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Ginsenoside-Mc1 reduces cerebral ischemia-reperfusion injury in hyperlipidemia through mitochondrial improvement and attenuation of oxidative/endoplasmic reticulum stress

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Abstract: The neuroprotective effect of ginsenoside-Mc1 (GMc1) in hyperlipidemic rats in the setting of cerebral ischemia-reperfusion injury (I/RI), as well as the role of mitochondrial ATP-sensitive potassium (mitoKATP) channels and oxidative/endoplasmic reticulum (ER) stress, was investigated. Hyperlipidemia (8 weeks) was induced by a high-fat diet in Sprague Dawley rats. GMc1 (10 mg/kg, i.p.) was given to hyperlipidemic rats daily for one month before I/RI. Rat brains were subjected to 2 h of local ischemia followed by 24 h reperfusion. The cerebral infarcted injury was measured by triphenyltetrazolium chloride staining and the levels of oxidative stress indicators were detected by ELISA and spectrophotometry. A fluorometric technique was employed to evaluate mitochondrial function. Western blotting was used to detect changes in the expression of ER stress proteins. GMc1 reduced cerebral infarct volume in hyperlipidemic rats in comparison to untreated ones ($P<0.01$). GMc1 reduced cerebral infarct volume in hyperlipidemic rats as compared to untreated rats ($P<0.01$). GMc1 significantly decreased mitochondrial membrane depolarization, mitochondrial reactive oxygen species (mitoROS) and malondialdehyde levels ($P<0.01$), while increasing the activity of superoxide dismutase (SOD), catalase (CAT) and glutathione-peroxidase (GPx) ($P<0.001$). GMc1 administration reduced the expression of ER stress markers, including phosphorylated (p)-endoplasmic reticulum kinase (PERK), p-eukaryotic translation initiation factor 2 subunit 1 (elF2α), and C/EBP homologous protein (CHOP). Inhibition of mitoKATP channels with hydroxydecanoate significantly eliminated the protective impacts of GMc1 in hyperlipidemic rats subjected to cerebral I/RI. The neuroprotective effect of GMc1 preconditioning was remarkably improved by increasing mitoKATP channel activity and decreasing oxidative and ER stress levels in hyperlipidemic rats, implying that this compound could be an appropriate candidate for reducing cerebral I/RI in comorbidities.

Keywords: ginsenoside-Mc1; ATP-sensitive potassium channel; endoplasmic reticulum stress; hyperlipidemia; ischemia-reperfusion injury

INTRODUCTION

Stroke, a life-threatening neurological disorder, is one of the leading causes of death globally. It is caused by the rupture of blood vessels or thrombosis and can result in the sudden death of brain cells due to the lack of oxygen and other nutrients [1]. Restoration of the blood supply to the ischemic zones (reperfusion) is the desired target therapy for stroke management [2]. However, when blood flow in the central nervous system is restored, the injury becomes more severe, leading to the development of cerebral ischemia-reperfusion injury (I/RI). Cerebral I/RI involves many complex pathological processes such as oxidative stress, inflammation, amino acid toxicity and release of endogenous substances and ultimately leads to apoptotic death of nerve cells [3]. Hyperlipidemia, also known as high cholesterolemia, is characterized by excessive amounts of lipids in the blood, which significantly raises the risk of stroke [4]. Hyperlipidemia has been shown to increase ischemic damage through endothelial cell destruction, oxidative stress, inflammation and loss of nervous system function [5]. Therefore, protecting nerve cells from I/RI in hyperlipidemic conditions is critical.

Increased oxidative stress is known as one of the pathophysiological mechanisms of hyperlipidemia-related complications in obesity that causes enhanced cerebral infarction and mortality [5]. More importantly, reactive oxygen species (ROS) produced during the ischemic and reperfusion phases of stroke play crucial roles in its pathogenesis and can assault brain cells and intracellular organelles [6]. As a result, reducing oxidative stress may be a promising route for protecting neural cells from I/RI in hyperlipidemic conditions. I/RI-triggered production of free radicals and ROS leads to the opening of mitochondrial permeability transition pores (mPTPs) and malfunctioning of mitochondrial ATP-sensitive potassium ($mitoK_{ATP}$) channels [7,8]. This condition in hyperlipidemic subjects, whose physiological ATP synthesis is also impaired, can worsen I/RI and prevent neural protection [9]. It has been demonstrated that the brain possesses more $mitoK_{ATP}$ channels than do other vital organs such as the heart and liver [10], and that the activation of these channels has protective outcomes on the brain under I/RI [8]. Therefore, maintaining the activity of $mitoK_{ATP}$ channels and achieving ionic balance at a physiologic level is essential in hyperlipidemic circumstances.

