 4, OGD significantly increased mRNA levels of Fasl, caspase-8, and caspase-9, compared with the control group, with levels rising to 3.03±0.97 ($P<0.05$), 2.45±0.90 ($P<0.05$), and 2.86±0.99 ($P<0.05$)-fold higher, respectively. However, although mRNA levels of Fas increased to 3.05±1.04-fold, no significant differences were detected ($P>0.05$). After ginsenoside Rd treatment, mRNA expression levels of caspase-8 and caspase-9 decreased to 1.34±0.50 and 1.32±0.33-fold higher, respectively. This was significantly decreased, compared with the HYPOXIA group ($P<0.05$). Moreover, Fasl expression decreased to 2.44±1.08-fold high-
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Results of Western blotting

Caspase-8, caspase-9, and FADD are important members of Fas signaling pathways and mitochondrial pathways, functioning in the initiation process of apoptosis. As shown in Figure 5, Western blot analysis revealed that hypoxia treatment increased caspase-8, caspase-9, and Fas-associated via death domain (FADD) protein expression in PC12 cells by 0.77±0.10 (P<0.05), 0.38±0.03 (P<0.05), and 0.69±0.09 (P<0.05), respectively, compared with controls. After ginsenoside Rd treatment, protein levels of caspase-8, caspase-9, and FADD decreased to 0.34±0.06 (P<0.05), 0.20±0.05 (P<0.05), and 0.24±0.03 (P<0.05), respectively, compared with the hypoxia group (Figure 5).

Results of mitochondrial membrane potential detection

As shown in Figure 6, the relative fluorescence rate of the ginsenoside Rd group was 3.70±1.11, significantly higher than that of the control group (2.34±0.35; P<0.05), indicating that ginsenoside Rd inhibited PC12 cell apoptosis. The relative fluorescence rate of the hypoxia group was 0.75±0.27, significantly lower than that of the control group (P<0.05), indicating that hypoxia lowered the mitochondrial membrane potential and increased PC12 cell apoptosis. After ginsenoside Rd treatment, the relative fluorescence rate of the hypoxia+Rd group was 2.12±0.55, significantly higher than that of the hypoxia group (P<0.05).

Results of plasma cytochrome C measurement

When mitochondrial permeability increases, cytochrome C is released from the mitochondria into plasma, initiating apoptosis. As shown in Figure 7, Western blot analysis revealed cytochrome C expression levels in the hypoxia group of 5.20±0.56. These were significantly higher than those in the control group (P<0.01). After ginsenoside Rd treatment, the cytochrome C level of the hypoxia+Rd group fell to 1.90±1.38, significantly lower than that in the hypoxia group (P<0.05), indicating decreased PC12 cell apoptosis (Figure 7).

Discussion

The major active component of ginseng, ginsenoside Rd provides anti-inflammatory effe-
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It can also decrease the infarct size in ischemic strokes. It, therefore, possesses better neuroprotective function than edaravone [7]. Consequently, it has attracted a great deal of

Figure 4. mRNA analysis of grouped PC12 cells. A. Effects of ginsenoside Rd on Fas mRNA expression; B. Effects of ginsenoside Rd on FasL mRNA expression; C. Effects of ginsenoside Rd on caspase-8 mRNA expression; D. Effects of ginsenoside Rd on caspase-9 mRNA expression. **, P<0.05 compared with the control group; #, P<0.05 compared with the hypoxia (HY, oxygen-glucose deprivation) group. ##, p<0.01 compared with the control group.

Figure 5. Effects of ginsenoside Rd on protein expression levels. A. Western blot analysis of caspase-8 and caspase-9; B. Effects of ginsenoside Rd on caspase-8 protein expression; C. Effects of ginsenoside Rd on caspase-9 protein expression; D. Western blot analysis of FADD; E. Effects of ginsenoside Rd on FADD protein expression. **, P<0.05 compared with the control group; ***, P<0.01 compared with the control group; ##, P<0.05 compared with the hypoxia (HY, oxygen-glucose deprivation) group; ###, P<0.01 compared with the HY group.
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attention, with the number of studies increasing annually. Ginsenoside Rd has also shown protective roles in the kidneys of SCII mice by downregulating the polarization process of M1 macrophages [10] and demonstrated cardioprotective effects through activating the nuclear factor erythroid 2-related factor 2/heme oxygenase-1 signal pathways, alleviating oxidative stress induced by SCII [8]. In establishing a rat model of spinal cord contusion, Cong et al. showed that ginsenoside Rd improved rat BBB scores and neuron survival rates, decreased regional malonaldehyde (MDA) production, increased superoxide dismutase activity and glutathione levels, and significantly decreased motor neuron apoptosis [11]. Current findings are in accord with the neuroprotective functions of ginsenoside Rd in spinal injuries.

