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RESEARCH ARTICLE

Protective effects of ginsenoside Rg1 against hydrogen peroxide-induced injury in human neuroblastoma cells

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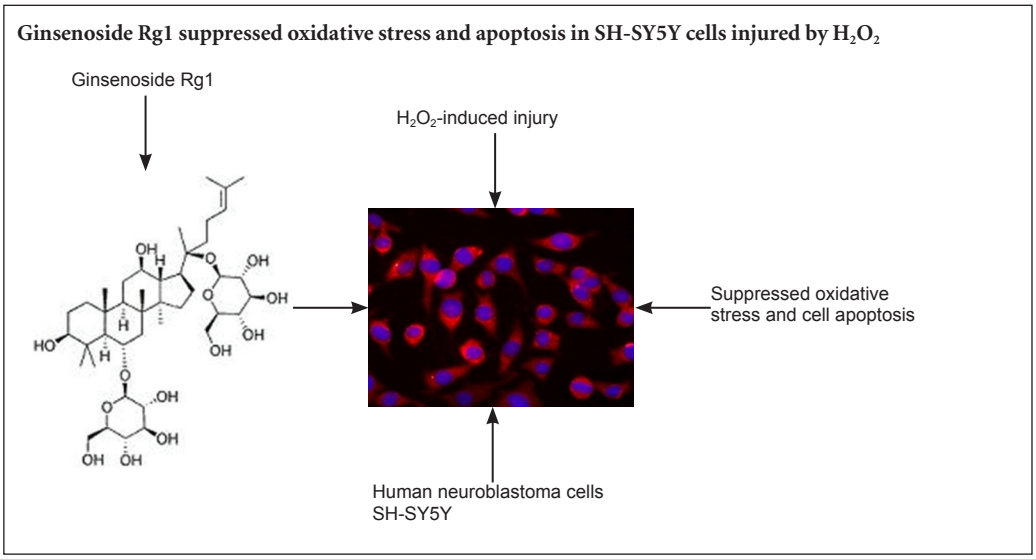
Graphical Abstract



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Abstract

The active ingredient of ginseng, ginsenosides Rg1, has been shown to scavenge free radicals and improve antioxidant capacity. This study hypothesized that ginsenosides Rg1 has a protective role in human neuroblastoma cells injured by H_2O_2. Ginsenosides Rg1 at different concentrations (50 and 100 μM) was used to treat H_2O_2 (150 μM)-injured SH-SY5Y cells. Results demonstrated that ginsenoside Rg1 elevated the survival rate of SH-SY5Y cells injured by H_2O_2, diminished the amount of leaked lactate dehydrogenase, and increased superoxide dismutase activity. Ginsenoside Rg1 effectively suppressed caspase-3 immunoreactivity, and contributed to heat shock protein 70 gene expression, in a dose-dependent manner. These results indicate that ginsenoside Rg1 has protective effects on SH-SY5Y cells injured by H_2O_2 and that its mechanism of action is associated with anti-oxidation and the inhibition of apoptosis.

Key Words: *nerve regeneration; traditional Chinese medicine monomer; ginsenoside Rg1; SH-SY5Y cells; H_2O_2; cerebral ischemia; cell apoptosis; lactate dehydrogenase; superoxide dismutase; caspase-3; heat shock protein 70; dose-effect relationship; neural regeneration*

Introduction

The effects of ischemic cerebrovascular disease on learning and memory and the neurobiological mechanisms involved are a hot topic in neuroscience (Dong et al., 2013). Ischemic cerebrovascular disease refers to a decrease in chronic blood flow caused by various factors, which promotes pathological and biochemical alterations, disorders energy metabolism (Liang et al., 2012; Chen et al., 2014; Ji et al., 2014), and causes oxygen free radical injury (Fraser et al., 2011), changes in neurotransmitters (Shen et al., 2011), cholinergic receptor deletion (Tracey et al., 2007), white matter damage and neuronal deletion (Xiong et al., 2012). These changes form the pathophysiological basis of chronic cerebral ischemia-induced dysfunction (Inoue et al., 2012). Jian et al.