When the $mitoK_{ATP}$ channels are closed, not only is the mitochondrial redox function disrupted, producing more free radicals but so is the mitochondrial interaction with other cellular organelles such as the endoplasmic reticulum (ER), leading to organelle dysfunction [7,8]. Mitochondrial dysfunction caused by the inactivation of the $mitoK_{ATP}$ channel indirectly activates the proteins and mediators involved in ER stress by disrupting cellular homeostasis. In addition, ER dysfunction in the setting of cerebral ischemia leads to the accumulation of unfolded proteins in the ER lumen, a state known as ER stress [7,11]. Consequently, ER stress stimulates a variety of nerve cell death pathways that are critical in the pathophysiology of stroke [12]. Interestingly, a higher incidence of ER stress also induces aberrant lipid metabolism and plays a key role in the pathogenesis of hyperlipidemia, highlighting the importance of preventing mitochondrial-ER physiological interaction failure [13].

Ginseng has been used as a traditional herbal medicine in Asia for thousands of years. Ginseng has been shown to be beneficial in patients suffering from diabetes, stroke and cardiovascular diseases [14]. Ginsenosides are the principal components of ginseng extract, and they have antioxidant, anticancer, anti-inflammation and physiological properties [15]. Previous research has shown that ginsenosides have neuroprotective benefits against I/RI through alleviating mitochondrial dysfunction [16,17]. Ginsenoside compound Mc1 (GMc1), a newly identified deglycosylated ginsenoside [18], is more pharmacologically active than other glycosylated ginsenosides [19]. Previous studies have shown that ginsenosides also affect ER stress in a variety of metabolic organs, including adipose tissue, heart and brain [14,20,21]. It was recently shown that GMc1 decreased ER stress and apoptotic damage in the liver of obese mice [22]. Furthermore, preliminary research suggests that GMc1 may reduce oxidative stress and apoptosis while increasing cell viability in the heart [23]. Due to the protective potential of GMc1, a full investigation of its effects on brain cells in stroke patients with major comorbidities such as hyperlipidemia needs to be undertaken.

Given the prevalence and importance of stroke, managing hyperlipidemic patients with cerebral I/RI to activate $mitoK_{ATP}$ channels and minimize oxidative/ER stress would have specific outcomes. Considering the beneficial impacts of $mitoK_{ATP}$ channels on the brain under I/RI settings and the protective potential of GMc1, it appears that administration of GMc1 may lessen complications of a stroke in the hyperlipidemic condition. Accordingly, this work aimed to investigate the neuroprotective potential of GMc1 on cerebral I/RI by focusing on the role of $mitoK_{ATP}$ channels and evaluating the oxidative and ER stress in hyperlipidemic rats.

MATERIALS AND METHODS

Animals

All animal experiments and experimental protocols were performed in accordance with the NIH Guide for the Care and Utilize of Laboratory Animals and were approved by the local Institutional Animal Ethical Committee (No: ARC-20191682). This work included 70 male Sprague-Dawley rats aged 8-10 months with an average weight of 250±20 g. All animals were kept in an animal center under standard conditions of

25±2°C with 55% humidity in a 12-h light/dark cycle with *ad libitum* access to food and water.

Experimental design and sample size calculation

After transport to the university animal center, the animals were given a week to adapt to the changing environment and transport stress. Following one week of acclimation (with free access to water and food), the animals were randomly allocated to either the control or hyperlipidemic groups (10 control rats and 60 hyperlipidemic rats, respectively). Control rats were used to compare changes in lipid profiles of hyperlipidemic rats after receiving a high-fat diet. The hyperlipidemic (HL) rats were randomly divided into five groups as follows: (i) HL group: hyperlipidemic rats (receiving a high-fat diet for 8 weeks); (ii) HL+IR group: hyperlipidemic rats subjected to cerebral I/RI (by closing the middle cerebral artery for 2 h and reperfusion for 24 h); (iii) HL+IR+GMC group: hyperlipidemic rats subjected to cerebral I/RI pretreated with GMc1 (10 mg/kg, daily) [23,24] by intraperitoneal (i.p.) injection for 4 weeks; (iv) HL+IR+5HD group: hyperlipidemic rats subjected to cerebral I/RI and pretreated with hydroxydecanoate (5HD); (v) HL+IR+GMC+5HD group: hyperlipidemic rats subjected to cerebral I/RI and pretreated with GMc1 and 5HD.

