

Neuroprotective effect of ginsenoside Rg1 prevents cognitive impairment induced by isoflurane anesthesia in aged rats via antioxidant, anti-inflammatory and anti-apoptotic effects mediated by the PI3K/AKT/GSK-3 β pathway

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Abstract. Ginsenoside Rg1 is the primary active substance in ginseng, and it has multiple pharmacological actions. Investigations on the pharmacologic action of ginsenoside Rg1 have developed, with a particular focus on the regulation of metabolism. The present study hypothesized that the neuroprotective effects of ginsenoside Rg1 prevent cognitive impairment induced by isoflurane anesthesia via antioxidant, anti-inflammatory and anti-apoptotic effects, mediated by the phosphoinositide 3-kinase (PI3K)/AKT/glycogen synthase kinase-3 β (GSK-3 β) pathway in aged rats. Sprague-Dawley rats were divided into isoflurane and ginsenoside Rg1 groups and were treated with 20 mg/kg ginsenoside Rg1 for 7 days. Morris water maze was performed to analyze the cognitive function of the rats. Enzyme-linked immunosorbent assays were used to analyze the levels of malondialdehyde, glutathione, interleukin (IL)-1 β , IL-6 and caspase 3. The protein expression levels of AKT, GSK 3 β , p21WAF1/CIP1 and p53 were measured using western blot analysis. Ginsenoside Rg1 significantly improved cognitive function, and exhibited antioxidant and anti-inflammatory effects, demonstrating the neuroprotective effects of ginsenoside Rg1 against the effect of isoflurane anesthesia in the rats. In addition, ginsenoside Rg1 significantly reduced caspase-3 activity, upregulated the expression of PI3K/AKT/GSK-3 β and downregulated the mRNA expression levels of p21WAF1/CIP1 and p53 in the

aged rats exposed to isoflurane anesthesia. The data obtained in the present study provided evidence that the neuroprotective effects of ginsenoside Rg1 prevented the cognitive impairment induced by isoflurane anesthesia via antioxidant, anti-inflammatory and anti-apoptotic effects, mediated by the PI3K/AKT/GSK-3 β pathway.

Introduction

A graying society is an indispensable and significant link during humthean indispensable (1). A graying society is a population structure in which the aging population has reached or exceeded a certain proportion (1). According to the traditional standard of the United Nations, a region where the population of individuals aged >60 years old is 10% of the total population can be regarded as entering into a graying society (2). However, in the current standard, it refers to a population of individuals, in which those aged >65 years old represent 7% of the total population (3). In 2002, China became a graying society. As age increases, the elderly are more susceptible to various types of diseases due to their unique physiological characteristics, which introduce severe challenges for medical treatment and the social insurance industry (4). It is important to seriously consider these problems, and there is interest in postoperative cognitive dysfunction (POCD), specifically in gerontal patients (5).

Isoflurane has lasting and severe effects on cognitive function. In particular, it can affect learning and memory function in sea horses (4,6). Isoflurane is a novel inhalation anesthetic, which is frequently used clinically (7). It can inhibit cholinergic neurons from transmitting signals and induces anesthetic effects via the N-methyl-D aspartic acid receptor, which affect learning and memory function (7,8).

Ginseng is a type of traditional Chinese medicine, which is widely used for the promotion of health and treatment of diseases (9). Ginseng, marten and cartialgenous are also collectively known as 'the three treasures' in northeast China. There are several ginsenosides, including Ro, Ra, Ral-2, Rbl-3, Rc, Rd, Re, Rf and Rg1 (10). Of the ginseng glycosides, ginsenoside Rg1

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has anti-oxidative and anti-apoptotic functions and other neuro-protective effects (10). Previous experiments have indicated that ginsenoside Rg1 can improve, consolidate and recover memory in cognitive impairment caused by quinolinic acid, Pb, dexamethasone, anisodine, ethylene and ethyl alcohol (11,12). In addition, it can improve impairment of learning and memory function caused by scopolamine and morphine (13). The aims of the present study were to detect whether the neuroprotective effect of ginsenoside Rg1 prevents cognitive impairment induced by isoflurane anesthesia via antioxidant, anti-inflammatory and anti-apoptotic effects, and whether these are mediated by the phosphoinositide 3-kinase (PI3K)/AKT/glycogen synthase kinase-3 β (GSK-3 β) pathway in aged rats.