(2013) suggested that free radical injury was a key factor in the injury to ischemic neurons. Ischemia and hypoxia in brain tissues induce a large amount of oxygen free radicals. Free radicals with a strong oxidative capacity attack vascular endothelial cells, destroy lipid membranes and cross-link membrane proteins to phospholipids, resulting in lipid peroxidation, increased permeability of the cell membrane to Ca^{2+}, destruction of the blood-brain barrier, and irreversible protein deactivation (Lu et al., 2012). Free radicals also increase proapoptotic gene caspase-3 expression and suppress anti-apoptotic gene heat shock protein 70 (HSP70) activity, causing cell membrane destruction, neuronal injury, and apoptosis (Tirapelli et al., 2012).

Recently, increasing numbers of studies have focused on the effects of traditional Chinese medicine to counter oxidative stress (Wang et al., 2013). Panax ginseng C.A.Meyer is a traditional Chinese herb that has been reported to regulate immunity, promote excitability, resist oxidation and fatigue, improve brain function, and contribute to the recovery of learning and memory functions (Zheng et al., 2011). Ginsenoside Rg1, a major component of Panax ginseng C.A.Meyer, has been shown to enhance superoxide dismutase (SOD) activity, inhibit the production of malondialdehyde, scavenge accumulating free radicals, and elevate antioxidative effects (Kim et al., 2009). Another study confirmed that a *Shenlong* decoction containing ginsenoside Rg1 reduced nitric oxide and inducible nitric oxide synthase contents, elevated the ability of learning and memory in rats with cerebral ischemia, and strengthened vascular endothelial growth factor expression in the rat hippocampus after cerebral ischemia (Zhang et al., 2011). Studies addressing the antioxidative mechanism of ginsenoside Rg1 for treatment of ischemic brain damage have mainly focused on the inhibitory effects of ginsenoside against neuronal apoptosis and its protective effects on neuronal cells (Li et al., 2015), but have seldom focused on the antioxidative mechanism of cells *in vitro* (Huang et al., 2016).

SH-SY5Y cells generated from human neuroblastoma have a low level of differentiation and are pyramidal with the presence of apparent axons (Lee et al., 2010). Some physiological functions of SH-SY5Y cells are similar to those of normal neurons (Waly et al., 2016). SH-SY5Y cells are commonly used in studies of the onset of nervous system disease and the mechanisms involved in the action of drugs (Ccy et al., 2014).

The current study investigated the regulatory effects of ginsenoside Rg1 on the survival rate, amount of leaked lactate dehydrogenase (LDH), SOD activity, caspase-3 expression, and HSP70 gene activity in SH-SY5Y cells injured by H_2O_2 to determine its protective effects and the mechanisms involved in its antioxidative and antiapoptotic effects.

Materials and Methods

Cells

Human dopaminergic neuroblastoma cell strain (SH-SY5Y) was a gift from the Sixth Institute of Academy of Military Medical Sciences, China.

Drugs

Ginsenoside Rg1 powder was purchased from Nanjing Zelang Medical Technology Co., Ltd., (Nanjing, Jiangsu Province, China, batch No. ZL201003; purity > 95%).

The experiments were approved by the Animal Ethics Committee, Chinese PLA General Hospital, China.

SH-SY5Y cell culture

SH-SY5Y cells were thawed and, digested with 0.25% trypsin and 0.02% ethylenediamine tetraacetic acid for 3 minutes, incubated with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Hyclone, Logan, UT, USA), 100 U/mL penicillin and 100 U/mL streptomycin in a 37°C 5% CO_2 incubator. The medium was replaced every 3 days. When cells reached 90% confluence, they were digested with 0.25% trypsin (Gibco, Carlsbad, CA, USA) for passage. Cells in the logarithmic phase were collected for further experiments.

