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Research Article

Neuroprotective effects of Korean White ginseng and Red ginseng in an ischemic stroke mouse model

Myungho Jin [a, 1], Kyung-Min Kim [a, 1], Chiyeon Lim [b], Suin Cho [c, *], Young Kyun Kim [a, **]

[a] College of Korean Medicine, Dong-Eui University, Busan, Republic of Korea
[b] Department of Medicine, College of Medicine, Dongguk University, Goyang, Republic of Korea
[c] Department of Korean Medicine, School of Korean Medicine, Pusan National University, Yangsan, Republic of Korea

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ABSTRACT

Background: Stroke is a neurological disorder characterized by brain tissue damage following a decrease in oxygen supply to brain due to blocked blood vessels. Reportedly, 80% of all stroke cases are classified as cerebral infarction, and the incidence rate of this condition increases with age. Herein, we compared the efficacies of Korean White ginseng (WG) and Korean Red Ginseng (RG) extracts (WGex and RGex, respectively) in an ischemic stroke mouse model and confirmed the underlying mechanisms of action.
Methods: Mice were orally administered WGex or RGex 1 h before middle cerebral artery occlusion (MCAO), for 2 h; the size of the infarct area was measured 24 h after MCAO induction. Then, the neurological deficit score was evaluated and the efficacies of the two extracts were compared. Finally, their mechanisms of action were confirmed with tissue staining and protein quantification.
Results: In the MCAO-induced ischemic stroke mouse model, WGex and RGex showed neuroprotective effects in the cortical region, with RGex demonstrating superior efficacy than WGex. Ginsenoside Rg1, a representative indicator substance, was not involved in mediating the effects of WGex and RGex.
Conclusion: WGex and RGex could alleviate the brain injury caused by ischemia/reperfusion, with RGex showing a more potent effect. At 1,000 mg/kg body weight, only RGex reduced cerebral infarction and edema, and both anti-inflammatory and anti-apoptotic pathways were involved in mediating these effects.

1. Introduction

Radix Ginseng, originating from *Panax ginseng*, is considered the most popular herb in traditional Korean medicine and is frequently used worldwide to treat various diseases [1]. Ginseng is used as a general tonic to promote health in several Asian countries owing to its pharmacological properties conferred by ginsenosides [2]. Ginseng extracts contain numerous physiologically active ingredients; ginsenosides, the main ingredients of ginseng, are reported to possess anti-allergic, antioxidant, and immunostimulating properties [3].

In traditional medicine, two traditional Korean ginseng preparations, White ginseng (WG) and Red ginseng (RG), are speculated to possess different biological activities. WG is produced by drying fresh ginseng, and RG is produced by steaming fresh or dried ginseng for a reasonable period, followed by drying until its moisture content is ˂15% [4].

Several researchers have reported that the steaming process increases the contents of bioactive ingredients in ginseng when

Abbreviations: ANOVA, analysis of variance; BSA, bovine serum albumin; CV, cresyl violet; H&E, hematoxylin and eosin; HPLC, high-performance liquid chromatography; iNOS, inducible nitric oxide synthase; IκBα, nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor alpha; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MCA, middle cerebral artery; Mn-SOD, manganese superoxide dismutase; NDS, neurological deficit scores; PBS, phosphate-buffered saline; rCBF, relative cerebral blood flow; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; tMCAO, transient middle cerebral artery occlusion; TTC, 2,3,5-triphenyl-tetrazolium chloride.

* Corresponding author. School of Korean Medicine, Yangsan Campus of Pusan National University, Yangsan, 50612, Republic of Korea.
** Corresponding author. College of Korean Medicine, Dong-Eui University, Busan, 47227, Republic of Korea.
E-mail addresses: sicho@pusan.ac.kr (S. Cho), lab3402201@gmail.com (Y.K. Kim).
[1] These authors contributed equally to this study.

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compared with those of non-steamed samples [4–6]. However, comparative studies evaluating the effects of WG and RG in animal models of stroke are limited.

Globally, cerebrovascular disorders (CVDs) remain a leading cause of death and have been attributed to abnormalities in the heart and blood vessels. Among CVDs, acute ischemic stroke is considered the leading cause of morbidity and mortality in modern society [7–9]. Ischemic stroke can be characterized by arterial embolic or thrombotic occlusion, resulting in brain inflammation and cell death. Notably, white blood cells, delivered via the blood, infiltrate the brain to activate inflammation, and then cell death signals are activated via the permeability of the outer mitochondrial membrane [10,11]. Thrombolytic drugs such as anticoagulants and antiplatelets are used as therapeutic agents to treat ischemic stroke. As this condition can result in morbidity and disability and impose a heavy burden on patients by increasing medical expenses and necessitating rehabilitation and long-term care [12,13], further in-depth investigations on the prevention and treatment of ischemic stroke are urgently needed [14,15].

Although several studies have reported [16–18] that ginseng or its constituents can potentially inhibit brain damage, its effects, as well as mechanisms of action, in animal models of cerebral infarction have rarely been reported. Herein, we investigated the differences in and mechanisms underlying the protective effects of WG and RG on cerebral injury induced by ischemia/reperfusion of middle cerebral artery (MCA) blood flow.

2. Materials and methods

2.1. Animals

Six-week-old C57BL/6 male mice (Samtako Bio, Osan, Korea), weighing 20–22 g, were housed in cages at 24 ± 4°C under a 12 h light/dark cycle. The mice were fed a standard pellet diet and provided water *ad libitum*. All experimental procedures performed in the present study were approved (approval no. PNU 2019–2485) by the Ethics Committee for Animal Care and Use at Pusan National University, certified by the Korean Association of Laboratory Animal Care.