Materials and methods

Reagents. Ginsenoside Rg1 ($\geq 90\%$ in high performance liquid chromatography) was purchased from Sigma-Aldrich (St. Louis, MO, USA; its chemical structure is shown in Fig. 1). Isoflurane, the interleukin (IL)-1 β kit (cat. no. H002), IL-6 kit (cat. no. H007), malondialdehyde (MDA) kit (cat. no. A003-1), caspase-3 kit (cat. no. H076) and glutathione (GSH) kit (cat. no. A006-2) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The bicinchoninic acid assay (BCA) assay was purchased from Wuhan Boster Bio-engineering Co., Ltd. (Wuhan, China).

Animals and ethical statement. Male, 3-month-old Sprague-Dawley (SD) rats were purchased from the Medical and Laboratory Animal Center of Beijing Jishuitan Hospital (Beijing, China) and housed at a temperature ($23 \pm 1^\circ\text{C}$) in a light-controlled room (12-h light/dark cycle), with a relative humidity of 50-70% and free access to water and food. All SD rats were treated, according to the guidelines of the Guide for the Care and Use of Laboratory Animals of Beijing Jishuitan Hospital and the study was approved by the ethics committee of Beijing Jishuitan Hospital.

Exposure to anesthesia and grouping. The rats were randomly divided into three groups: Control, isoflurane and ginsenoside Rg1 ($n=10$ in each group). The SD rats in the ginsenoside Rg1 group were treated with 20 mg/kg ginsenoside Rg1 for 7 days (14) by intraperitoneal injection. After 12 h, the rats in the isoflurane and ginsenoside Rg1 groups were exposed to 1.3% isoflurane (Nanjing Jiancheng Bioengineering Institute) in a humidified 30% oxygen carrier gas for 4 h. The SD rats in the control group were exposed to humidified 30% oxygen without isoflurane for 4 h.

Morris water maze performance. A round pool, with a diameter of 150 cm and depth of 50 cm, was filled with 24°C opaque water to the top of a movable, clear 15-cm-diameter platform (1.5 cm above water). Motion detection software (Actimetrics software, Evanston, IL, USA; version ACT-556) was used to record the swimming motions and analyze the results. Each rat was wiped dry and kept warm, and returned to their cage with free access to food. Following isoflurane exposure, trials were performed for 4 days, and the spatial information of every rat was analyzed. The pool had a dark black surround, which was used to prevent confounding visual cues. All rats were placed

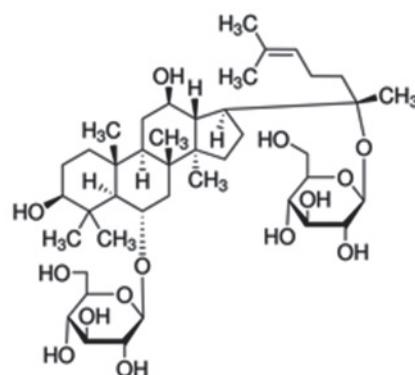


Figure 1. Chemical structure of ginsenoside Rg1.

in a fixed position in the swimming pool, facing the wall, on the 4 days. The rats were allowed to find the platform (in the third quadrant) for 20 sec, prior to being removed from the pool. The swim speed and the time of escape latency were recorded. Less time taken for a rat to reach the platform indicated a higher learning ability.

Detection of levels of MDA, GSH, IL-1 β , IL-6 and caspase-3 using ELISA. Following isoflurane exposure, the rats in each group were sacrificed and the hippocampi were dissected for measurement of the protein levels of IL-1 β , IL-6, MDA, GSH and caspase-3 using ELISA kits (Nanjing Jiancheng Bioengineering Institute).