Establishment of a cell model of H_2O_2-induced injury

SH-SY5Y cell concentrations in each group were adjusted to 1×10^6/mL. After removal of primary medium, cells in each well were incubated in complete medium containing 50, 100, 150, or 200 μM H_2O_2 in a 5% CO_2, 37°C incubator (Thermo, American) for 12 hours. The experimental cells were allocated to control, model (H_2O_2 150 μM), 50 μM ginsenoside Rg1 (H_2O_2 150 μM + ginsenoside Rg1 50 μM) and 100 μM ginsenoside Rg1 (H_2O_2 150 μM + ginsenoside Rg1 100 μM) groups.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to measure rate of cell survival

Cells from each group were adjusted to 1×10^6/mL. MTT (5 g/L; Sigma, St. Louis, MO, USA) 20 μL was added to each well of a tissue culture plate in a 5% CO_2 incubator at 37°C (Shellab, Cornelius, NC, USA) for 4 hours. When blue-purple crystals formed, the supernatant was removed. The samples were incubated with 150 μL dimethyl sulfoxide (Sigma) in each well, and shaken in a shaking bed for 10 minutes to dissolve the blue-purple crystals in cells completely. Optical density values were measured at 570 nm with a microplate reader (Polar star Galaxy; BMG, Offenburg, Germany). The average optical density value of cells from six wells was calculated by the following formula: survival rate = optical density$_{\text{experimental group}}$/optical density$_{\text{control group}}$ × 100%. The experiment was performed in triplicate. Cell viability was determined by MTT assay to identify the optimal H_2O_2 concentration (150 μM in this study). Different doses of ginsenosides Rg1 (10, 50, and 100 μM) combined with 150 μM H_2O_2 were used for 12 hours to observe the protective effects of different concentrations of cells.

Measurement of LDH leakage and SOD activity in cells

The cells (method described above) were treated with 150 μM H_2O_2. SH-SYSY cells were additionally treated with 10, 50, or 100 μM ginsenoside Rg1 in a 5% CO_2 incubator at

37°C for 24 hours following H_2O_2 (150 μM) treatment. The amount of leaked LDH and SOD activity in supernatants were examined using an LDH assay kit and SOD activity assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu Province, China).

Immunofluorescence histochemistry for caspase-3 expression in cells

The experimental procedure followed the instructions of the caspase-3 fluorescence detection kit (Beijing Boaosen Bioengineering Institute, Beijing, China). SH-SY5Y cells (1×10^6/mL) were washed three times with PBS, fixed with 4% paraformaldehyde for 20 minutes, washed three times with PBS, blocked with normal goat serum at 37°C for 20 minutes, incubated with primary antibody (rabbit anti-caspase-3 polyclonal antibody; Bioss, Woburn, MA, USA) at 4°C overnight, rewarmed for 10 minutes, and washed three times with PBS (each for 5 minutes). Subsequently, the samples were incubated with secondary antibody (goat anti-rabbit IgG, 1:20–1:100) at 37°C for 90 minutes, washed three times with PBS (each for 5 minutes), mounted with glycerol buffer, and then observed under a fluorescence (fluorescein isothiocyanate, Cy3 labeled) microscope (BX-60; Olympus, Tokyo, Japan). The Image-Pro Plus 5.1 image analytical system (Media Cybernetics, Seattle, WA, USA) was used to measure the number of caspase-3-positive cells and the fluorescence intensity.

Reverse transcription-polymerase chain reaction (RT-PCR) to measure HSP70 mRNA expression

Cells were adjusted to 1×10^6/mL, and total RNA was extracted (Liu et al., 2006). An ultraviolet spectrophotometer was utilized to measure nucleic acid concentrations. Total RNA (0.5 μg) was treated with DNaseI (EN0521, Fermentas, Canada), and reverse transcribed (K1622, Fermentas) into cDNA. cDNA (1 μL) was mixed with 8.2 μL ddH_2O. HSP70 and β-actin were amplified on a quantitative PCR device. Primers were prepared as previously described (Liu et al., 2006).

