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ORIGINAL ARTICLE

The beneficial effects of 18β-glycyrrhetinic acid following oxidative and neuronal damage in brain tissue caused by global cerebral ischemia/reperfusion in a C57BL/J6 mouse model

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Abstract This study investigated the effects of 18β-glycyrrhetinic acid (GA) on neuronal damage in brain tissue caused by global cerebral ischemia/reperfusion (I/R) in C57BL/J6 mice. All subjects ($n = 40$) were equally divided into four groups: (1) sham-operated (SH), (2) I/R, (3) GA, and (4) GA+I/R. The SH group was used as a control. In the I/R group, the bilateral carotid arteries were clipped for 15 min, and the mice were treated with the vehicle for 10 days. In the GA group, mice were given GA (100 mg/kg) for 10 days following a median incision without carotid occlusion. In the GA+I/R group, the I/R model was applied to the mice exactly as in the I/R group, and they were then treated with the same dose of GA for 10 days. Cerebral I/R significantly induced oxidative stress via an increase in lipid peroxidaitons and a decrease in elements of the antioxidant defense systems. However, GA treatment was protective against the oxidative effects of I/R by inducing significant increases in antioxidant defense systems and a significant decrease of lipid peroxidations. Additionally, cerebral I/R increased the incidence of histopathological damage and apoptosis in brain tissue, but these neurodegenerative effects were eliminated by GA treatment. Therefore, the current study demonstrated that GA treatment effectively prevents oxidative and histological damage in the brain caused by global I/R. In this context, GA may be useful for the attenuation of the negative effects of global cerebral I/R and, in the future, it may be a viable and safe alternative treatment for ischemic stroke in humans.

Keywords Global cerebral I/R · Oxidative stress · Neuronal damage · Glycyrrhetinic acid · C57BL/J6 mice

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Introduction

Cerebral stroke is among the most common causes of death in developing countries and is thought to typically originate from global cerebral ischemia/reperfusion (I/R) [1]. Cerebral I/R can lead to neurological damage and severe physical impairments or disabilities in patients [2]. Diabetes, hypertension, and hypercholesterolemia are the main risk factors for cerebral ischemia, and various types of preventive treatments have been attempted to mitigate these factors. However, the preventive treatment approach is generally inadequate, so it must be supported by protective agents [3]. The oxidative stress that results from reactive oxygen species (ROS) is a critical neurotoxic factor that underlies cerebral ischemic injury and which can easily cause neuronal death and damage in brain tissue through the oxidization of intracellular molecules such as lipids, proteins, and DNA [4, 5]. Previous studies [6, 7] have demonstrated that antioxidant agents can be beneficial for the amelioration of cerebral I/R. For example, Xu et al. [3] found that Baicalin, a flavonoid, attenuates the

neurological damage caused by I/R via its ROS-scavenging properties. Similarly, the total oligomeric flavonoids of *Cyperus rotundus* reduce the neurological deficits, excitotoxicity, and behavioral alterations that are induced by cerebral I/R [1]. Therefore, antioxidant pharmacotherapy may be an important method with which to treat global cerebral I/R injury.

18β-Glycyrrhetinic acid (GA), a hydrolyzed metabolite of glycyrrhizin, is a triterpenoid saponin and can be found in *Glycyrrhiza glabra* [8]. Licorice root contains a significant amount of GA and is commonly used in traditional Chinese and Japanese medicine for many pathological conditions, such as hepatitis, ulcers, and neurological disorders [8, 9]. Recent investigations [10, 11] have demonstrated that GA possesses strong antioxidant capabilities in addition to its anti-inflammatory and anti-cancer properties. Furthermore, GA reduces the oxidative stress caused by substances such as carbon tetrachloride and tert-butyl hydroperoxide, reduces pro-inflammatory cytokine levels, and represses immune function via its anti-inflammatory effects. For example, Li et al. [12] demonstrated that the anti-inflammatory properties of GA make it a potent drug for the prevention and treatment of allergic rhinitis.

Because cerebral I/R induces oxidative stress and inflammation in brain tissue, it was hypothesized that the antioxidative and anti-inflammatory properties of GA would ameliorate the neurological damage caused by I/R in mice. Thus, the current study evaluated the oxidative stress status and histopathological changes in the brain tissue of C57BL/6J mice.