2.2. Reagents

Cresyl violet (CV) and 2,3,5-triphenyl-tetrazolium chloride (TTC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol, phosphate-buffered saline (PBS), and saline were purchased from JW Pharmaceutical Co., Ltd. (Seoul, Korea). Optimal cutting temperature (OCT) compound was obtained from Thermo Fisher Scientific (Waltham, MA, USA). The protein extraction solution was procured from iNtRON (Seongnam, Gyeonggi-do, Korea). Primary antibodies against rabbit anti-mouse phospho-JNK (p-JNK), JNK, p-IκBα, IκBα, Mn-SOD, and β-actin were obtained from Cell Signaling Technology (Danvers, MA, USA). The goat anti-rabbit IgG and pAb secondary antibodies were purchased from Enzo Life Sciences, Inc. (Farmingdale, NY, USA). The BCA reagent, bovine serum albumin (BSA) standard, and enhanced chemiluminescence (ECL) western blotting chemiluminescent substrate were obtained from Thermo Fisher Scientific.

2.3. Preparation and high-pressure liquid chromatography (HPLC) analysis of WG and RG extracts

Standardized WG and RG, manufactured from a 5-year-old ginseng sample, were obtained from Gwangmyung Natural Pharmaceutical Co. (Busan, Korea), and voucher specimens (No. 202KWG and 202KRG) were deposited at the Herbarium of Pusan National University. For the preparation of both WG and RG extracts, 200 g of each sample was roughly ground and extracted with 10 volumes of methanol at 25°C for 24 h; this step was repeated thrice. After filtration, methanol was removed using a vacuum evaporator (Eyela, Japan) at 45°C, and the filtrate was stored at −20°C until further use. The final lyophilized WG extract (WGex) and RG extract (RGex) from WG and RG weighed 24.3 g and 25.2 g, yielding 12.2% and 12.6%, respectively.

For confirming the quality of WGex and RGex, each extract was subjected to HPLC analysis. To obtain a fingerprint of the extracts used in this experiment, we determined the presence of ginsenoside Rg1, a component of ginseng, using an HPLC system (Shimadzu, Kyoto, Japan) equipped with an LC-20AD pump, a SIL-20A sampler, an SPD-M20A detector, and a CTO-20A column oven. The samples were separated using a YMC Triart C18 column. Details of the mobile phase and elution system are listed in Supplementary Table 1 (Table S1).

2.4. Induction of transient MCAO (tMCAO)

In the present study, a *tMCAO* model was established using the modified Koizumi's method; detailed protocols and materials have been previously reported [19]. Briefly, inhalation anesthesia was induced with 1.5% isoflurane in N_2O/O_2 (70%/30%), and mice were then placed on a heating pad (Harvard Instruments, Boston, MA, USA). During surgery, the body temperature of mice was monitored using a rectal temperature probe and maintained at 37 ± 1°C. The left side of the skull was exposed, and an optic fiber was attached to the skull to monitor relative cerebral blood flow (rCBF). A midline incision was made on the neck, and soft tissues over the trachea were gently retracted. The external carotid artery (ECA) and common carotid artery (CCA) were knotted with 4–0 suture silk sutures. The ECA and CCA were ligated before clamping the internal carotid artery. Then, an 8–0 monofilament suture line (Ethicon, CA, USA), coated with silicon, was inserted into the MCA through the perforated CCA. For induction of tMCAO, the monofilament was retracted after the predetermined occlusion time was completed (2 h tMCAO). The incision region was sutured and disinfected to complete the surgical procedure. All surgeries were performed using laser Doppler flowmetry (Moor Instruments Ltd., Devon, UK) to detect rCBF and confirm successful occlusion and reperfusion.

2.5. Pretreatment with WGex and RGex

Mice were randomly divided into eight groups as follows: 100, 300, and 1000 mg/kg WGex-treated; 100, 300, and 1000 RGex-treated; PBS-treated control; sham-operated (sham) group. Each group consisted of 15 mice (Supplementary Fig. 1 and Fig. S1). In Korean medicine, 30 mg/kg is the recommended daily dose of WG and RG extracts in humans; in mice, the equivalent dose was 400 mg/kg. Thus, the dose ranged from 100-1000 mg/kg. For each WGex and RGex concentration prepared in PBS, the solution was orally administered using an oral zonde 1 h before MCAO induction; sham and control mice were orally administered the same volume of PBS. After 24 h of MCAO induction, the mice were sacrificed to perform several assays. A schematic of the experimental protocol is presented in Fig. 1.

2.6. Infarct and edema area measurements

The mice were anesthetized using CO_2 respiration, the brains were harvested to measure the total infarct volume, and the olfactory bulb and cerebellum were isolated. Serial coronal sections (1 mm thick) were stained with a 2% TTC solution to assess infarct