Western blot analysis of the protein expression of AKT, GSK-3 β , p21WAF1/CIP1 and p53. Hippocampal tissues were collected and were homogenized with phenylmethanesulfonyl fluoride containing radioimmunoprecipitation assay buffer and protease inhibitor cocktail (EDTA-free) on ice. The supernatant were collected and centrifuged at 13,000 x g at 4°C for 30 min, following which the BCA method (Wuhan Boster Bio-engineering, Wuhan, China) was used to analyze protein concentrations. The protein (50 mg) was separated by SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Wuhan Boster Biological Technology, Wuhan, China). The membranes were blocked using 5% skim milk powder and incubated overnight at 4°C with anti-AKT (1:500; cat. no. sc-5298; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-phosphorylated (p)-AKT (1:500; cat. no. sc-293125; Santa Cruz Biotechnology, Inc.), anti-GSK-3 β (1:2,000; cat. no. 9337; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-p21WAF1/CIP1 (1:2,000; cat. no. 2947; Cell Signaling Technology, Inc.), anti-p53 (1:2,000; cat. no. 2524; Cell Signaling Technology, Inc.) and anti- β -actin antibodies (1:500; cat. no. BM0626; Wuhan Boster Biological Technology). The membranes were then incubated with anti-mouse secondary antibody (1:5,000; cat. no. BM2002; Wuhan Boster Biological Technology) at 37°C for 1 h and visualized using an enhanced chemiluminescence detection system (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and expression was quantified using a gel image analysis system (Thermo Fisher Scientific, Inc.).

Statistical analysis. All data are presented as the mean \pm standard error of the mean, and analysis was performed using

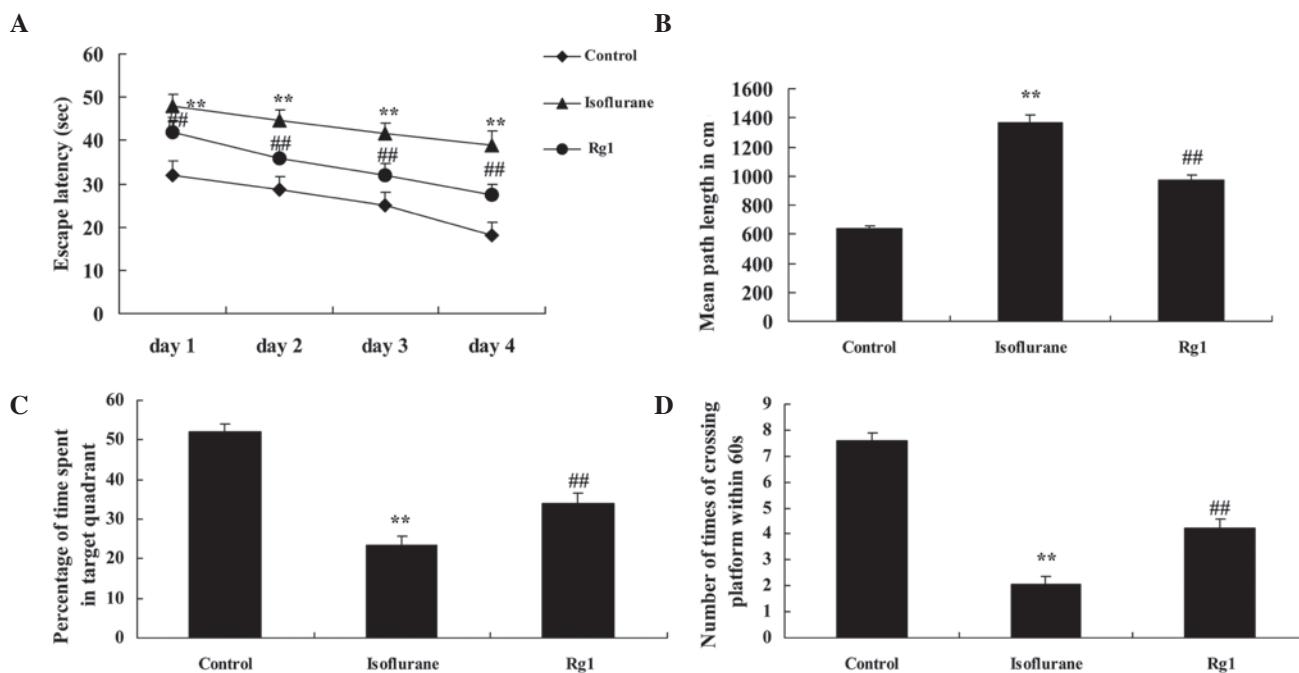


Figure 2. Neuroprotective effect of ginsenoside Rg1 on cognitive function in aging rats exposed to isoflurane anesthesia. Neuroprotective effects of ginsenoside Rg1 on (A) escape latency, (B) path length, (C) mean swim speed and (D) duration of swimming were examined in aging rats exposed to isoflurane anesthesia. Data are presented as the mean \pm standard error of the mean. **P<0.01, compared with the control group; ##P<0.01, compared with the isoflurane group.