Materials and methods

Animals and experimental protocol

The present study was approved by the Ethical Committee on Animal Research of Inonu University and carried out in accordance with The Guidelines for Animal Research from the National Institutes of Health (NIH). C57BL/6J male mice (clean grade) weighing 18–22 g were supplied by the Inonu University Laboratory Animals Research Center (Malatya, Turkey), housed in sterilized polypropylene cages, and given an ad libitum diet of standard commercial food pellets and water. All mice were kept under a 12 h light/dark cycle at an ambient temperature of 21 ± 2 °C and a humidity level of 60 ± 5 %. A total of 40 animals were randomly divided into four groups ($n = 10$): (1) sham-operated (SH), (2) global cerebral I/R, (3) GA, and (4) GA+I/R.

GA (100 mg/kg) was dissolved in 0.1 % carboxymethyl cellulose (CMC) and administered intraperitoneally (i.p.) for ten consecutive days. The dose of GA was based on preliminary dose-finding experiments from our lab, and GA treatment was initiated concomitantly with the induction of global cerebral I/R. Mice in the SH group and the I/R group were treated with only the 0.1 % CMC solution as a vehicle. In the GA and GA+I/R groups, mice were treated with GA (100 mg/kg/day) for 10 days following the I/R procedure, after which all animals were killed under anesthesia, and tissue samples were obtained for laboratory analyses.

Surgical procedure

For the induction of global cerebral ischemia, the mice were anesthetized with xylazine (5 mg/kg, i.p.) and ketamine (100 mg/kg, i.p.), and the procedure was performed according to the methods of Yonekura et al. [13]. Briefly, after a midline cervical incision, the bilateral common carotid arteries of animals in the I/R and GA+I/R groups were isolated and occluded simultaneously for 15 min using two vascular miniclips. The same surgical procedure was applied to the SH and GA groups except that the carotid arteries were not clipped. Following surgery, all mice were placed in a thermal room until they recovered from anesthesia.

Biochemical analyses

The homogenization of tissue was briefly described in our previous study [14]. The levels of thiobarbituric acid reactive substances (TBARS), total glutathione (GSH) levels and catalase (CAT), CuZn-superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities were determined by spectrophotometric methods and these methods were briefly given in our previous study [14, 15].

Histopathological examination

Prior to evaluation using a light microscope, the brain samples were fixed in 10 % formalin and embedded in paraffin. The paraffin-embedded specimens were cut into 5-μm-thick sections, mounted on slides, and stained with hematoxylin and eosin (H&E). The tissue samples were examined using a Leica DFC280 light microscope and a Leica Q Win Image Analysis system (Leica Micros Imaging Solutions Ltd., Cambridge, UK).

For immunohistochemical analysis, the sections were mounted on polylysine-coated slides. Following rehydration of the samples, they were transferred to a citrate buffer (pH 7.6) and heated in a microwave oven for 20 min, cooled for 20 min at room temperature, and then washed with phosphate buffered saline (PBS). The sections were immersed in 0.3 % H_2O_2 for 7 min, washed with PBS, and then incubated with a primary rabbit

Table 1 Levels of SOD, CAT, GPx, GSH, and TBARS in the brain tissue of C57BL/J6 mice (mean ± SD)

	TBARS (nmol/g) tissue	Reduced GSH (nmol/ml)	CAT k/mg protein	SOD U/mg protein	GPx U/mg protein
SH	9.05 ± 0.65^{a}	194.6 ± 7.37^{a}	0.019 ± 0.0014^{a}	27.03 ± 1.52^{a}	303.2 ± 22.4^{a}
I/R	11.7 ± 1.35^{b}	134.5 ± 18.0^{b}	0.011 ± 0.0017^{b}	16.65 ± 2.14^{b}	205.2 ± 19.6^{b}
GA	8.35 ± 1.19^{a}	189.8 ± 17.8^{a}	0.019 ± 0.0012^{a}	26.67 ± 2.23^{a}	296.6 ± 38.5^{a}
I/R+GA	8.24 ± 1.20^{a}	188.6 ± 12.6^{a}	0.015 ± 0.0017^{c}	19.22 ± 2.99^{c}	245.0 ± 29.1^{c}