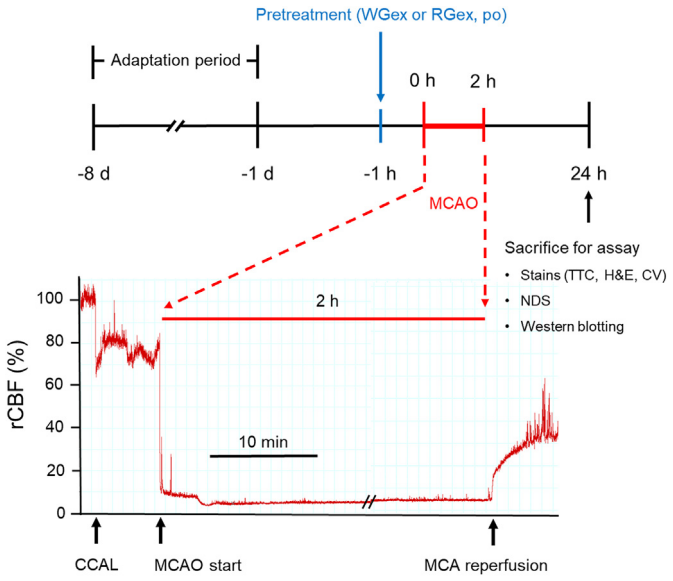

Fig. 1. Experimental design to induce a 2 h transient middle cerebral artery occlusion (tMCAO) model. Ischemic stroke model in mice. Mice were acclimatized for seven days at animal facilities. Pretreatment (*per os*, po) was performed to compare the efficacies of ginseng extracts (WGEX and RGex). CCAL, common carotid artery ligation.

volume. Finally, ischemic and non-infarct tissues were separated using an image analysis system (Digimizer, Ostend, Belgium).

Areas of brain edema were calculated by dividing the total infarction volumes in the ipsilateral hemispheres by brain edema indices, using TTC-stained brain sections. The formula used to calculate brain edema indices was as follows:

Brain edema index = total volume of the ipsilateral hemisphere/total volume of the contralateral hemisphere

2.7. Neurological deficit score (NDS) measurement

Post-MCAO induction, behavioral changes were measured using a 5-scale assessment; the score was defined as follows: Score 0, mice presenting behavior similar to that of normal mice; Score 1, spontaneous exercise could be performed, but behavioral responses were slow; Score 2, reduced body temperature and weight, with mice moving toward the contralateral direction; Score 3, reduced body temperature and weight, with mice demonstrating sensitivity to mechanical stimuli, including pain, walking, or circling in one direction; Score 4, reduced body temperature and weight, with mice failing to exhibit spontaneous behavior or demonstrating extreme sensitivity to mechanical stimuli.

2.8. Cardiac perfusion for brain harvesting

We next performed cardiac perfusion using PBS. Accordingly, the left ventricle was pierced with a needle, and the needle was fixed in the ascending aorta. Immediately after perfusion, the right atrium was incised using scissors. PBS and a 4% paraformaldehyde (PFA) solution were used for perfusion and fixation, respectively. After fixation, the brain was soaked in 10% PFA with sucrose, at 4°C, for 3 days.

2.9. Frozen sections of mice brain

Mice brains were sequentially placed in 10-30% sucrose solutions before freezing (in OCT compound) and then stored at −80°C. For each group, brain sections were obtained using a cryostat (Leica, Wetzlar, Germany) at a thickness of 30 μm; the sections were placed on glass slides for 12 h and stored at −80°C until use.

2.10. Hematoxylin and eosin (H&E) staining

Brain sections placed on slides were allowed to dry on a slide warmer; then, the slides were dipped into 80% ethanol. The slides were immersed in a hematoxylin solution for 5 min, followed by gentle washing with distilled water. Next, the slides were soaked in acid alcohol (1%) and immersed in a lithium carbonate solution. Sections were then washed, and the slides were dipped in eosin solution for 30 s. The slides were immersed in graded ethanol concentrations (95% and 100% sequentially) for 1 min, placed in xylene, mounted, and then observed under a light microscope. The density of H&E-positive cells was measured in the cortical regions of mice brains using the ImageJ program (NIH, MD, USA).

2.11. Nissl staining

The sections on glass slides, obtained from each group, were allowed to dry on a slide warmer. The slides were then immersed in an alcohol/chloroform solution for 30 min. Next, the slides were incubated in a 0.1% CV solution for 10 min at 40°C in an incubator. Then, the slides were quickly washed with distilled water, placed in 95% ethanol for 5 min, sealed with a coverslip and mounting solution, and then observed under a light microscope. The density of neuronal cells in the cortical region of the mouse brain was measured using ImageJ.

2.12. Western blot analysis

Proteins in the ipsilateral hemisphere of the brain were isolated using a protein extraction solution. Brain tissue lysates were obtained by centrifugation at 15,871 × *g* and 4°C for 10 min. Total protein levels in the supernatant were quantified using the BSA method, and the quantified protein was used for western blot analysis. In brief, 30 μg protein was separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes. Following protein transfer, the membranes were blocked with 5% skim milk in TBST buffer (mixture of Tris-buffered saline and 0.1% Tween 20) for 1 h, at 25°C, and then incubated at 4°C with primary antibodies against iNOS, p-IkBα, IkBα, p-JNK, JNK, Mn-SOD, and β-actin. After incubation for 12 h, the goat anti-rabbit IgG, pAb, and goat anti-mouse IgG pAb secondary antibodies were added for 1 h at 25°C. The membranes were then treated with an ECL kit, and the expression levels of proteins were detected using a luminescent analyzer system (Amersham™ Imager 600, Buckinghamshire, UK). The densities of all detected bands were analyzed using ImageJ.

2.13. Statistical analysis

Data were analyzed using analysis of variance (ANOVA), followed by the Holm-Sidak test, to determine the significance of differences among groups. Data analyses were performed using Sigmaplot 12.0 (Systat Software Inc., CA, USA). Data values are expressed as mean ± standard deviation (SD), and a value of $p < 0.05$ was considered significant.