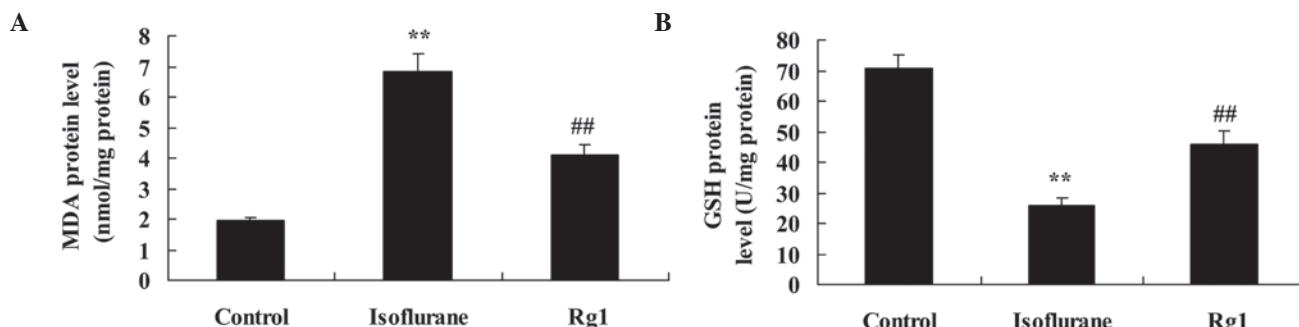


Figure 3. Antioxidant effect of ginsenoside Rg1 in aging rats exposed to isoflurane anesthesia. Effects of ginsenoside Rg1 on the protein levels of (A) MDA and (B) GSH were examined in aging rats exposed to isoflurane anesthesia. Data are presented as the mean \pm standard error of the mean. **P<0.01, compared with the control group; ##P<0.01, compared with the isoflurane group. MDA, malondialdehyde; GSH, glutathione.

SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance was used for comparison of mean values across the groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Neuroprotective effects of ginsenoside Rg1 on cognitive function in isoflurane anesthesia-exposed aged rats. As shown in Fig. 2A and B, the escape latency and path length of the control group were lower, compared with those of the isoflurane anesthesia group following the Morris water sessions. Ginsenoside Rg1 effectively inhibited the increased escape latency and path length in the aged rats exposed to isoflurane anesthesia (Fig. 2A and B). The mean swim speed and swimming durations were significantly inhibited in the aged rats exposed to isoflurane anesthesia, compared with the control

group (Fig. 2C and D). However, ginsenoside Rg1 effectively elevated the suppression of mean swim speed and extended the duration of swimming in the rats exposed to isoflurane anesthesia (Fig. 2C and D).

Antioxidant effects of ginsenoside Rg1 in aging rats exposed to isoflurane anesthesia. As shown in Fig. 3A and B, the rats in the isoflurane groups had significantly increased protein levels of MDA and decreased protein levels of GSH, compared with the control group. Treatment with ginsenoside Rg1 markedly reduced the elevated MDA protein level and inhibited GSH protein level in the rats exposed to isoflurane anesthesia (Fig. 3A and B).

Anti-inflammatory effect of ginsenoside Rg1 in aging rats exposed to isoflurane anesthesia. The results of the present study revealed increases in the protein levels of IL-1 β and IL-6