Mean values bearing different superscripts within the same column are significantly different ($P < 0.01$)

polyclonal caspase-3 antibody (PA1302; Boster; Pleasanton, CA, USA) for 2 h. The sections were then were rinsed in PBS before incubation with biotinylated goat anti-polyvalent for 10 min and then with streptavidin peroxidase for 10 min at room temperature. Staining was completed after the substrate was incubated with a chromogen for 15 min and the slides were counterstained with Mayer's hematoxylin for 1 min, rinsed in tap water, and dehydrated. The caspase-3 kit was used according to the manufacturer's instructions, and caspase-3 positive cells were stained a brown color.

Statistical analysis

SPSS 13.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. For biochemical values, the statistical analyses were conducted using one-way analysis of variance (ANOVA) and post hoc Tukey's Honestly Significant Differences test. The degree of significance was set at $P \leq 0.01$.

Results

Biochemical results

TBARS, GSH, CAT, GPx, and SOD levels in mice brain tissue are provided (Table 1). There was a significant increase in the level of TBARS and a significant decrease in GSH, CAT, GPx, and SOD levels in the I/R groups compared with the GA and SH groups. There were no significant changes between the GA and SH groups. In the GA+I/R group, there was an attenuated increase of TBARS levels and an enhancement of the diminished GSH levels. These two parameters were very similar to the SH group such that there were no significant differences between these two groups. Similarly, the GA+I/R group exhibited an increase in CAT, GPx, and SOD activities compared with the I/R group. However, the activities of these enzymes partially reversed the normal values, and a significant difference was found between the SH and GA+I/R groups.

Histopathological results

H&E staining was performed to detect any histological changes in the cerebral cortices of mice following ischemic injury. Histopathological examination of the SH (Fig. 1a) and GA (Fig. 1b) groups revealed normal cytoarchitecture absent of any abnormalities of the brain parenchyma as evidenced by normal cell morphology with large round nuclei located in the cell center. In contrast, the I/R group exhibited significant histological damage. Focal ischemic areas were detected in the cerebral cortex (Fig. 2a), and cell infiltration, vascular congestion, shrinkage of the cytoplasm, and extensively dark pyknotic nuclei were observed in the neurons of this tissue. There were lower levels of cell infiltration (Fig. 3a) and neuronal degeneration (Fig. 3b) in the I/R+GA group, and the change in degenerated neurons was significantly different from that of the I/R group (Fig. 2d).

The current findings also demonstrated that global cerebral I/R causes cell death via induction of caspase-3-dependent apoptosis (Fig. 4). There was significantly high percentage of caspase-3-stained cells in I/R group. Caspase-3-stained cells were not observed in the SH (Fig. 4a) or GA (Fig. 4b) groups, and the percentage of caspase-3-positive cells was higher in the I/R group (Fig. 4c, d) than others. The density of caspase-3-stained cells was minimal in the I/R+GA group (Fig. 4e, f).

Discussion

Ischemic stroke is a leading cause of severe complications such as mortality, morbidity, and disability in humans worldwide. Therefore, experimental ischemic models have been developed to determine the efficacy of new therapeutic agents, such as flavonoids, for the prevention and treatment of factors associated with stroke [1, 3, 6]. In this context, the efficacy of GA in ameliorating the neuronal damage caused by global cerebral I/R was examined in a C57/BL6 mouse model. The present study found that treatment with GA (100 mg/kg) protected the central nervous system against I/R-induced damage via a significant