3. Results

3.1. Analytical comparisons of WGex and RGex and their standard, ginsenoside Rg1

The structure of Rg1 is shown in Supplementary Fig. 1A (Fig. S1A), and the presence of Rg1 in WGex and RGex was confirmed with HPLC (Fig. S1B, S1C). Following quantitative measurement, a three-fold higher concentration of Rg1 was detected in WGex than in RGex (Table 1).

3.2. Measurement of total infarct volume, brain edema area, and NDS

Pretreatment with WGex and RGex was performed 1 h before the induction of ischemic stroke, to select the effective dose. Single doses of 100, 300, and 1,000 mg/kg/day were administered (treatment on the day of MCAO induction; representative images of TTC-stained brain slices are shown in Fig. 2A). Among these, the most effective dosage of RGex was 1,000 mg/kg body weight (bw) (Fig. 2A). Although WGex had no effect on the infarct volume within the investigated dose range, the infarct volume tended to decrease in a dose-dependent manner (Fig. 2A). Following pretreatment with 1,000 mg/kg bw RGex, the percentage of the infarct volume in the ipsilateral brain hemisphere was 41.40 ± 5.28% and that in the MCAO control group was 56.67 ± 10.05%.

Brain edema was reduced in the RGex groups pretreated with 300 and 1,000 mg/kg/day bw (16.73 ± 3.39 mm^3 and 18.60 ± 1.82 mm^3, respectively) when compared with that in the MCAO control group (28.92 ± 6.85 mm^3) (Fig. 2B).

Regarding NDS, no significant differences were observed among the MCAO-induced groups (Fig. 2C); however, pretreatment with WGex and RGex (1,000 mg/kg bw) resulted in lower scores than those in the low-dose treatment groups.

We assessed rCBF during MCAO induction and reperfusion using laser Doppler flowmetry. Assuming that the entire blood flow before CCA ligation was 100%, CCA occlusion was found to reduce rCBF to approximately 59 ± 6% of baseline levels. Following MCAO induction, rCBF values decreased to approximately 10 ± 8%, and no significant difference was observed among groups (Supplementary Fig. 3, Fig. S3).

3.3. Morphological changes in neuronal cells

In the sham group, H&E-stained neuronal cells were intact and showed morphologically well-arranged cytoplasm and nucleus. The MCAO group showed aberrant morphology, displaying an H&E-negative area. Animal groups pretreated with ginseng extracts presented marginal recovery of neuronal damage (WGex, 74.33 ± 5.86%; RGex, 79.67% ± 5.03%), demonstrating a similar cellular structure to that observed in the sham group; however, upon comparing the H&E color intensity between groups, the groups treated with ginseng extracts showed no significant change when compared with the MCAO control group (Fig. 3A).

Table 1
Quantitative measurement of the standard compound, Ginsenoside Rg1, in Ginseng extracts

Extract	λ_{max} (nm)	t_R (min)	r^2	Amount of ginsenoside Rg1 (ppm)
WGex	203	14.86	0.9982	145.03
RGex				48.71

WGex, methanolic extract of white ginseng; RGex, methanolic extract of red ginseng.

Fig. 3B shows the cell density in the CV-stained ipsilateral brain hemispheres of all groups. The cell density of the sham group was 100.00 ± 5.00%, while that of the MCAO control group was 65.33 ± 7.02%. Following pretreatment of MCAO-induced mice with 1,000 mg/kg bw of WGex or RGex, the cell densities were 81.67 ± 3.06% and 85.33 ± 5.51%, respectively (Fig. 3B). The cell densities in mice brains pretreated with ginseng extracts were significantly higher than those in the control group (Fig. 3B). The MCAO control group showed cellular damage, with shrinkage and pyknotic nuclei (red arrowheads, Fig. 4B), whereas the ginseng extract-treated groups showed neuronal cell recovery (arrows, normal neurons; black arrowheads, glial cells). Overall, the ipsilateral cell density of all ginseng-treated groups was significantly recovered (Fig. 4C and D).

3.4. Expression of cell death-related proteins in the ipsilateral cerebral cortex

Reportedly, iNOS (inducible nitric oxide synthase) promotes inflammation and exerts synergistic effects with other inflammatory mediators. Post-ischemia, the increased iNOS protein levels induce NO production and DNA damage; thus, inhibiting iNOS activity could be a key target for regulating inflammatory responses in the ischemic brain [20–22]. In the current study, pretreatment with 1,000 mg/kg bw RGex decreased (1.44 ± 0.18-fold) the MCAO-induced increase in iNOS protein expression following brain damage (2.16 ± 0.44-fold) (Fig. 5A).

Mitogen-activated protein kinase (MAPK), an important intracellular signal-mediating molecule, is involved in various pathways of cellular activities such as proliferation, differentiation, death, and deformation [23–25]; as a downstream signal of MAPK, Jun N-terminal kinases (JNKs) activate the apoptotic pathways by upregulating pro-apoptotic genes [25]. Increased JNK expression in the MCAO control group was inhibited by the administration of both WGex and RGex extracts. The ratio of p-JNK/JNK expression was 1.00 ± 0.15 in the sham group, 10.06 ± 3.49 in the MCAO control group, and 5.82 ± 1.26 and 4.22 ± 1.47 in the WGex- and RGex-pretreated groups, respectively (Fig. 5B).