Primer sequences used in this study:

Primer	Sequence	Product size (bp)
HSP70	Upstream: 5'-CGC GAC CTG AAC AAG AGC AT-3' Downstream: 5'-TCG AAG GTC ACC TCG ATC TG-3'	375
β-Actin	Upstream: 5'-TG GGG CGC CCC AGG CAC CA-3' Downstream: 5'-CTC CTT AAT GTC ACG CAT TT-3'	226

Amplification conditions were as follows: predenaturation at 94°C for 5 minutes, denaturation at 94°C for 30 seconds, annealing at 57°C for 45 seconds, extension at 72°C for 20 seconds, for 40 cycles, followed by 72°C for 10 minutes. PCR products were electrophoresed on a 2% agarose gel, and photographed using a gel imaging system (Media Cybernetics). Results were expressed as the relative optical density value (HSP70/β-actin).

Statistical analysis

Measurement data, expressed as the mean ± SD, were analyzed with SPSS 13.5 software (SPSS, Chicago, IL, USA).

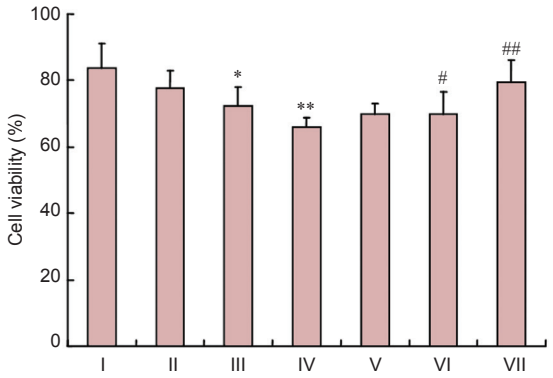

Figure 1 Effect of ginsenoside Rg1 on the viability of H_2O_2-treated SH-SY5Y cells.

*$P < 0.05$, **$P < 0.01$, *vs.* I; #$P < 0.05$, ##$P < 0.01$, *vs.* IV (mean ± SD, $n = 6$, one-way analysis of variance and least significant difference *post hoc* test). I: Control group; II: H_2O_2 50 μM group; III: H_2O_2 100 μM group; IV: 150 μM group; V: H_2O_2 (150 μM) + ginsenoside Rg1 (10 μM) group; VI: H_2O_2 (150 μM) + ginsenoside Rg1 (50 μM) group; VII: H_2O_2 (150 μM) + ginsenoside Rg1 (100 μM) group.

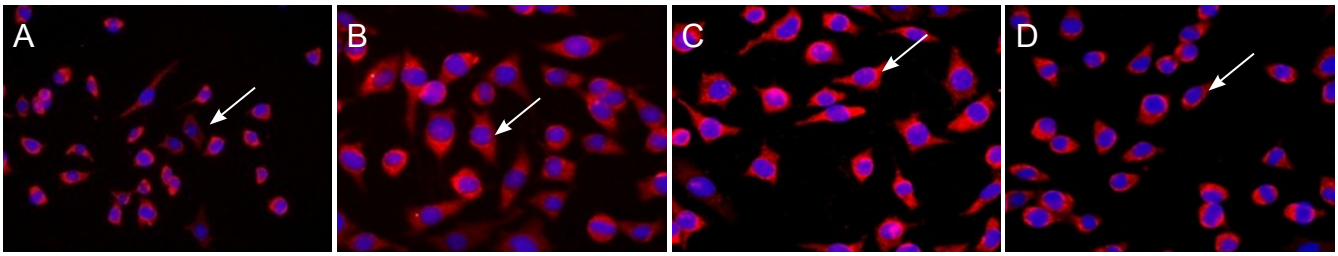

Figure 2 Effect of ginsenoside Rg1 on caspase-3 expression in SH-SY5Y cells injured by H_2O_2 (red immunofluorescence staining, inverted fluorescence microscope, magnification × 200).