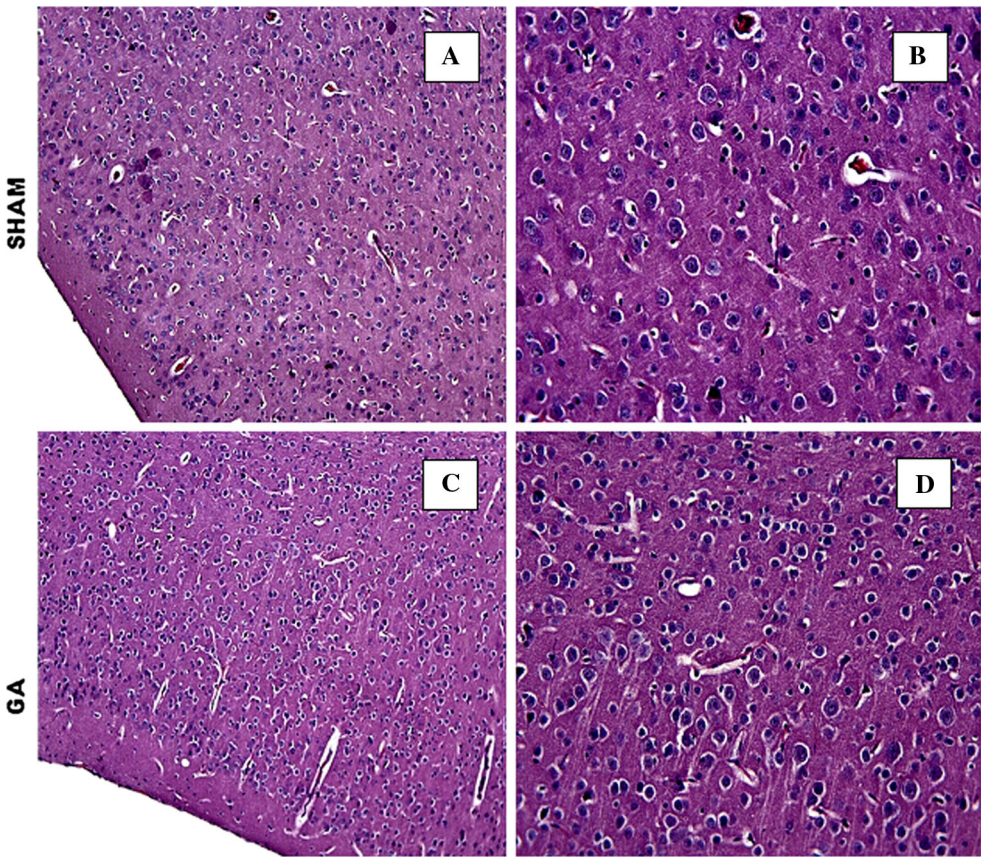

Fig. 1 In the SH and GA groups, brain tissue exhibited a normal histological appearance. **a**, **c**: H&E (×10). **b**, **d**: H&E (×20)

reduction in lipid peroxidation and a significant induction of the enzymatic and non-enzymatic antioxidant defense systems.

Oxidative stress in the brains of mice following global cerebral I/R is a primary pathological consequence that causes irreversible injury to neurons due to enhanced lipid peroxidation by ROS. This is evidenced by elevated TBARS levels following the ischemic process [16]. ROS are produced in excess following the reperfusion process and cause a downregulation in elements of the antioxidant defense systems, including SOD, CAT, GPx, and GSH [16]. In this instance, oxidative stress, which is caused by an imbalance between TBARS and the antioxidant defense systems, is induced by the I/R process and may play an important role in ischemic stroke injury [14, 15]. In this study, the I/R-induced lipid peroxidation caused by a significant increase in TBARS levels led to irreversible neuronal damage. Additionally, both the enzymatic (SOD, CAT, and GPx) and non-enzymatic (GSH) antioxidant defense systems were suppressed in ischemic mice, as there were significant decreases in SOD, CAT, and GPx activities and in the levels of GSH, a specific ROS scavenger. Similarly, Chen et al. [17] demonstrated that cerebral I/R generates a significant increase in TBARS levels and a significant decrease in SOD activity in C57 BL/6J mice. Furthermore, Zhan and Yang [18] found that TBARS levels significantly increase and that SOD, CAT, and GPx levels significantly decrease following experimental ischemic stroke. These, and many other findings, support the present results [7, 19].