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) participates in inflammasome regulation; this protein is present in the cytoplasm along with regulatory proteins called inhibitors of κB (IkBs). IkB proteins are phosphorylated and subsequently degraded in the proteasome, and degradation of IkBs allows translocation of NF-κB into the nucleus, thus promoting inflammatory responses [26,27]. The increased expression of p-IkBα in the MCAO control group was inhibited following RGex administration. The ratio of p-IkBα/IkBα expression was 1.00 ± 0.26 in the sham group, 2.06 ± 0.36 in the MCAO control group, and 1.48 ± 0.23 in the RGex-treated group (Fig. 5C).

Manganese superoxide dismutase (Mn-SOD) is specifically localized in the inner membrane of mitochondria and is related to antioxidant pathways [28–30]; it shows neuroprotective effects against oxidative stress [31,32], thus presenting a potential target for determining herbal agents with activity against ischemic brain injury. In the present study, MCAO significantly increased the protein expression of Mn-SOD, while pretreatment with 1,000 mg/kg bw RGex significantly inhibited this increase in Mn-SOD expression. The expression values were 1.00 ± 0.13 in the sham group, 1.54 ± 0.11 in the MCAO control group, and 1.08 ± 0.31 in the RGex pretreated group (Fig. 5D).

4. Discussion

P. ginseng, also known as Korean ginseng, is a well-known functional health food used for vitalizing energy and eliminating

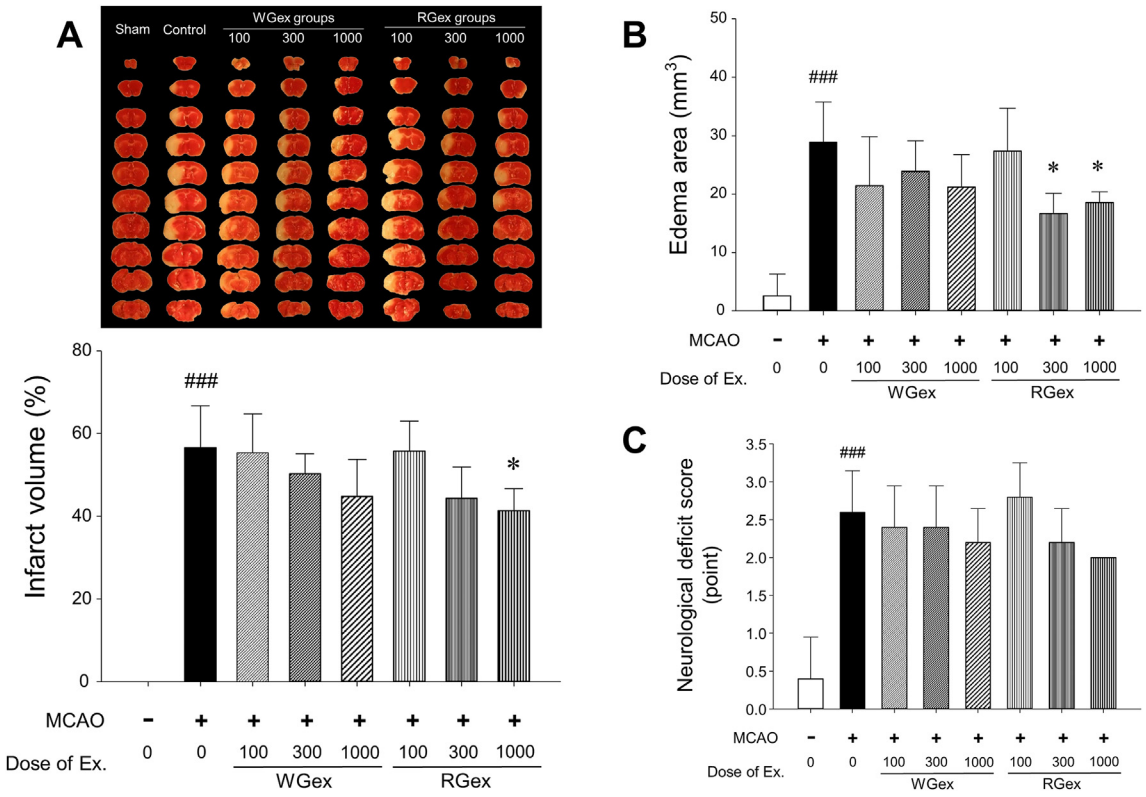

Fig. 2. Measurement of the infarct volume, edema area, and neurological deficit score (NDS). A, Representative photographs of TTC-stained brain slices (1 mm) showing the infarct area 24 h after MCAO induction (upper column) and quantitative analysis of the total infarct volume (lower column). B, Quantitative analysis of the edema area, calculated by dividing total infarct volumes in ipsilateral hemispheres by brain edema indices, using TTC-stained brain slices. C, Quantitative analysis of NDS. All data are expressed as mean ± standard deviation (SD) (n = 5). ### $p < 0.001$ *vs.* sham group and * $p < 0.05$ *vs.* MCAO control group. MCAO, middle cerebral artery occlusion; TTC, 2,3,5-triphenyltetrazolium chloride.