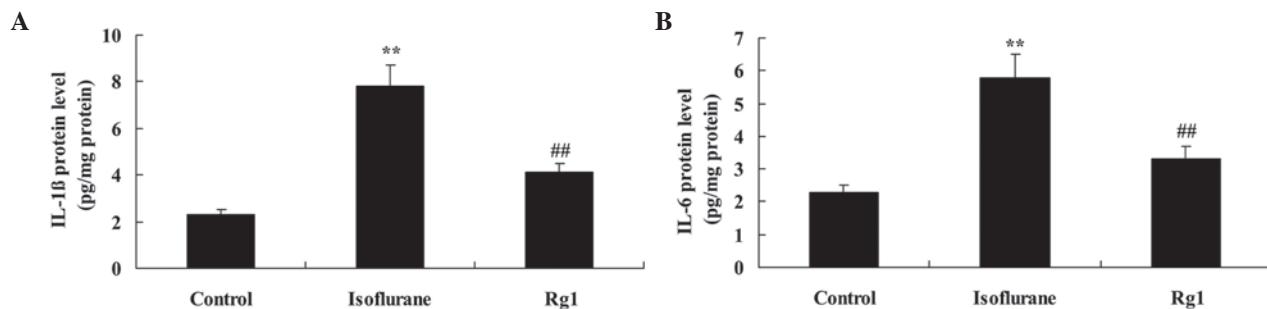


Figure 4. Anti-inflammatory effect of ginsenoside Rg1 in aging rats exposed to isoflurane anesthesia. Effects of ginsenoside Rg1 on the protein expression of (A) IL-1 β and (B) IL-6 were examined in aging rats exposed to isoflurane anesthesia. Data are presented as the mean \pm standard error of the mean. **P<0.01, compared with the control group; ##P<0.01, compared with the isoflurane group. IL, interleukin.

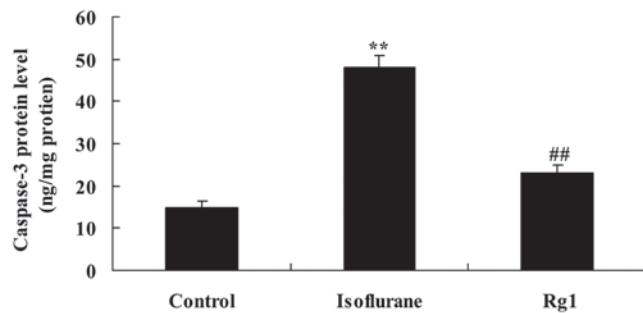


Figure 5. Anti-apoptotic effects of ginsenoside Rg1 in aging rats exposed to isoflurane anesthesia. The protein levels of caspase-3 were determined. Data are presented as the mean \pm standard error of the mean. **P<0.01, compared with the control group; ##P<0.01, compared with isoflurane group.

in the isoflurane anesthesia group, compared with the control group (Fig. 4A and B). However, the isoflurane-induced protein levels of IL-1 β and IL-6 were significantly weakened by pretreatment with ginsenoside Rg1 (Fig. 4A and B).

Anti-apoptotic effect of ginsenoside Rg1 in aging rats exposed to isoflurane anesthesia. Isoflurane exposure significantly increased the protein level of caspase-3 in the aging rat, compared with the rats in the control group (Fig. 5). This increase was attenuated by ginsenoside Rg1 (Fig. 5).

Neuroprotective effect of ginsenoside Rg1 on p-AKT/AKT in isoflurane anesthesia-exposed aging rats. Compared with the rats in the control group, there was a significant decrease in the level of p-AKT/AKT of the aging rat following isoflurane exposure (Fig. 6). However, treatment with ginsenoside Rg1 effectively increased the levels of p-AKT/AKT in the aging rats exposed to isoflurane (Fig. 6).

Neuroprotective effect of ginsenoside Rg1 on GSK-3 β in aging rats exposed to isoflurane anesthesia. Compared with the rats in the control group, the protein expression of GSK-3 β was reduced in the aging rats following exposure to isoflurane (Fig. 7). This inhibited protein expression of GSK-3 β was elevated by treatment with ginsenoside Rg1 (Fig. 7).

Neuroprotective effect of ginsenoside Rg1 on p21WAF1/CIP1 in isoflurane anesthesia-exposed aging rats. As shown

in Fig. 8, the protein expression of p21WAF1/CIP1 in the isoflurane anesthesia group was higher, compared with that of the control group. Ginsenoside Rg1 treatment significantly inhibited the promotion in the protein expression of p21WAF1/CIP1 in the aging rat exposed to isoflurane anesthesia (Fig. 8).

Neuroprotective effect of ginsenoside Rg1 on p53 in isoflurane anesthesia-exposed aging rats. Compared with the control group, the protein expression of p53 was activated by isoflurane (Fig. 9). However, the isoflurane-induced protein expression of p53 was suppressed by pretreatment with ginsenoside Rg1 in the aging rats exposed to isoflurane anesthesia (Fig. 9).