The high-fat diet contained 35% normal rat pellet, 32% lard, 27% casein, 4% sucrose, 1% cholesterol, 0.05% cholate and 0.3% DL-methionine (Sigma Aldrich, St. Louis, USA) with total calories of 4.6 kcal/g. Each group contained a total of 12 rats, with 6 rats used for infarct volume evaluation and 6 rats used for other quantifications.

Measurement of blood glucose, plasma hormones and lipid profiles

The tail vein blood sample was used to measure the levels of blood glucose, plasma insulin, leptin, adiponectin and lipid profiles. The concentration of glucose was quickly measured after sampling with a glucose test meter (Glutest EII; Kyoto First Scientific, Kyoto, Japan). The collected samples were centrifuged to separate plasma. The plasma concentrations of insulin, leptin and adiponectin were determined with appropriate rat-specific enzyme-linked immunosorbent assay (ELISA) kits (Cayman Chemical, Ann Arbor, Michigan, USA; Morinaga, Kanagawa, Japan; Assaypro, St Charles, MO, USA, respectively). The concentrations of cholesterol and triglyceride were measured using commercially available assay kits (Wako Pure Chemical Industries, Osaka, Japan), and calculated according to the manufacturer's instructions.

Establishment of cerebral ischemia-reperfusion injury model

A model of cerebral I/RI was employed with minor modifications according to the previous report [25]. During the experiment, room temperature was kept at 27°C. All hyperlipidemic rats were anesthetized with an i.p. injection of sodium pentobarbital (40 mg/kg). Then, the right common carotid artery (CA), internal CA and external CA were exposed through a ventral midline neck incision. Following this procedure, a 4-0 monofilament nylon filament (Beijing Sunbio Biotech Co. Ltd., Beijing, China) with a rounded tip was inserted into the internal CA from the common CA through the external CA trunk and slightly advanced 18 to 20 mm to close the middle cerebral artery. Internal body temperature was kept between 37°C and 37.5°C with a heating pad. After 120 min of middle cerebral artery occlusion, the filament was removed for reperfusion. Sham-operation rats, hyperlipidemic rats that were not subjected to cerebral I/RI, underwent the same surgery except for artery occlusion. During the tests, Laser-Doppler flowmetry (MoorDRT4; Biopac Systems, Inc., CA, USA) was applied to monitor cerebral blood flow before and after middle cerebral artery occlusion. A flexible 0.5-mm fiber optic probe was perpendicularly placed at 1 mm over the skull surface of the occlusion region (4 mm lateral and 2 mm posterior of the bregma). This model was regarded as successful only when the drop in cerebral blood flow was ≥70% of baseline during the time of obstruction [15]. The overall mortality rate after surgery was close to 20% for all groups, and these rats were replaced with new rats.

Measurement of infarct volume

After 24 h of reperfusion, the brains were quickly extracted and gradually frozen to preserve the morphology before slicing. The infarct volume was calculated

as previously described [26]. Briefly, the brain was quickly dissected and sectioned into five coronal blocks in the brain matrix with an approximate thickness of 2 mm and stained with 2% (w/v) 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma Aldrich, St. Louis, USA) for 30 min at 37°C, followed by overnight immersion in 4% (w/v) paraformaldehyde. The infarct areas on each slice were analyzed by Image J software (National Institutes of Health, Bethesda, MD, USA). The infarct volumes of the brains were calculated as the infarct area percentage and were normalized to the total brain volume of the animals. The infarct volumes of five brain sections were added together and expressed as the total infarct volume.

Measurement of brain water content

To estimate the brain water content, the contralateral hemispheres of the brains of six rats were quickly harvested after reperfusion and the wet tissue was weighed, then dried in an oven for 48 h at 105°C and weighed again. The brain water content (%) was calculated as: [(wet weight – dry weight) / wet weight] × 100%.

Tissue sampling and brain mitochondria isolation

Brain tissues obtained from penumbra surrounding the ischemic core were obtained, dissected and one section was removed to -80°C deep freezer, while another section was transferred to a homogenizer containing mitochondrial isolation buffer (containing 70 mM sucrose, 210 mM mannitol, one mM EDTA in 50 mM Tris-HCl, pH 7.4). Following homogenization and centrifugation at 1,300 × g for 10 min at 4°C, the resultant supernatant was put aside and the pellet was centrifuged at 12,000 × g under the same condition. The mitochondrial pellet was suspended in storage buffer (containing 70 mM sucrose and 210 mM mannitol in 50 mM Tris-HCl, pH 7.4, at a final volume of 100 µL). Nanodrop was used to quantify and assess protein purity.