The signaling pathways underlying I/R remain largely unknown, but oxidative stress plays an important role in global cerebral I/R. It was thought that oxidative stress causes significant cell death and neuronal damage in the brain, because the brain has a large amount of oxidizable unsaturated fatty acids and low antioxidant enzyme activity. In this context, the effects of I/R in the brain may depend primarily on increases in oxidative stress. To our knowledge, there are no reports investigating the beneficial effects of GA following experimental cerebral I/R in mice. It is well known that GA has strong anti-inflammatory, antitumorigenic, and antihepatotoxic activities [20, 21]. However, the findings from this study demonstrate that GA also exhibits strong antioxidant activity based on the determination of TBARS, SOD, CAT, GSH, and GPx levels. TBARS and GSH levels were affected positively in groups receiving GA treatment compared with the SH group. Moreover, GA treatment for 10 days following experimental I/R resulted in a significant decrease in the elevated TBARS levels and a significant increase in

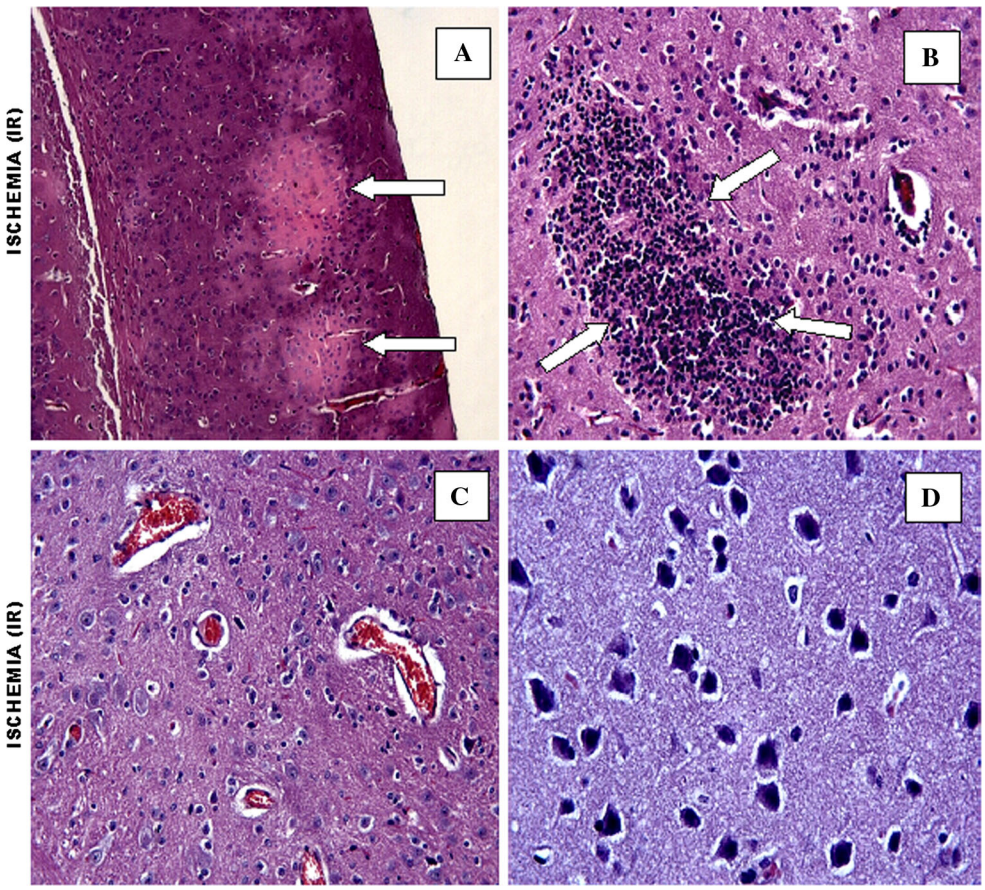

Fig. 2 Focal ischemia areas (H&E, ×10), mononuclear cell infiltration (H&E, ×20), vascular congestion (H&E, ×20), and neuronal degeneration (H&E, ×40) were observed in the brain tissues of the I/R group