Discussion

POCD is an acute reversible abalienation syndrome that occurs following surgery with anesthesia (15). It often occurs in the elderly several days or several weeks following surgery, with a decline in memory, comprehension and concentration, and a decrease in social adaptation ability, possibly leading to the loss of independent living and a permanent cognitive disorder (16). POCD was originally identified in gerontal patients following cardiac surgery (17), however, it has been found in patients who have not had cardiac surgery with higher rates of morbidity. There are several risk factors for POCD during the early post-operative period, including history of surgery with anesthesia, low levels of education, secondary surgery, postoperative infection and respiratory system complications, whereas advanced age is the predominant risk factor during the later post-operative period (18). Compared with younger adults, the brain cells of the elderly are atrophied, with seriously decreased cranial capacity, enlarged ventricles, and alterations in cranial nerve distribution and neurotransmitter types (19). As a result, gerontal patients are more sensitive to nerve cell damage caused by surgery and anesthesia (20). Furthermore, postoperative pain can affect cognitive function during early period, however, chronic pain prior to surgery has no effect on the results of cognitive function assessments (21). The results of the present study suggested that the neuroprotective effects of ginsenoside Rg1 improved cognitive function in the aging rats exposed to isoflurane anesthesia.

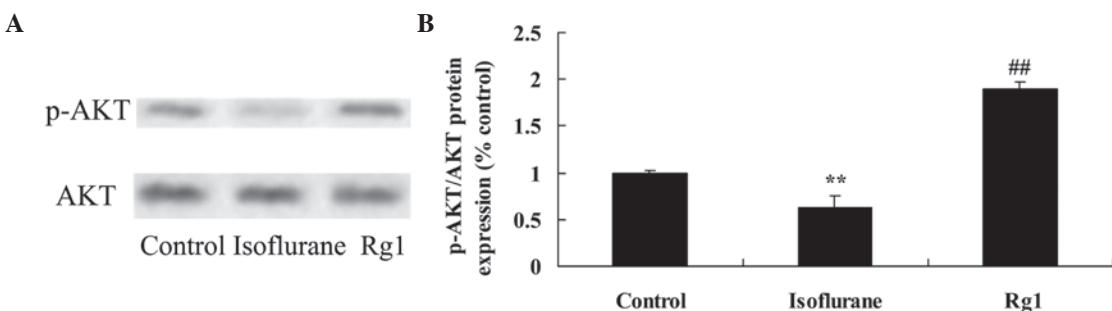


Figure 6. Neuroprotective effect of ginsenoside Rg1 on p-AKT/AKT in isoflurane anesthesia exposed aging rats. The effects of ginsenoside Rg1 on the protein expression levels of p-AKT and AKT were determined using (A) western blot analysis, and (B) statistical analysis of the protein expression levels of p-AKT and AKT was performed. Data are presented as the mean \pm standard error of the mean. **P<0.01, compared with the control group; ##P<0.01, compared with the isoflurane group.

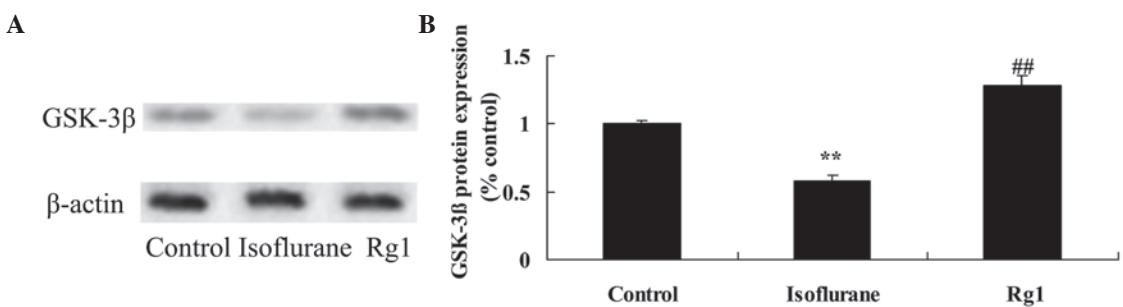


Figure 7. Neuroprotective effect of ginsenoside Rg1 on GSK-3 β in aging rats exposed to isoflurane anesthesia. (A) Effect of ginsenoside Rg1 on the protein expression of GSK-3 β , determined using western blot analysis. (B) Statistical analysis of the protein expression levels. Data are presented as the mean \pm standard error of the mean. **P<0.01, compared with the control group; ##P<0.01, compared with the isoflurane group. GSK-3 β , glycogen synthase kinase-3 β .

