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ORIGINAL PAPER

Neuroprotective Effects of β-Myrcene Following Global Cerebral Ischemia/Reperfusion-Mediated Oxidative and Neuronal Damage in a C57BL/J6 Mouse

Osman Ciftci · M. Namik Oztanir · Aslı Cetin

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Abstract The aim of this study was to investigate the effects of β-myrcene (MYR) on oxidative and histological damage in brain tissue caused by global cerebral ischemia/reperfusion (I/R) in C57BL/J6 mice. Mice (n = 40) were equally divided into four groups: (1) sham-operated (SH), (2) global cerebral I/R, (3) MYR, and (4) MYR + I/R. The SH group was used as a control and received 0.1 % carboxymethyl cellulose (CMC) as a vehicle following a medial incision without carotid occlusion. In the I/R group, the bilateral carotid arteries were clipped for 15 min, and treated with the vehicle intraperitoneally (i.p.) for 10 days. In the MYR group, mice were given 200 mg/kg MYR dissolved in 0.1 % CMC for 10 days following a medial incision without carotid occlusion. In the MYR + I/R group, the I/R procedure was performed exactly as in the I/R group, and they were then treated with the same dose of MYR for 10 days. Cerebral I/R induced oxidative stress via an increase in thiobarbituric acid reactive substances (TBARS) formation and a decrease in the antioxidant defense systems, including glutathione (GSH), catalase, glutathione peroxidase (GPx) and superoxide dismutase (SOD). However, MYR treatment protected against the oxidative effects of I/R by inducing significant increases in GSH, GPx, and SOD and a significant decrease in the formation of TBARS. Additionally, cerebral I/R increased the incidence of histopathological damage and apoptosis in brain tissue, but these neurodegenerative effects were eliminated by MYR treatment. This study has demonstrated that MYR effectively attenuates oxidative and histological damage in the brain caused by global I/R. The beneficial effects of MYR probably contribute to its strong antioxidant and radical scavenging properties. In conclusion, MYR may be useful for the attenuation of the negative effects of global cerebral I/R and, in the future, may be a viable and safe alternative treatment for ischemic stroke in humans.

Keywords Global cerebral I/R · Oxidative stress · Neuronal damage · β-Myrcene · C57BL/J6

O. Ciftci (✉)
Department of Medical Pharmacology, Faculty of Medicine, University of Inonu, 44280 Malatya, Turkey
e-mail: osmciftci@gmail.com

M. N. Oztanir
Department of Brain and Neurosurgery, Faculty of Medicine, University of Inonu, 44280 Malatya, Turkey

A. Cetin
Department of Histology and Embryology, Faculty of Medicine, University of Inonu, 44280 Malatya, Turkey

Introduction

Normal brain function requires sufficient blood flow to supply oxygen and glucose, and the reduction or interruption of brain blood flow caused by global or focal ischemic stroke can lead to fatal brain damage [1]. Ischemic stroke has increased in developing countries over the past four decades, and is among the most common causes of death and disability in humans [2]. Following ischemic stroke, several acute metabolic disturbances are caused by depletion of energy stores, excitotoxic amino acid release, ion homeostasis distribution and free radical formation [3]. Although the exact mechanism of stroke is unclear, free radical formation as a result of ischemic stroke has a critical role in neuronal damage from ischemia, as well as the reperfusion process [4, 5]. Oxidative

stress resulting from free radical accumulation in tissue can lead to neuronal cell death and damage brain tissue through the oxidization of intracellular molecules such as lipids, proteins, and DNA [4–6]. Our previous study [6] showed that 15 min of ischemic stroke in C57BL/J6 mice caused significant oxidative and histological damage in brain tissue, leading to neuronal apoptotic cell death. It is thought that antioxidant agents, which have the ability to scavenge reactive oxygen species (ROS), could attenuate the neurological damage caused by ischemia/reperfusion (I/R). Our previous studies [6, 7] demonstrated that the antioxidant agents 18β-glycyrrhetinic acid and hesperidin have beneficial effects on experimental cerebral I/R via a decrease in thio barbituric acid reactive substances (TBARS) and an increase in antioxidant defense systems. Aras et al. [8] found that Ebselen, a synthetic seleno-organic compound, prevented neuronal I/R injuries as a result of oxidative and histological damage, via its ROS-scavenging properties. Therefore, antioxidant pharmacotherapy may be an important method to treat global cerebral I/R injury.