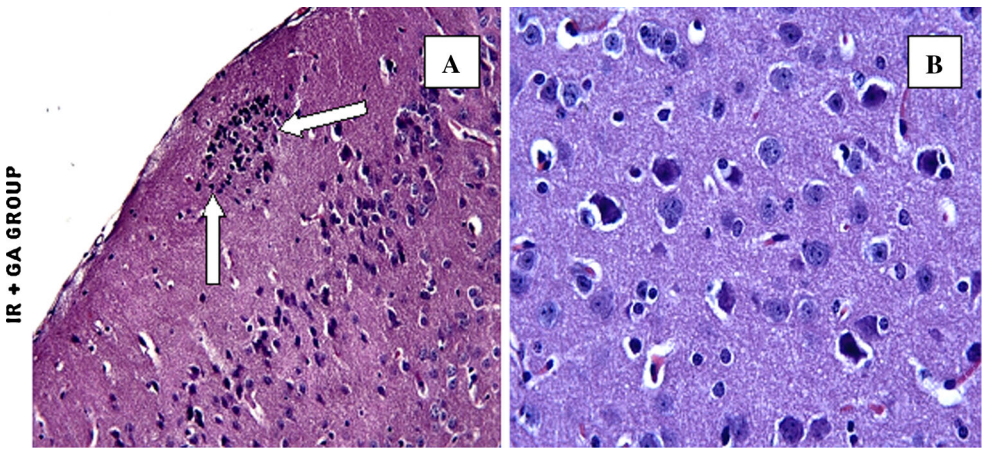

Fig. 3 Decreases in mononuclear cell infiltration (*arrows*; H&E, ×20) and neuronal degeneration (H&E, ×40) were observed in the GA+I/R group

elements of the antioxidant defense systems that were negatively affected by I/R, including SOD, CAT, and GPx activity and GSH levels. It is thought that GA treatment significantly reduces lipid peroxidation in the brain and reverses the neuronal damage caused by I/R via its antioxidant properties. Although there is a lack of studies investigating the relationship between GA and cerebral I/R in C57BL/J6 mice, the effects of GA treatment have been evaluated in organs such as the heart, liver, and spinal cord following I/R [22–24].