Mitochondrial ROS generation

A fluorometry test was used to evaluate mitochondrial ROS generation of brain samples by the dichlorohydrofluorescein diacetate (DCFDA) method according to the manufacturer's instructions (Sigma Aldrich, St. Louis, USA). After incubation of the mitochondrial pellets in a storage buffer with 2 µM of DCFDA dye for 30 min, their fluorescence was measured at $\lambda_{excitation}$=480 nm and $\lambda_{emission}$=530 nm with a fluorescent microplate reader. Mitochondrial ROS levels were determined using fluorescence intensities (FI) and expressed as FI/mg protein.

Mitochondrial membrane potential

Changes in the mitochondrial membrane potential ($\Delta\Psi m$) were measured by the JC-1 method, according to the manufacturer's protocol (Sigma-Aldrich, St. Louis, USA). The isolated mitochondrial pellets (3 µg) were diluted in 100 µL of JC-1 assay buffer and incubated for 15 min at 25°C in the dark. Following membrane potential dissipation, red fluorescence converts to green. Red fluorescence (JC-1 aggregates) was exited at 525 nm and emitted at 590 nm; green fluorescence (JC-1 monomers) was exited at 485 nm and emitted at 530 nm utilizing a fluorometer. The $\Delta\Psi m$ was controlled by the ratio of red/green intensity and normalized to the sample protein. Elevation of red to green JC-1 intensity indicates mitochondrial membrane depolarization.

Oxidative stress marker assessment

The oxidative stress markers in samples were evaluated by measuring the amounts of malondialdehyde (MDA) content, superoxide dismutase (SOD) activity, catalase (CAT) and glutathione peroxidase (GPX). Briefly, the tissues were cut into small pieces and mixed with a 10-fold volume of cold saline. Then, the mixtures were homogenized at 4°C and the homogenates were centrifuged at 4,000 × g for 10 min (at 4°C). The activity of oxidative stress markers in supernatants was measured with assay kits (Randox Laboratories, United Kingdom). MDA levels were expressed as nmol/mg protein and SOD, CAT and GPX activities were expressed as U/mg of protein.

Western blotting for proteins expression

The expression of p-PERK, p-eIF2a and CHOP proteins was identified by Western blotting. Proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (30 µg protein from penumbra) were transferred to a PVDF membrane, which was then blocked in 5% skim milk containing

0.1% Tween-20 for 1 h. The blocked membranes were incubated overnight individually with a primary antibody for each target protein and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (for all antibodies, 1:1000, Cell Signaling Technology, USA) at 4°C. After washing with Tris-buffered saline, horseradish peroxidase (HRP)-conjugated secondary antibody (1:2500, Cell Signaling Technology, USA) was added for 1 h. Then, the membrane was washed and incubated with increased enhanced chemiluminescence (ECL) reagents in the dark and exposed to an X-ray film. The protein bands were visualized, and the related intensities were quantified with Image J software (1.46r version, NIH, USA) and normalized to the intensity of GAPDH in each sample.

Statistical analysis

All data are presented as the mean±SEM. The data were statistically analyzed and compared using the Student's t-test, one-way ANOVA and Tukey's *post hoc* tests. P<0.05 was statistically significant.

RESULTS

General characteristics of the animals

Following an 8-week high-fat diet, all hyperlipidemia (HL) rats had significantly higher plasma concentrations of insulin, leptin, triglycerides and cholesterol, and considerably lower levels of adiponectin. In addition, HL rats had higher body weights than control rats. However, there was no significant difference in blood glucose levels between control and HL rats (Table 1).

Table 1. General information of control and hyperlipidemic rats.

Groups	Control	HL
Blood glucose (mg/dlL	83 ± 4	92 ± 6
Plasma insulin (ng/mL)	3.56 ± 0.63	8.23 ± 1.27**
Plasma leptin (ng/mL)	4.67 ± 0.33	7.46 ± 1.6*
Plasma adiponectin (ng/mL)	6.03 ± 0.51	4.3 ± 0.17*
Plasma cholesterol (mg/dL)	97 ± 8	176 ± 21*
Plasma triglyceride (mg/dL)	104 ± 9	136 ± 16
Body weight (g)	326 ± 11	458 ± 15*

The data were expressed as mean±SEM. (n=10 per group; * P<0.05 and **P<0.01 vs. control rats). HL – hyperlipidemic.