β-myrcene (7-methyl-3-methylene-1,6-octadiene) (MYR) is an olefinic natural compound classified as a pleasant-smelling monoterpene [9A large variety of botanical species, including bay, ylang-ylang, wild thyme, parsley and hops, contain MYR, which is also a major constituent in lemongrass and bay oils [9, 10]. It is a important ingredient used in the manufacturing of cosmetics, fragrance products in shampoos, soaps, detergents, and it is also a constituent of essential oils that have been used in the food industry as a flavoring additive [11]. MYR has many important pharmacological effects as an analgesic and anti-inflammatory with anti-ulcer activity and antioxidant properties [9–12]. Ciftci et al. [12] showed that MYR treatment caused a decrease in lipid peroxidation induced by 2,3,7,8-tetracholorodibenzo-p-dioxin (TCDD) toxicity via antioxidant and radical scavenger properties. In previous studies (6,7) flavonoids such as hesperidin and 18β-glycyrrhetinic acid were tested for brain injury caused by stroke and others, but a monoterpene such as MYR not investigated for this area. It was thought that MYR is a strong antioxidative agent and with this property it can be prevents oxidative and histological damage in tissues. Therefore MYR were selected in this study for treatment of stroke. Thus, we hypothesized that MYR can prevent neurodegeneration caused by conditions such as I/R and can promote healthy brain functions.

Based on these previous studies, the current study hypothesized that MYR treatment ameliorates the oxidative and histological neurological damage caused by global cerebral I/R in C57BL/6 J mice. To determination this, we evaluated changes in oxidative stress and brain histopathology during I/R in C57BL/6 J mice.

Materials and Methods

Chemicals

MYR and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). All chemicals were either analytical grade or the highest grade available.

Experimental Animal Protocol

The present study was approved by the Ethical Committee on Animal Research of Inonu University (Malatya, Turkey) and carried out in accordance with the Guidelines for Animal Research of the National Institutes of Health (NIH). C57BL/6 J male mice (clean grade), weighing 18–22 g, were supplied by the Inonu University Laboratory Animals Research Center, 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) MYR, and (4) MYR + I/R.

This experimental design was described in our previous study [6]. MYR (200 mg/kg) was dissolved in 0.1 % carboxymethyl cellulose (CMC) and administered intraperitoneally (i.p.) for 10 consecutive days. The dose of MYR was based on preliminary experiments in our laboratory, and MYR treatment was initiated concomitantly with the induction of global cerebral I/R. Mice in the SH and I/R group were treated with the 0.1 % CMC solution as a vehicle. In the MYR and MYR + I/R groups, mice were treated with 200 mg/kg/day MYR for 10 days following the I/R procedure, after which all animals were sacrificed under anesthesia, and tissue and blood samples were obtained for laboratory analyses.

Surgical Procedure

This experimental design was described in our previous study [6]. 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 MYR + 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 MYR groups, except that the carotid arteries were not clipped. Following surgery, all mice were placed in a thermal room until they recovered from anesthesia.

Table 1 The levels of SOD, CAT, GPx, GSH and TBARS in brain tissue of C57 BL/J6 mice. (Mean ± SD)

	TBARS nmol/g tissue	Reduced GSH nmol/ml	CAT k/mg protein	SOD U/mg protein	GPx U/mg protein
Sham	9.17 ± 0.93^{a}	203.7 ± 5.2^{a}	0.028 ± 0.0011^{a}	21.99 ± 1.92^{a}	254.9 ± 19.5^{a}
I/R	13.4 ± 1.09^{b}	155.8 ± 7.81^{b}	0.018 ± 0.0007^{b}	15.16 ± 2.09^{b}	185.7 ± 21.2^{b}
MYR	8.71 ± 0.99^{a}	198.1 ± 9.2^{a}	0.029 ± 0.0012^{a}	22.21 ± 1.61^{a}	269.3 ± 16.9^{a}
I/R + MYR	11.01 ± 1.17^{c}	176.9 ± 6.3^{c}	0.020 ± 0.0010^{b}	18.90 ± 1.83^{c}	225.0 ± 18.8^{c}

Means bearing different superscripts within same column were significantly different ($P < 0.01$)

Biochemical Analyses

Tissue was homogenized as described previously [14]. The levels of thiobarbituric acid reactive substances (TBARS) and total glutathione (GSH), together with the activities of catalase (CAT), CuZn-superoxide dismutase (SOD) and glutathione peroxidase (GPx), were determined using spectrophotometric methods, as described previously [14, 15].