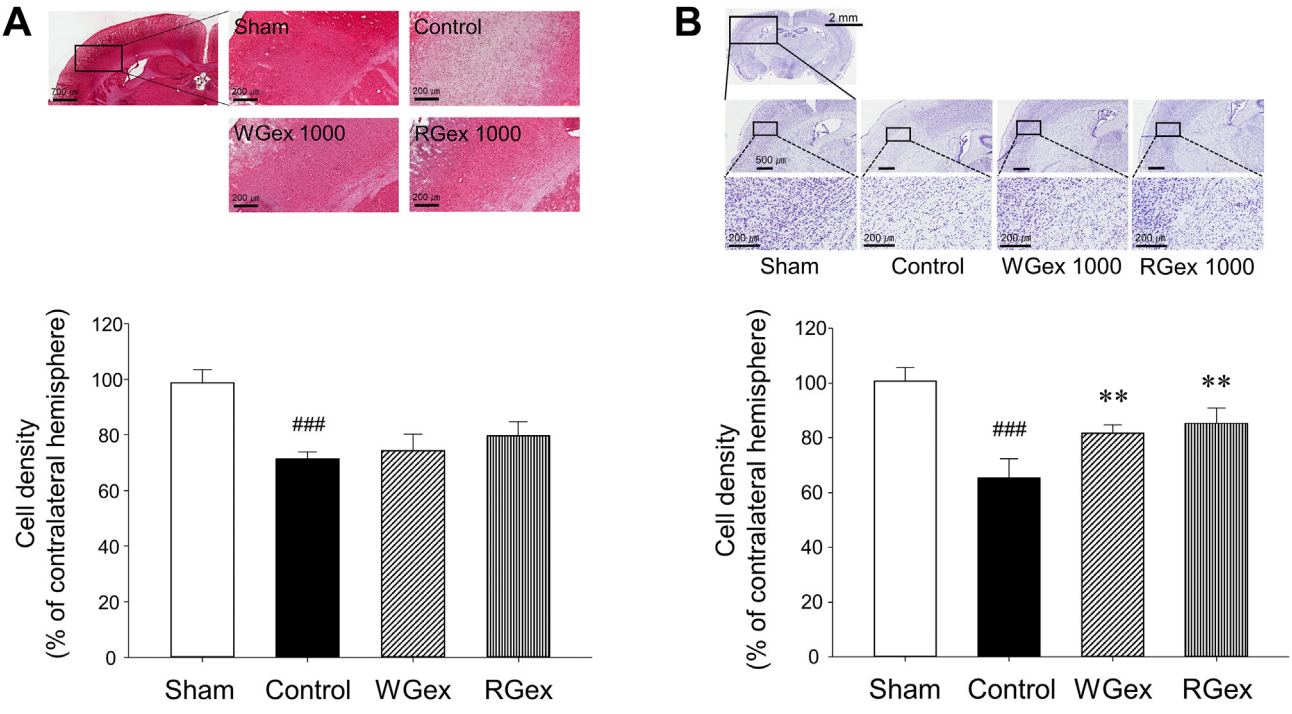

Fig. 3. Neuroprotective effects of ginseng extracts against MCAO-induced cell death. A, Representative photomicrographs of H&E-stained sections (upper column) and quantitative analysis showing changes in the number of cells (lower column). B, Representative photomicrographs of CV-stained sections (upper column) and quantitative analysis showing changes in the number of cells (lower column). All data are expressed as mean ± standard deviation (SD) (n = 5). ### $p < 0.001$ *vs.* sham group and ** $p < 0.01$ *vs.* MCAO control group. CV, cresyl violet.

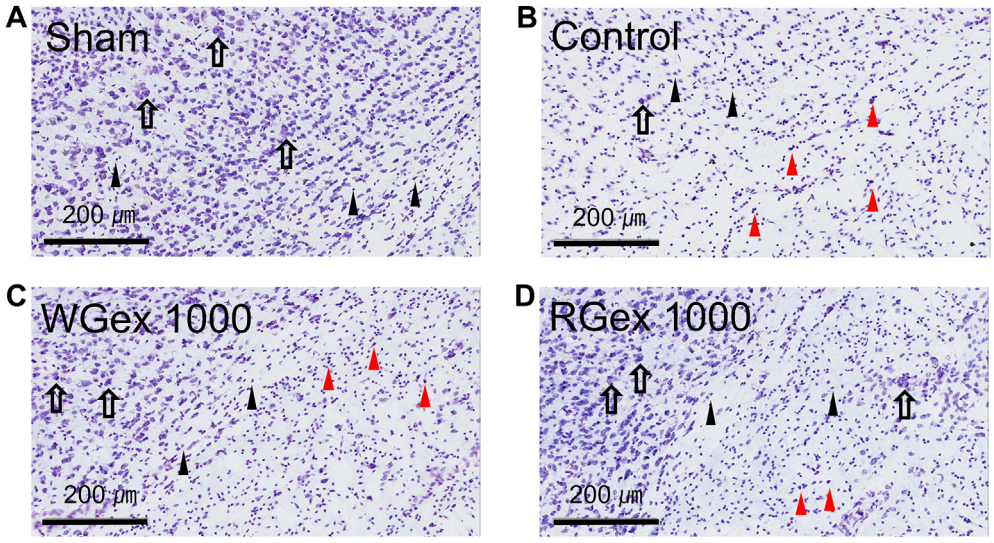

Fig. 4. Morphological changes in the cortical regions of ischemic ipsilateral hemispheres. Each photomicrograph represents CV-stained cortical regions. Arrows indicate normal neurons, black arrowheads represent glial cells in the resting state, and red arrowheads represent activated glial cells. Scale bars, 200 μm.

chronic fatigue while improving health. In several Asian countries, it has been employed as a dietary supplement for over 2000 years [33]. RG is obtained by steaming and drying WG [4], during which the chemical composition of ginsenosides is altered and various bioactive compounds are produced that can potentially induce unique physiological activities *in vivo* [33]. More than 30 ginsenosides from *P. ginseng* have been isolated and characterized, and these ginsenosides are known to possess diverse pharmacological effects [33]. However, comparative studies on the effects of WG and RG in animal models have not been extensively reported in terms of pharmaceutical differences. Using network pharmacology research tools, we recently reported that WG and RG could be efficacious in ischemic brain diseases [18]; however, comparative studies on their efficacy and underlying mechanisms remain scarce in ischemic stroke animal models.