GMc1 reduced cerebral infarct volume and water content following cerebral I/RI, while 5HD reversed these effects

Fig. 1 shows that the extent of cerebral injury indicated by infarct volumes (Fig. 1A) and brain water content (Fig. 1C) was significantly increased in I/RI HL rats as compared with the non-injured HL group (P=0.001). Pre-administration of GMc1 was capable of reducing the cerebral infarction (P=0.001) and water content (P=0.004) when compared to the HL+IR group. Treatment of IR-injured rats with 5HD as the inhibitor of mitoK_{ATP} channels had no significant effect, but it did significantly decrease the beneficial effects of GMc1 on infarct volumes (P=0.004) and brain water content (P=0.049), as compared with the HL+IR+GMC group.

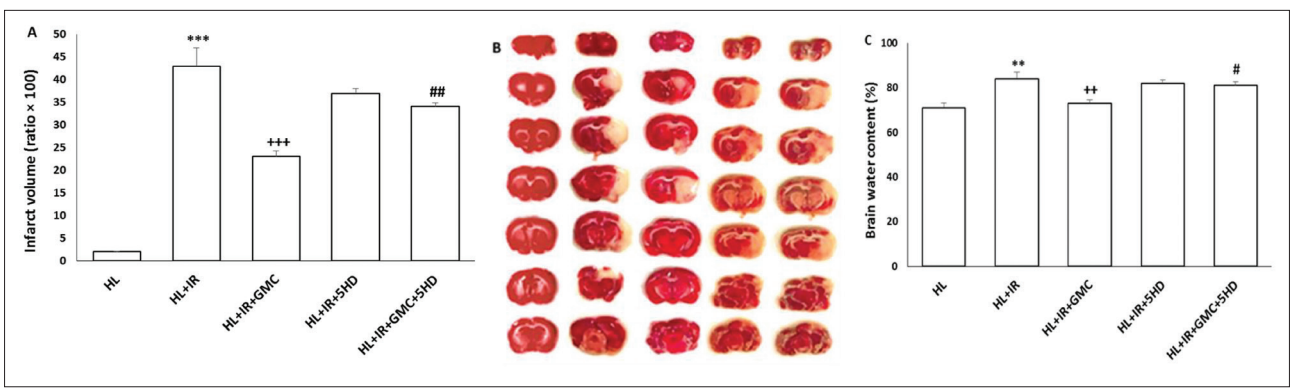

Fig. 1. Cerebral injury evaluated by infarct volumes (**A** and **B**) and brain water content (**C**) in different experimental groups. The data were expressed as the mean±SEM. (n=6 per group).** P<0.01 and ***P<0.001 vs. the HL group; ++P<0.01 and +++P<0.001 vs. the HL+IR group; #P<0.05 and ##P<0.01 vs. the HL+IR+GMc1 group. HL – hyperlipidemia; IR – ischemia-reperfusion; GMC – ginsenoside Mc1; 5HD – 5-hydroxydecanoate.

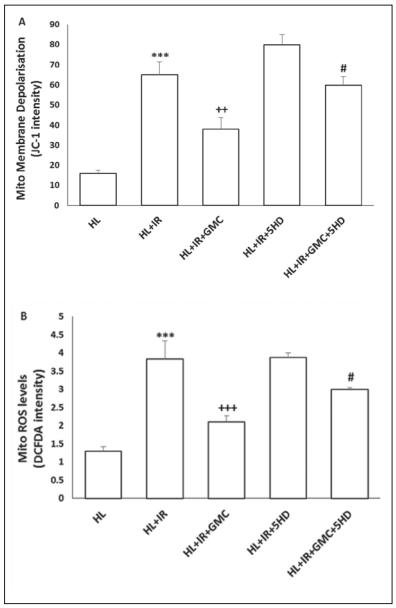

Fig. 2. Mitochondrial membrane depolarization (**A**) and mitoROS production (**B**) in different experimental groups. The data were expressed as the mean±SEM (n=6 per group).*** P<0.001 vs. HL group, ++P<0.01; +++P<0.01 vs. the HL+IR group; #P<0.05 vs. the HL+IR+GMc1 group. HL – hyperlipidemia; IR – ischemia-reperfusion; GMC – ginsenoside Mc1; 5HD – 5-hydroxydecanoate; mitoROS – mitochondrial reactive oxygen species.