(A) Control group; (B) model group; (C) 50 μM ginsenoside Rg1 group; (D) 100 μM ginsenoside Rg1 group. Arrows represent caspase-3-immunoreactive cells.

Table 1 Effect of ginsenoside Rg1 on the release of LDH and SOD activity in SH-SY5Y cells injured by H_2O_2

Group	LDH (U/L)	SOD (U/mg protein)
Control	277.00±62.00	637.59±26.02
Model	642.00±53.00**	307.72±30.43**
50 μM ginsenoside Rg1	447.00±67.00$^{\#}$	510.87±22.71$^{\#}$
100 μM ginsenoside Rg1	374.00±59.00$^{\#\#\&}$	594.70±19.06$^{\#\#}$

$^{**}P < 0.01$, *vs.* control group; $\#P < 0.05$, $\#\#P < 0.01$, *vs.* model group; $\&P < 0.05$, *vs.* 50 μM ginsenoside Rg1 group (mean ± SD, $n = 6$, one-way analysis of variance and least significant difference *post hoc* test). LDH: Lactate dehydrogenase; SOD: superoxide dismutase.

Table 2 Effects of ginsenoside Rg1 on caspase-3 immunoreactivity in H_2O_2-treated SH-SY5Y cells

Group	Fluorescence intensity	Number of immunoreactive cells
Control	0.542±0.013	5.961±3.827
Model	1.879±0.061**	24.079±4.010*
50 μM ginsenoside Rg1	1.503±0.029$^{\#}$	14.905±2.764$^{\#}$
100 μM ginsenoside Rg1	1.337±0.057$^{\#\#}$	10.256±3.907$^{\#}$

$^{*}P < 0.05$, $^{**}P < 0.01$, *vs.* control group; $\#P < 0.05$, $\#\#P < 0.01$, *vs.* model group (mean ± SD, $n = 6$, one-way analysis of variance and least significant difference *post hoc* test).

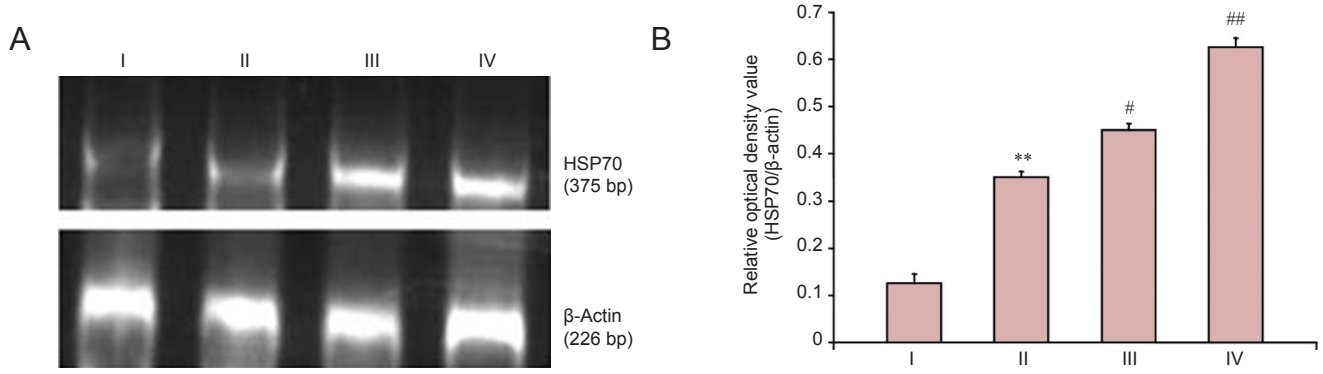

Figure 3 Effects of ginsenoside Rg1 on HSP70 mRNA expression in H_2O_2-treated SH-SY5Y cells.
$^{**}P < 0.01$, *vs.* control group; $\#P < 0.05$, $\#\#P < 0.01$, *vs.* model group (mean ± SD, $n = 6$, one-way analysis of variance and least significant difference *post hoc* test). HSP70: Heat shock protein 70. I: Control group; II: model group; III: 50 μM ginsenoside Rg1 group; IV: 100 μM ginsenoside Rg1 group.