Stroke is considered the second leading cause of death, with ischemic stroke being the most common type. Ischemic stroke is a major neurological disorder that causes physical and psychological disability [7–9]. Patients suffering from post-ischemic cerebral dysfunction should be prescribed suitable therapeutic agents to counteract ongoing risk factors. Moreover, it is imperative to develop a therapeutic agent to prevent neuronal cell death, excitotoxicity, and oxidative stress, all of which are known pathways that lead to brain cell damage [34]. Ischemic stroke is primarily attributed to metabolic diseases, such as atherosclerosis, and its pathogenesis involves oxidative stress, inflammatory response, apoptosis, and autophagy [7–9]. Given the various pathogenic processes involved in ischemic stroke, multicomponent and multitarget agents, including traditional herbal medicine, can be effectively employed to treat ischemic stroke at various stages.

Several studies have reported that treatment with ginseng or ginsenosides improves general symptoms, physical exercise capacity, and fluid metabolism in patients with coronary and myocardial ischemia and reperfusion diseases [35,36]. Zheng et al reported that total ginseng saponins improve neurological function deficits following focal cerebral ischemia, by inducing endogenous neural stem cell activation and enhancing central nervous system regeneration in an MCAO rat model [37]. Ban et al demonstrated that Korean RG extract exerts a neuroprotective effect in rats with ischemia/reperfusion-induced brain injury by reducing lipid peroxidation and enhancing the endogenous antioxidant enzymatic activity [38]. Recently, Liu et al reported that ginseng pretreatment affords protection against acute sensorimotor deficits, thereby promoting long-term recovery after proximal-distal MCAO in mice via activation of nuclear factor erythroid 2-related factor 2 (Nrf2) [39]. These lines of evidence indicate that WG, RG, and ginsenosides protect the brain from ischemic brain injury; however, no previous study has compared the effects of WG and RG using an animal model of ischemic stroke. Thus, in the present study, the MCAO mouse model was used to compare the distinct effects and measure the contents of Rg1, a representative component of ginseng, between WGex and RGex to determine whether the differences in their efficacies are Rg1 dependent.

We determined the effects of WGex and RGex after 2 h of MCAO in mice. Pretreatment with 1,000 mg/kg bw of RGex significantly reduced the infarct volume, but WGex showed no significant change in the dose range investigated (Fig. 2A). Following pretreatment with WGex or RGex, the infarct volumes were reduced in a concentration-dependent manner. Although a high concentration of WGex elicited a response, the effect of RGex at the same concentration was superior to that of WGex. Upon measuring the content of Rg1 (Table 1), we found that WGex contained three times more Rg1 content than RGex; this finding indicated that Rg1 is not involved in the neuroprotective effect of RGex in the MCAO mouse model. A previous study has shown that total ginsenosides protect the brain from ischemic injury [37]; however, in the present study, specific ginsenosides were found to play a critical role in the unique pharmacological effects. The pharmacological activities of these individual constituents need to be further investigated. RGex pretreatment suppressed brain edema, but did not affect NDS changes; conversely, WGex did not affect brain edema, NDS, and rCBF changes (Fig. 2B and C, and Fig. S3).

To evaluate the neuroprotective effects of WGex and RGex in the cortical regions of brain tissues, we used H&E– or CV-stained frozen sections. In tissues stained with H&E, the effects of WGex and RGex could not be confirmed (Fig. 3A); however, CV staining revealed that both WGex and RGex exerted significant neuroprotective effects in ischemic ipsilateral cortical neuronal cells (Fig. 3B). These results can be attributed to the characteristic of CV, which is capable of explicitly staining neuronal cells; on magnification, it was observed that RGex significantly inhibited cellular changes related to the inflammatory response (Fig. 4).