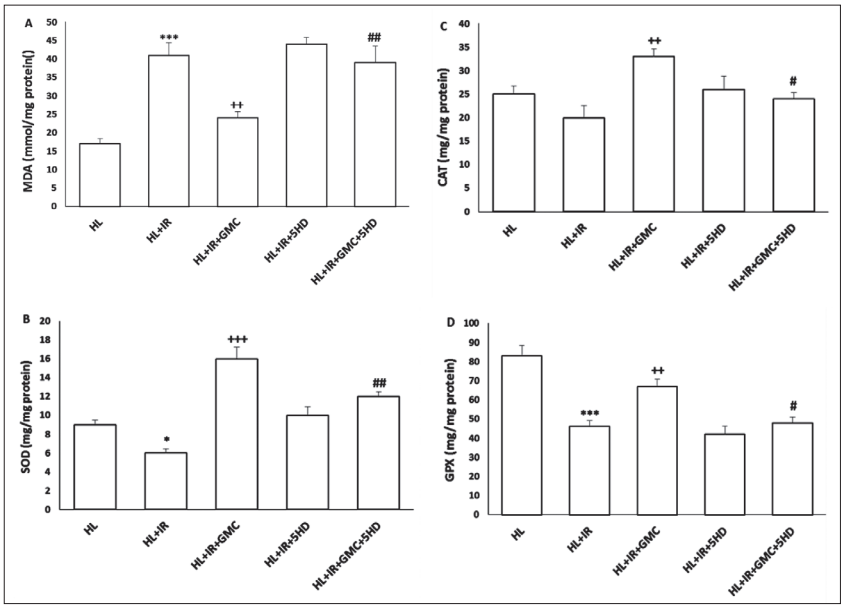

Fig. 3. Assessment of oxidative stress indicators in different groups: MDA (**A**); SOD (**B**); CAT (**C**); GPx (**D**). The data were expressed as the mean±SEM (n=6 per group). *P<0.05 and ***P<0.001 vs. the HL group; ++P<0.01 and +++P<0.001 vs. the HL+IR group; #P<0.05 and ##P<0.01 vs. the HL+IR+GMc1 group. HL – hyperlipidemia; IR – ischemia-reperfusion; GMC – ginsenoside Mc1; 5HD – 5-hydroxydecanoate; MDA – malondialdehyde; SOD – superoxide dismutase; CAT – catalase; GPX – glutathione peroxidase.

GMc1 improved cerebral mitochondrial function following cerebral I/RI, while 5HD reversed this effect

Mitochondrial membrane depolarization. As shown in Fig. 2A, mitochondrial membrane depolarization was significantly higher in the HL+IR group than in intact HL rats (P=0.001). GMc1-pretreatment significantly decreased mitochondrial membrane depolarization as compared with the HL+IR group (P=0.009). 5HD administration increased mitochondrial membrane depolarization but not significantly, as compared with the HL+IR group. Inhibiting mitoK_{ATP} channels in GMc1-treated HL rats significantly increased the mitochondrial membrane depolarization as compared to the HL+IR+GMc1 group (P=0.048).

Mitochondrial ROS production. Induction of cerebral I/RI in HL rats significantly increased mitochondrial ROS levels in comparison with the HL group (P=0.001) (Fig. 2B). GMc1 significantly decreased mitochondrial ROS as compared with IR-treated HL rats (P=0.001). The level of mitochondrial ROS was also significantly increased following 5HD administration in GMc1-treated HL rats as compared with the HL+IR+GMC group (P=0.046).

GMc1 attenuated cerebral oxidative stress following cerebral I/RI, while 5HD reversed this effect

As can be seen in Fig. 3, the cerebral levels of MDA, as a lipid peroxidation marker, were significantly raised (P=0.001), while the levels of SOD (P=0.039) and GPX (p=0.001) as two essential enzymatic endogenous antioxidants, were diminished in IR-treated HL rats as compared to the HL group. Treatment of rats with GMc1 reversed these changes, resulting in lower MDA levels (P=0.003) and increased activities of SOD (P=0.001), CAT (P=0.003) and GPX (P=0.006) in comparison with the HL+IR group. Importantly, when mitoK_{ATP} channels were blocked using 5HD, the antioxidative effects of GMc1 on reducing MDA levels (Fig. 3A) and increasing antioxidant activities (Fig.