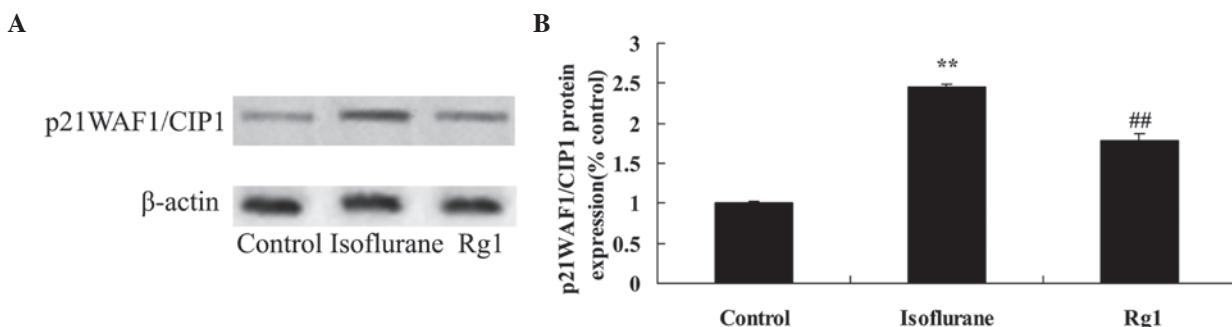


Figure 8. Neuroprotective effect of ginsenoside Rg1 on p21WAF1/CIP1 in aging rats exposed to isoflurane anesthesia. (A) Neuroprotective effect of ginsenoside Rg1 on the protein expression of p21WAF1/CIP1, determined using western blot analysis. (B) Statistical analysis of the protein expression of p21WAF1/CIP1. Data are presented as the mean \pm standard error of the mean. **P<0.01, compared with the control group; ##P<0.01, compared with the isoflurane group.

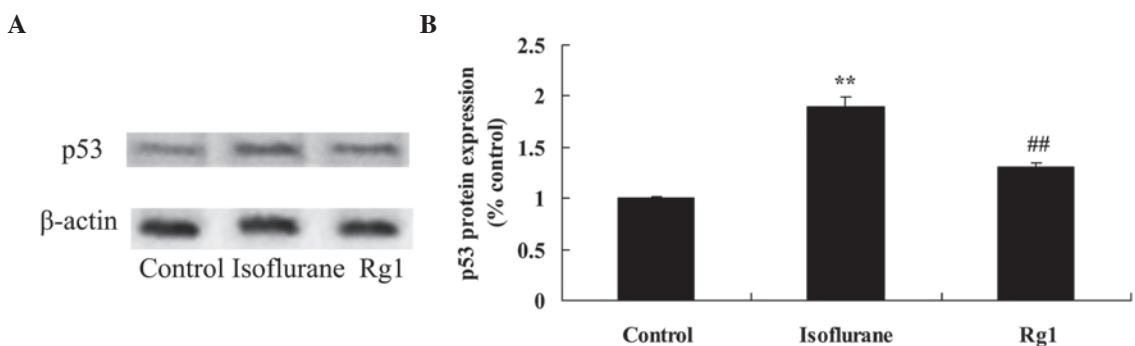


Figure 9. Neuroprotective effect of ginsenoside Rg1 on p53 in aging rats exposed to isoflurane anesthesia. (A) Neuroprotective effect of ginsenoside Rg1 on the protein expression of p53, determined using western blot analysis. (B) Statistical analysis of the protein expression of p53. Data are presented as the mean \pm standard error of the mean. **P<0.01, compared with the control group; ##P<0.01, compared with the isoflurane group.

In previous years, a number of studies have indicated that the inflammatory response inside the brain and oxidative stress are of significant importance in the pathogenesis of cognitive impairment (22). Several reports have confirmed that inflammation contributes to pathological injury in various ways. For example, inflammation damages the integrity of vascular function and contributes to the pathogenesis of cognitive impairment diseases, including POCD (23). Treatment of this disease through targeting this pathomechanism can significantly improve neuronal injury and modify nerve fiber damage to relieve the inflammatory response within the brain, improving learning and memory abilities (23,24). Therefore, there has been substantial investigation on the association between oxidative stress and cognitive impairment (25). Free radicals have high levels of activity and potent oxidative effects, which can injure biomacromolecules and multiple components of cells. In addition, they are harmful to nerve cells, causing alterations, including lipofuscin deposition, age pigment increase and vacuolar degeneration within the cells (23). The present study found that ginsenoside Rg1 exhibited antioxidant and anti-inflammatory effects in the treatment of isoflurane-induced cognitive impairment in aging rats. Yang *et al* also reported that ginsenoside Rg1 exerts anti-inflammatory and anti-apoptotic properties in rats with ischemia-reperfusion injury (26), and Yu *et al* (9) suggested that ginsenoside Rg1 ameliorates oxidative stress in streptozotocin-induced diabetic rats.