One-way analysis of variance and *post hoc* least significant difference test were used at $\alpha = 0.05$.

Results

Effects of ginsenoside Rg1 on the survival rate of H_2O_2-treated SH-SY5Y cells

As shown in **Figure 1**, the survival rate of SH-SY5Y cells was gradually decreased with an increasing concentration of H_2O_2. When SH-SY5Y cells were treated with 150 μM H_2O_2 for 12 hours, the optical density of cells was significantly decreases compared with the control group ($P < 0.01$). This indicated that the ability of cells to reduce MTT decreased, and the cell survival rate was diminished. Therefore, 150 μM H_2O_2 was used in all the following experiments. After 12 hours of H_2O_2 treatment, 10 μM ginsenoside Rg1 had no significant effects on the cell survival rate compared with the model group ($P > 0.05$). However, 50 and 100 μM ginsenoside Rg1 had significant protective effects on SH-SY5Y cells injured by 150 μM H_2O_2 compared with the model group ($P < 0.05$, $P < 0.01$, respectively). Thus, 50 and 100 μM ginsenoside Rg1 were utilized in the following experiments.

Effects of ginsenoside Rg1 on the amount of leaked LDH from H_2O_2-treated SH-SY5Y cells

LDH leakage was significantly greater in SH-SY5Y cells injured by 150 μM H_2O_2 (model group) compared with the control group ($P < 0.01$). In addition, 50 and 100 μM ginsenoside Rg1 effectively significantly inhibited the LDH leakage in SH-SY5Y cells injured by H_2O_2 compared with the model group ($P < 0.05$, $P < 0.01$, respectively). The inhibitory effect was significantly enhanced with an increased dose of ginsenoside Rg1. Significant differences in LDH leakage were observed between the 50 and 100 μM ginsenoside Rg1 groups ($P < 0.05$; **Table 1**).

Effects of ginsenoside Rg1 on SOD activity in SH-SY5Y cells injured by H_2O_2

SOD activity was significantly lower in SH-SY5Y cells injured by H_2O_2 (model group) compared with the control group ($P < 0.01$). SOD activity was significantly higher in the 50 and 100 μM ginsenoside Rg1 groups in a dose-dependent manner compared with the model group ($P < 0.05$, $P < 0.01$, respectively). Nevertheless, no significant differences in SOD activity were detectable between the 50 and 100 μM ginsenoside Rg1 groups ($P > 0.05$; **Table 1**).

Effects of ginsenoside Rg1 on caspase-3 expression in SH-SY5Y cells injured by H_2O_2

As shown in **Figure 2** and **Table 2**, immunofluorescence staining revealed that caspase-3 expression was significantly higher in SH-SY5Y cells injured by H_2O_2 (model group) than in hippocampal neurons of the control group ($P < 0.05$, $P < 0.01$, respectively). In addition, 50 and especially 100 μM ginsenoside Rg1, significantly diminished the caspase-3 expression in

injured cells ($P < 0.05$, $P < 0.01$, respectively).

Effects of ginsenoside Rg1 on HSP70 mRNA expression in SH-SY5Y cells injured by H_2O_2

Clear bands of different brightness at 377 bp RT-PCR indicated the presence of HSP70 mRNA expression in cells from each group. Compared with the control group, HSP70 was obviously activated in cells injured by H_2O_2 for 12 hours. Furthermore, 50 and 100 μM ginsenoside Rg1 enhanced HSP70 expression in the injured cells. The optical density ratio of HSP70 to β-actin was considered a measurable indicator of the expression of HSP70 mRNA. The ratios in the control, model, 50, and 100 μM ginsenoside Rg1 groups were 0.630, 0.351, 0.457, and 0.630, respectively. Significant differences in the above ratios were detectable between the 50 and 100 μM ginsenoside Rg1 groups and the model group ($P < 0.05$, $P < 0.01$, respectively; **Figure 3**).