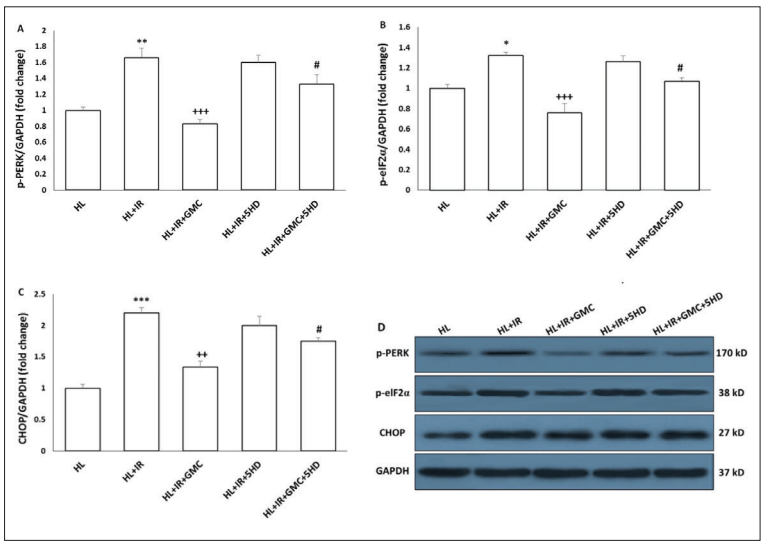

Fig. 4. Evaluation of the expression of ER stress markers in different groups. **A** – The levels of p-PERK/GAPDH. **B** – The levels of p-elF2α/GAPDH. **C** – The levels of CHOP/GAPDH. **D** – Representative immunoblots. The data were expressed as the mean±SEM (n=3 per group). *P<0.05, **P<0.01 and ***P<0.001 vs. the HL group; $^{++}$P<0.01 and $^{+++}$P<0.001 vs. the HL+IR group; $^{\#}$P<0.05 vs. the HL+IR+GMc1 group. HL – hyperlipidemia; IR – ischemia-reperfusion; GMC – ginsenoside Mc1; 5HD – 5-hydroxydecanoate.

3B, C, D) were reduced significantly when compared with those of rats treated with GMc1 alone (P=0.005 for MDA, P=0.003 for SOD, P=0.041 for CAT and P=0.021 for GPX).

GMc1 downregulated cerebral ER stress following cerebral I/RI, while 5HD reversed this effect

Induction of I/RI in HL rats significantly upregulated the expression of ER stress markers p-PERK (p=0.003), p-elF2α (p=0.015) and CHOP (p=0.001) as compared with HL group (Fig 4A-C). Administration of GMc1 significantly downregulated the expression of these proteins in comparison with the HL+IR group (p=0.001). Inhibition of mitoK$_{ATP}$ channels did not affect the expression of ER stress proteins as compared with the hyperlipidemia group. However, this inhibition significantly reduced the lowering effects of GMc1 on the protein expression of p-PERK (P=0.011), p-elF2α (P=0.028) and CHOP (P=0.050) when compared to the HL+IR+GMc1 group (Fig. 4).

DISCUSSION

The current study demonstrated the neuroprotective effects of GMc1 preconditioning in cerebral I/RI injury in hyperlipidemic rats. Preconditioning with GMc1 reduced brain damage, mitochondrial membrane depolarization and mitochondrial ROS generation. In addition, GMc1 reduced MDA levels and the expression of proteins regulating ER stress while increasing the levels of enzymatic antioxidants SOD, CAT and GPX. Moreover, our findings showed that the reduction of cerebral I/RI-induced oxidative/ER stress and mitochondrial dysfunction by GMc1 was partly mediated by its activating action on mitoK$_{ATP}$ channels. Accordingly, preconditioning with GMc1 has a neuroprotective influence in HL rats by enhancing mitoK$_{ATP}$ channel activity and attenuating oxidative/ER stress.

Studies have revealed that hyperlipidemia induces atherosclerosis and is linked to an increased risk of cerebral infarction [27]. Lipids and lipid mediators are required for brain tissue to retain its normal structure and activity. It has been extensively documented that hyperlipidemia has a close relationship with ischemic neurovascular injury [28]. Higher cholesterol levels trigger a number of vascular events, including oxidative stress, endothelial dysfunction, blood-brain barrier damage and vascular inflammation, all of which contribute to increased brain infarction and mortality [5]. Consequently, despite remarkable advances in therapeutic strategies for reducing cerebral post-ischemic damage in recent years, an effective strategy for protecting the brain against I/RI under HL conditions has yet to be developed.

In the present work, we showed that treatment of HL rats with GMc1 reversed the cerebral I/RI-induced changes in brain injury and biochemical/molecular mediators. These findings showed that GMc1 confers considerable neuroprotective influences in HL rats. GMc1 belongs to the ginseng family, which acts on a wide range of intracellular signaling molecules [29]. It has been demonstrated that by modulating

the mitochondrial unfolded protein response, this chemical increases mitochondrial activity and protects mitochondria against Parkinson's disease-associated oxidative stress [30]. Also, GMc1 significantly decreases oxidative stress in cardiomyocytes via an AMP-activated protein kinase-dependent mechanism [23]. Similarly, administration of GMc1 to HL rats had substantial antioxidative impacts following cerebral I/RI in our study. Thus, GMc1 may act mostly through scavenging oxidative stress and inducing mitochondrial promotion following cerebral I/RI.