Caspase-3 is the most important protease during apoptosis and is widely used for detecting cell apoptosis (27). Used as an apoptotic detection indicator, caspase-3 has confirmed that sevoflurane and isoflurane exposure in juvenile rats may lead to increased brain cell apoptosis, and that prolonging the duration of anesthesia increases the number of apoptotic cells (28). These findings indicate that there is causal association between the severity of brain cell apoptosis and the duration of drug treatment under these experimental conditions. Of note, ginsenoside Rg1 exerted anti-apoptotic effects and inhibited the expression of caspase-3 in the aging rats exposed to isoflurane anesthesia in the present study. Yang *et al* also showed that ginsenoside Rg1 suppresses the inflammation and level of caspase-3 in neuron cells in rats with cerebral ischemia-reperfusion injury (26).

The PI3K/AKT signal transduction pathway is important for membrane receptor signal transduction into cells, and it reduces cell apoptosis due to ischemia-reperfusion injury in organs, including the heart, kidney and liver (29). GSK-3 is a multifunctional serine/threonine protein kinase involved in cell differentiation, proliferation and apoptosis, with the exception of glycometabolism (30). The PI3K/AKT pathway is involved in regulating the gene expression of myeloid cell leukemia-1 (Mcl-1) in multiple types of tumor and cell line (30). AKT can phosphorylate B cell lymphoma (Bcl)-associated X protein and forms a heterodimer with Bcl-extra large and Mcl-1 in the cytoplasm, leading to decreased mitochondrial membrane translocation and cell apoptosis. GSK-3 β can phosphorylate Mcl-1 to increase the ubiquitination of Mcl-1 and decrease the induction of apoptosis. In addition, AKT affects the stability of Mcl-1 by negatively regulating GSK-3 β to inhibit apoptosis (31). In the present study, ginsenoside Rg1 activated the PI3K/AKT/GSK-3 β pathway in aging rats exposed to

isoflurane anesthesia. Wang *et al* also found that ginsenoside Rg1 regulates innate immune responses via the PI3K/AKT pathway (32), and Song *et al* reported that ginsenoside Rg1 attenuates spatial memory impairment through the GSK3 β signaling pathway in rats (33).

The p53 gene is a reactive gene located upstream and affected by cell DNA damage. If cells are exposed to external injury, the p53 gene is activated, and the protein expression of p53 mediates cell cycle arrest by activating a series of downstream cells, or by immediately combining with single or double stranded DNA to induce self-repair of DNA in cells (34). As the direct downstream mediator of p53, p21WAF1/CIP1 is important in adjusting the cell cycle by combining and restraining cyclin dependent kinase (35). In addition to cell cycle arrest, p21WAF1/CIP1 also inhibits p53 mediation and the apoptosis mediated by p53, and under certain specific conditions, it also promotes cell apoptosis (36). On analyzing the expression and behavior changes of nerve cell apoptosis and p21WAF1/CIP1, p53 protein, the results indicate that it presents negative correlation about the expression and behavior changes of the former two, otherwise, cell apoptosis and p21WAF1/CIP1, p53 protein express more, the injury of cognitive function is more serious (37). In the present study, treatment with ginsenoside Rg1 suppressed the protein expression of p21WAF1/CIP1 in aging rats exposed to isoflurane anesthesia. Zhu *et al* also reported that ginsenoside Rg1 prevented cognitive impairment through the inhibition of p21WAF1/CIP1 in D-galactose-induced aging rat (11).

In conclusion, the present study demonstrated the neuroprotective effects of ginsenoside Rg1 in preventing the cognitive impairment induced by isoflurane anesthesia via antioxidant, anti-inflammatory and anti-apoptotic effects mediated by the PI3K/AKT/GSK-3 β pathway in aging rats.

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