Discussion

The pathogenesis of neurons injured by cerebral ischemia is complicated, and is associated with oxygen free radical injury, inflammatory factor damage, excitatory amino acid injury, and intracellular Ca^{2+} overload (Nakase et al., 2008; Sierra et al., 2011). Of these, oxidative stress-induced oxygen free radical injury has become the focus of most attention (Allen et al., 2009). Brain tissues contain abundant unsaturated fatty acids and are therefore more susceptible to damage by free radicals (Kim et al., 2008). A recent study confirmed that oxygen free radical injury to ischemic neurons was correlated with caspase-3 and HSP70 expression in the brain (Ueda et al., 2002). When brain tissues experienced oxidative stress, such as during ischemia or hypoxia, caspase-3, a key executor of neuronal apoptosis, *i.e.*, apoptotic effector molecule (Awasthi et al., 2013), becomes activated. Caspase-3 destroys collagen, intervenes in mRNA splicing, blocks DNA replication and repair, and induces cell apoptosis (Broughton et al., 2009). Simultaneously, caspase-3 activation was reported to exhaust intracellular nicotinamide adenine dinucleotide/adenosine triphosphate, resulting in cell loss (Jie et al., 2011). HSP70 is expressed at low levels in normal cells, but this expression is increased during stress (Dong et al., 2012). Under ischemic/hypoxic conditions, intracellular nucleoproteins are denatured, heat shock factors bind to heat shock elements, and many molecules of HSP70 are synthesized (Franklin et al., 2005). HSP70 inhibits caspase-3 activation, cleaves caspase cascade reactions, and prevents cell apoptosis. HSP70 also prevents protein aggregation or incorrect folding under stress, maintains protein homeostasis, and prevents degeneration-induced disorders of DNA (Wang et al., 2012).

LDH leakage reflects the degree of cell membrane injury (Ya et al., 2013). Increased LDH concentrations in the extracellular fluid are a marker for irreversible damage or cell necrosis (Noh et al., 2011). SOD is a scavenger enzyme of superoxide free radical anions *in vivo* (Park et al., 2011). During oxidative stress, increased SOD consumption led to a reduction in SOD activity (Cui et al., 2013). Another study showed that ginsenoside Rg1 elevated SOD production and scavenged oxygen free radicals (Li et al., 2010). In this study, when SH-SY5Y cells were treated with 150 μM H_2O_2 for 12 hours, the amount of LDH leaked was markedly increased, but the SOD concentration was decreased. Ginsenoside Rg1 increased the survival rate of H_2O_2-injured SH-SY5Y cells, diminished the amount of leaked LDH and increased SOD activity. These results indicated that ginsenoside Rg1 strongly inhibited oxidative stress injury. Furthermore, ginsenoside Rg1 reduced caspase-3 immunoreactivity, promoted HSP70 gene expression, and reduced oxygen free radical injury in SH-SY5Y cells injured by H_2O_2.

Ginsenoside Rg1 had a dose-dependent mechanism involved in improving cell apoptosis because the protective effect of ginsenoside Rg1 increased with an increasing dose of ginsenoside Rg1.

In conclusion, cerebral ischemia-induced nerve cell apoptosis is a key neuropathological process. After cerebral ischemia, multiple factors and mechanisms interact, participate in the occurrence and development of nerve cell apoptosis, finally resulting in apoptosis. Ginsenoside Rg1 resists oxidative stress and free radical injury, increases the survival rate of damaged cells, reduces the amount of leaked LDH and caspase-3 activation, increases SOD activity and HSP70 expression, and finally suppresses cell apoptosis, in a dose-dependent manner.

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Conflicts of interest: *None declared.*

Plagiarism check: *This paper was screened twice using Cross-Check to verify originality before publication.*

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