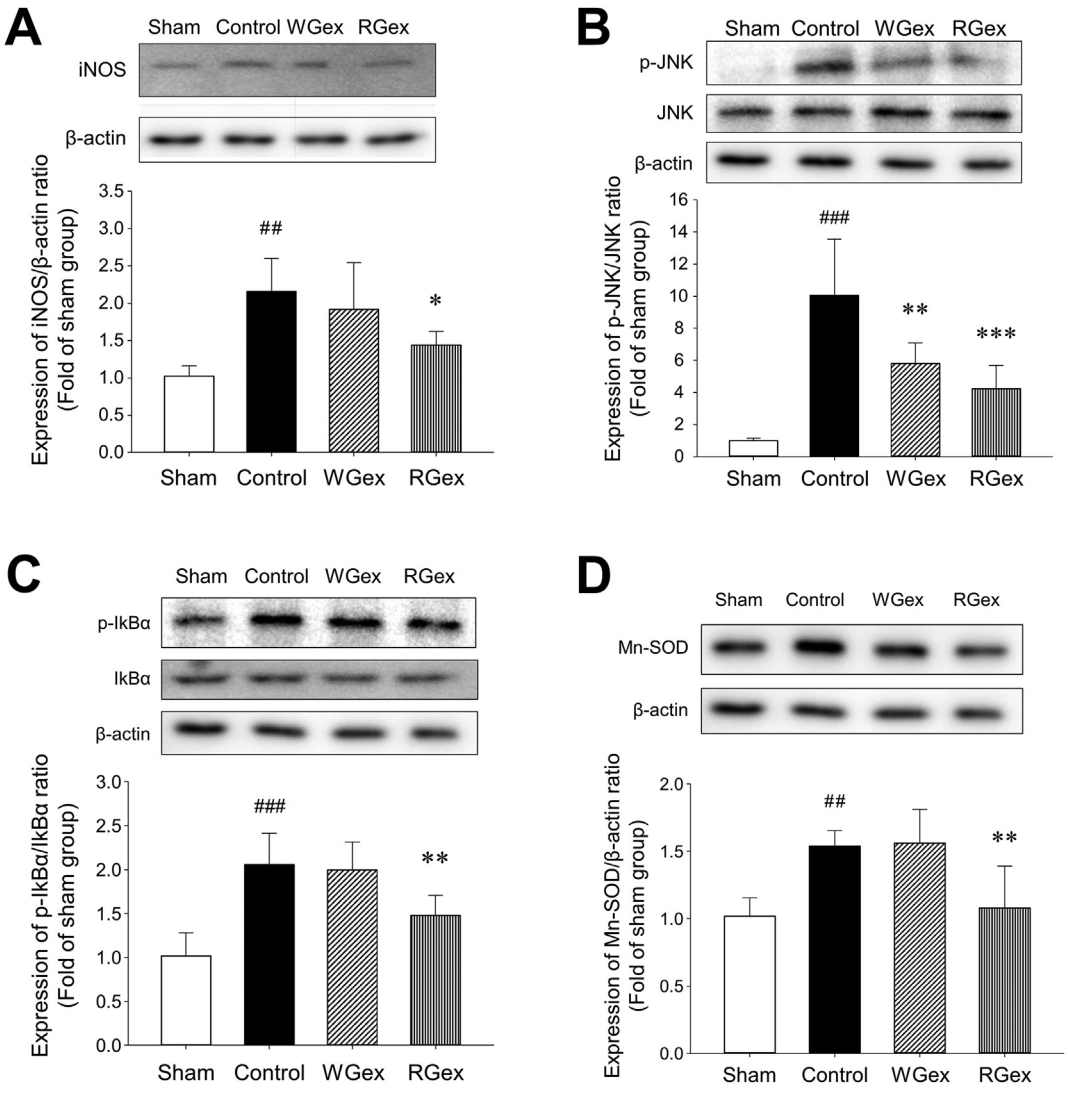

Fig. 5. Effects of pretreatment with WGex and RGex on the iNOS, p-JNK, p-IkBα, and Mn-SOD proteins in MCAO-induced mice brains. Representative images (upper column of A, B, C, and D) and relative densitometry (lower column of A, B, C, and D) of western blot analysis of the expression level of each protein and β-actin in brain tissues (n = 5 per group). All data are expressed as mean ± standard deviation (SD). ## $p < 0.01$ *vs.* sham group, ### $p < 0.001$ *vs.* sham group, * $p < 0.05$ *vs.* MCAO control group, ** $p < 0.01$, and *** $p < 0.001$ *vs.* MCAO control group.

iNOS plays a critical role in inflammation and apoptosis, and MAPK is an intracellular signaling molecule involved in cell migration, proliferation, and differentiation. Moreover, the MAPK pathway plays an essential role in the induction of iNOS expression [40–42]. JNK activation is associated with growth factor-mediated pathways [25]. In the present study, the expression of JNK was increased following MCAO induction, and pretreatment with both WGex and RGex reduced the expression of JNK (Fig. 5B), whereas the expression of iNOS was regulated by RGex pretreatment (Fig. 5A). Overall, MCAO promoted the iNOS and MAPK pathways, and pretreatment with 1,000 mg/kg bw RGex significantly altered their expression levels. Phosphorylated IκB proteins allow the translocation of NF-κB into the nucleus, thereby promoting inflammatory responses [26,27]. The increased expression of p-IkBα in the MCAO control group was inhibited by RGex administration (Fig. 5C). Mn-SOD is an important target for identifying neuroprotective agents with potential efficacy against ischemic brain injury [31]. Herein, MCAO significantly increased the protein expression of Mn-SOD, and pretreatment with 1,000 mg/kg bw/day RGex significantly inhibited this increase (Fig. 5D), indicating that antioxidative signals were activated by MCAO induction, and RGex reduced cerebral damage, thus inhibiting Mn-SOD protein expression.

In summary, both WGex and RGex protected neuronal cells in the MCAO mouse model, but the efficacy of RGex was superior to that of WGex, and ginsenoside Rg1 was not involved in the efficacy of RGex. Mice treated with 1,000 mg/kg bw of RGex showed reduced neuronal cell death in the MCAO-induced mice brains.

5. Conclusions

Both WGex and RGex exerted neuroprotective effects in the cortical regions of the MCAO-induced ischemic stroke mice; however, the overall efficacy of RGex was higher than that of WGex. Pretreatment with 1,000 mg/kg bw RGex significantly reduced the total infarct volume and edema area; these effects were not observed with WGex. The effects of WGex and RGex were found to be independent of Rg1. The protective effect of RGex in the MCAO mouse model was related to the anti-inflammatory and anti-apoptotic pathways.

Author contributions

Conceptualization: SC and YKK. Methodology: MJ and SC. Investigation: MJ, KMK, and SC. Data analysis: KMK and CL. Writing-Original Draft: MJ and SC. Writing-review and editing: KMK and YKK. Supervision: KMK, SC, and YKK. All authors read and approved the final version of the manuscript.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2021.06.012.

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