Although a neuroprotective role in SCII has been seen for several medicines [12-15], ginsenoside Rd possesses unique advantages. Its hydrophobicity enables it to penetrate the blood-brain barrier. The long half-time of ginsenoside Rd (19 hours) [16] reduces the dosing frequency. Thus, ginsenoside Rd has been considered a promising neuroprotective agent [17]. Studies of PC12 cells under hypoxia conditions have shown that ginsenoside Rd antagonized the cytotoxicity caused by hydrogen peroxide, reduced cell injury caused by oxidative stress, reduced MDA production, increased antioxidant activity, and enhanced the oxygen free radical scavenging activity of neurons. In this study, anti-apoptosis mechanisms of ginsenoside Rd were examined. Effects on members of the classical Fas and mitochondrial pathways were observed. Ginsenoside Rd downregulated caspase-8 and caspase-9 expression at both the mRNA and protein levels, reducing

Figure 6. Effects of ginsenoside Rd on PC12 cell mitochondrial membrane potential. A. Results observed using the JC-1 kit; B. Mitochondrial membrane potential changes in grouped PC12 cells. &&&, P<0.05 compared with the control group; ***, P<0.05 compared with the Rd group; ###, P<0.05 compared with the hypoxia (HY, oxygen-glucose deprivation) group. Scale bar=20 µm.

Figure 7. Effects of ginsenoside Rd on cytochrome C in PC12 cell plasma. A. Western blot analysis of cytochrome C; B. Plasma cytochrome C changes in grouped PC12 cells. ***, P<0.01 compared with the control group; **, P<0.05 compared with the hypoxia (HY, oxygen-glucose deprivation) group.
neuronal apoptosis. This is an important cause of motor function impairment after SCII.

Two types of anoxic cell models have been used in in vitro experiments [18]. One is a physical anoxic model created by a hypoxia culture environment with glucose. It contains nutrient deficiencies, such as low oxygen and high nitrogen, in a three-gas incubator, using a medium containing no serum or glucose. Although this model has been used extensively, expensive instruments are required. The second type is a chemical anoxic model established by chemical reagents or metabolites under anoxic conditions. It is known for its stability [19]. The hypoxia model, in the present study, combined these two methods by culturing PC12 cells in a three-gas incubator, then applying Na₂S₂O₃ to consume dissolved oxygen. This was to simulate a hypoxic environment that resembles ischemia-reperfusion injuries of spinal cord neurons. Because most ischemic reperfusion injuries are secondary lesions, the neuroprotective roles of ginsenoside Rd provide a theoretical basis for the clinical practice of alleviating secondary lesions.

Although the mechanisms of SCII remain unclear, neuronal apoptosis is considered an important part [20-24]. Many anti-apoptosis studies have been reported [25-27]. This study showed that ginsenoside Rd pretreatment provided neuroprotective effects by significantly decreasing PC12 cell apoptosis rates, suggesting it is a promising medicine for treatment of SCII.

Caspase-8 is a key initiating factor in Fas pathways. In the classical Fas pathway, Fas is located on the target cell membrane. It forms into a tri-polymer and recruits FADD in the plasma. FADD then recruits the self-activated caspase-8 precursor, which also activates downstream caspase-3 [28-30]. The current study found that ginsenoside Rd had different effects on different members of the Fas pathway. Ginsenoside Rd inhibited caspase-8 expression at mRNA and protein levels, decreased FADD protein expression, and had no significant influence on Fas/FasL mRNA levels. Present results indicate that, during SCII, ginsenoside Rd appears to downregulate plasma caspase-8 expression and decrease neuronal apoptosis through other pathways, rather than acting directly on Fas and FasL. However, detailed mechanisms should be elucidated in future studies.

Mitochondria plays a vital role in cell energy metabolism by providing ATP through the mitochondrial respiratory chain. The release of cytochrome C from the mitochondrial membrane into the plasma is a key part of the mitochondrial pathway [31-33]. The current study found that ginsenoside Rd played inhibiting roles at several stages of the mitochondrial pathway, ultimately decreasing cell apoptosis. Ginsenoside Rd also alleviated mitochondrial membrane potential reduction, decreased the plasma concentration of cytochrome C, as well as its release, and downregulated caspase-9 expression at mRNA and protein levels.

The current study had several limitations, however. First, there was no dose-response relationship between ginsenoside Rd and the neuroprotective effects for SCII. There were no significant differences in OD values of the Rd 10.0 µmol/L group, compared with the hypoxia group, while the Rd 1.0 µmol/L group showed significant differences. This indicates that high ginsenoside Rd concentrations might weaken its protective role. Thus, adverse effects should be further explored. Second, although the neuroprotective effects of ginsenoside Rd in SCII have been shown in both cell and animal models, human responses remain unclear. Traditional Chinese Medicine has reported that different patients respond differently to ginseng treatment. However, the contraindications of ginsenoside Rd have not been elucidated, requiring further investigation.

Conclusion

In summary, ginsenoside Rd decreased neuron apoptosis and was proven to be an effective neuroprotective agent in SCII. Its neuroprotective effects are related with Fas and mitochondrial pathways. Since SCII is a complicated pathophysiological process, whether other singing pathways are involved requires further exploration.

Acknowledgements

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Disclosure of conflict of interest
None.

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