Besides provoking the peroxidation of lipids, proteins and other important cellular elements and disrupting their function, higher levels of oxidative stress during cerebral I/RI in HL rats cause mitoK$_{ATP}$ channels to shut down upon initiation of reperfusion [31]. Their closure disrupts the mitochondrial electron transport chain and the phosphorylation-oxidative process because of an ionic imbalance in the mitochondria, and thereby reduces the biogenesis and efficiency of mitochondria in oxygen consumption and ATP production [32]. These events, in turn, can further reduce the activity of mitoK$_{ATP}$ channels. This defective feedback loop will eventually propel cardiomyocytes toward cell death. Apart from lowering oxidative stress indices, in this investigation the use of GMc1 improved mitochondrial function following I/RI and also raised cellular ATP production by defeating mitochondrial ROS and preventing mitochondrial membrane depolarization. Inhibiting mitoK$_{ATP}$ channels completely neutralized these beneficial effects of GMc1. Therefore, the presence of GMc1 in brain cells can significantly prevent mitochondrial dysfunction by opening these channels early in reperfusion. This could be a turning point in cellular function since mitochondrial improvement not only reduces the inhibitory events of cellular activity such as apoptosis, inflammation and oxidative stress but also promotes or facilitates intracellular organelle interactions [33].

The ER is one of the most critical cellular organelles that interacts closely with mitochondria and its activity in the HL condition deals with changing metabolic environments [13]. This event triggers the ER stress-related misfolded protein response. I/RI enhanced ER stress, as evidenced by higher phosphorylation and expression of PERK, elF2α, and CHOP. The elevated levels of ER stress following cerebral I/RI were significantly suppressed by prior application of GMc1. Similar to our finding, it was recently documented that GMc1 decreased ER stress as well as apoptotic injury in the liver of obese mice [22]. Higher ER stress interferes with mitochondrial activity and may hinder neuroprotection [11,34]. In the association between the ER and mitochondria, the mitochondria-associated ER membrane (MAM) facilitates highly effective transport of Ca^{2+} from the ER to the mitochondria under both physiological and pathological situations [34]. Many studies showed that the resulting mitochondrial Ca^{2+} excess is one of the main causes of cell death following I/RI [35]. Misfolded PERK, elF2α and CHOP protein accumulation in the ER lumen can induce mitochondrial dysfunction and increase mitochondrial ROS production [34]. Mitochondrial ROS and Ca^{2+} overload at reperfusion drive the cell to ER stress and apoptotic death, and hyperlipidemia may exacerbate this scenario [36]. However, mitoK$_{ATP}$ channel openers afford significant neuroprotection and hence the activation of mitoK$_{ATP}$ channels has been reported to reduce Ca^{2+} overload and ROS production and to hinder the depolarization of mitochondrial membrane potential [37]. Loss of the protective effects of GMc1 on ER stress protein expression as well as mitochondrial function after blocking mitoK$_{ATP}$ channels, suggests that the mitochondrial-ER link mediates the neuroprotective and antioxidative potential of GMc1 in HL rats. Besides this pathway, the protective impact of GMc1 in cerebral I/RI may be achieved through the activation of other important pathways and cellular mediators such as the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway, 5' AMP-activated protein kinase (AMPK)-related autophagy and mPTPs that require more exploration. Further research is needed to determine the precise role of GMc1 in lowering ionic currents across the mitochondrial membrane and ionic transport in the ER lumen following I/RI, as well as their contribution to GMc1-induced neuroprotection.

CONCLUSION

Pretreatment of HL rats with GMc1 improved cerebral mitochondrial function and decreased oxidative and ER stressors, resulting in a strong neuroprotective effect following cerebral I/RI. These effects of GMc1

were mediated through the activation of mitochondrial K/ATP channels. Thus, GMc1 preconditioning appears to be a potentially effective strategy for ischemic brain disease management in hyperlipidemic patients.

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Author contributions: All authors designed the project, performed the experimentations and analyzed and interpreted the data. MW was a major contributor to writing the manuscript. All authors read and approved the final manuscript.

Conflict of interest disclosure: The authors do not have any conflicts of interest to disclose.

Data availability: All data underlying the reported findings have been provided as part of the submitted article and are available at: https://www.serbiosoc.org.rs/NewUploads/Uploads/Wang%20and%20Li_7493_Data%20Report.pdf

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