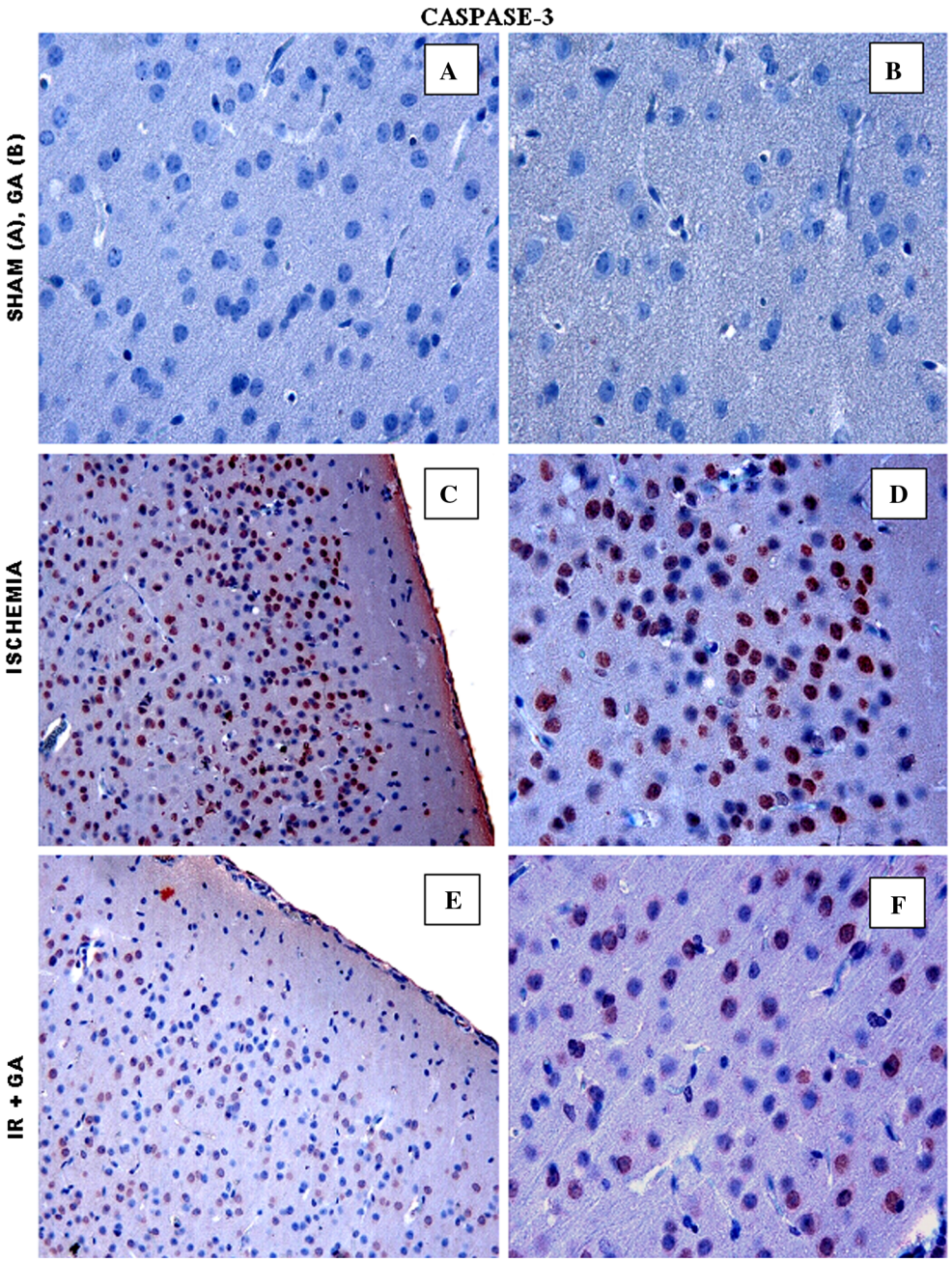

Fig. 4 Immunohistochemical expression of caspase-3 in all experimental groups. The number of caspase-3-positively stained cells decreased in the GA+I/R group (**e**, **f**) compared with the I/R group (**c**, **d**). SH group (**a**), GA group (**b**), caspase-3 (×40; **c**), caspase-3 (×20; **d**), caspase-3 (×40; **f**)

Haleagrahara et al. [22] determined that GA acts as a powerful antioxidant and reduces myocardial lipid peroxidation through decreases in 8-isoprostane levels and increases in SOD and GSH levels following myocardial ischemia in rats. Moreover, Ogiku et al. [24] demonstrated that glycyrrhizin prevents warm I/R-induced injuries during hepatobiliary surgery due to its antioxidant properties. Such findings are in agreement with the present findings and confirm our results. Based on such results, it is thought that GA treatment can be protective against ischemia due to its strong antioxidant properties and may prevent damage and lipid degeneration in neurons.

Histopathological and immunohistopathological examinations conducted in the present study revealed that I/R caused major structural changes in brain tissue compared with the sham-operated animals. The primary damage included diffuse focal ischemic areas in the cerebral cortex as well as secondary issues, such as cell infiltration,

vascular congestion, shrinkage of the cytoplasm, and extensively dark pyknotic nuclei in neurons of the cerebral cortex tissue. Yonekura et al. [1] also determined that 14 min of global cerebral ischemia leads to injury in all brain regions of C57BL/6J mice. However, in the present study, GA treatment partially alleviated the histological changes caused by I/R, and there were significantly higher numbers of caspase-3-stained cells, which are indicative of the apoptotic rate in neurons, in the I/R group compared with the GA+I/R group. This demonstrates that GA protects neurons against cell death. The results of Liu et al. [23] confirm the present findings and indicate that I/R injury to the spinal cord may cause notable increases in neuronal apoptosis. The findings of Liu et al. [23] also demonstrate that diammonium glycyrrhizinate, a GA analog, reduces neuronal apoptosis via the inhibition of the expression of nuclear factor kappa B. In this context, it was assumed that there is a correlation between oxidative status in the brain and histopathological alterations in terms of neurodegeneration and that these may be attributed to I/R. For this reason, it is proposed here that GA may be protective against ischemic damage by decreasing elevations in oxidative stress and preventing histological damage in brain tissue.

Conclusion

The current study demonstrated that 15 min of cerebral I/R in C57BL/J6 mice results in neurodegenerative effects associated with increases in oxidative stress and histopathological changes in brain tissue. Additionally, treatment with GA (100 mg/kg/day) for ten consecutive days following cerebral I/R generally reversed the potentially negative effects of I/R on brain tissue, likely due to its strong antioxidant and radical scavenging properties. Therefore, based on the present results, it is proposed that GA attenuates the neuronal damage caused by global cerebral I/R in the brain.

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Conflict of interest The authors have declared no conflict of interest.

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