Histopathological Examination

For light microscopic evaluation, brain samples were fixed in 10 % formalin and embedded in paraffin. Paraffin-embedded specimens were cut into 5-μm thick sections, mounted on slides and stained with hematoxylin and eosin (H–E). 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 (IHC) analysis, sections were mounted on polylysine-coated slides. After rehydrating, samples were transferred to citrate buffer (pH 7.6) and heated in a microwave oven for 20 min. After cooling for 20 min at room temperature, the sections were washed with phosphate-buffered saline (PBS). Sections were submerged in 0.3 % H_2O_2 for 7 min and washed with PBS. Sections were then incubated with a primary rabbit polyclonal antibody against caspase-3 (Boster, PA1302) for 2 h. After the primary incubation, sections were rinsed in PBS and incubated with biotinylated goat anti-polyvalent for 10 min and streptavidin peroxidase for 10 min at room temperature. Immunostaining was completed with a chromogen and substrate for 15 min, and slides were counterstained with Mayer's hematoxylin for 1 min, rinsed in tap water, and dehydrated. The caspase-3 antibody was used according to the manufacturer's instructions, and caspase-3-positive cells showed brown staining.

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). Global cerebral I/R caused a significant increase in TBARS levels and a significant decrease in GSH, CAT, GPx, and SOD levels compared with the SH and MYR groups. There were no significant changes between SH and MYR groups in all parameters. In contrast, in the MYR + I/R group, there was an attenuated increase in TBARS levels and an enhancement of the diminished GSH to levels in the SH group. Similarly, the MYR + I/R group exhibited an increase in GPx and SOD activities compared with the I/R group. Only CAT activity was not significantly affected by MYR treatment compared with I/R group, and did not return to normal values. However, the activities of GPx and SOD partially reversed the normal values, and a significant difference was found between the SH and MYR + I/R groups.

Histopathological Results

In control (Fig. 1a) and MYR (Fig. 1b) groups, a normal histological appearance was seen in the cerebral cortex. In the I/R group, some histological changes were observed, including focal ischemia in the cerebral cortex (Fig. 2a). In addition to this, mononuclear cell infiltration (Fig. 2b), cytoplasm shrinkage, extensive dark picnotic nuclei (Fig. 2c, d) and hemorrhage (Fig. 2c) were detected in neurons of the cerebral cortex in the I/R group. MYR treatment induced morphological alterations, mononuclear cell infiltration, cytoplasm shrinkage, extensive dark picnotic nuclei and hemorrhage. In contrast, histological damage was reduced significantly in the IR + MYR group (Fig. 3a, b) and the histological appearance of the brain tissue improved. Caspase-3 immunopositive cells were not observed in control (Fig. 4a) and MYR (Fig. 4b) groups. The number of caspase-3 positive cells was high in the IR group (Fig. 4c) and caspase-3 immunopositive neurons in

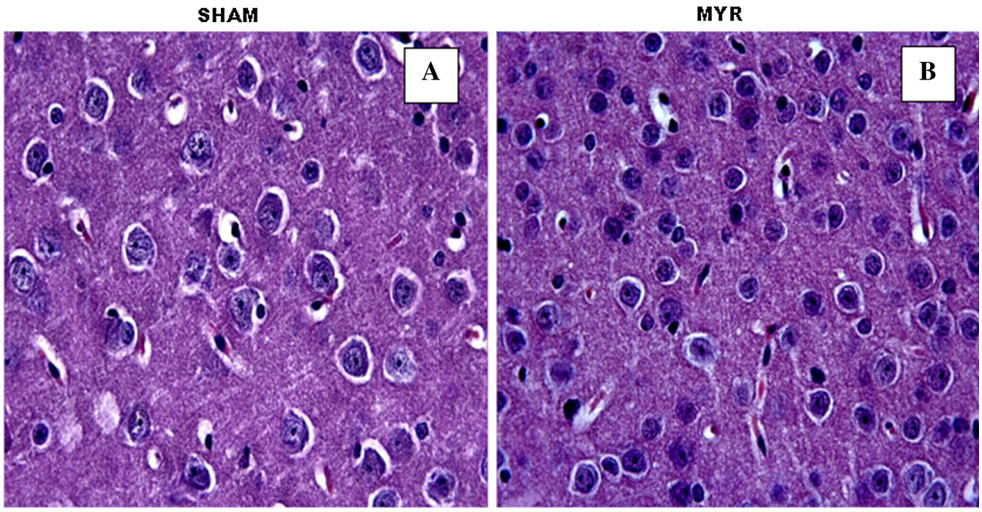

Fig. 1 Brain tissue from SH (**a**) and MYR (**b**) groups displayed normal histological appearance. H–E; ×40

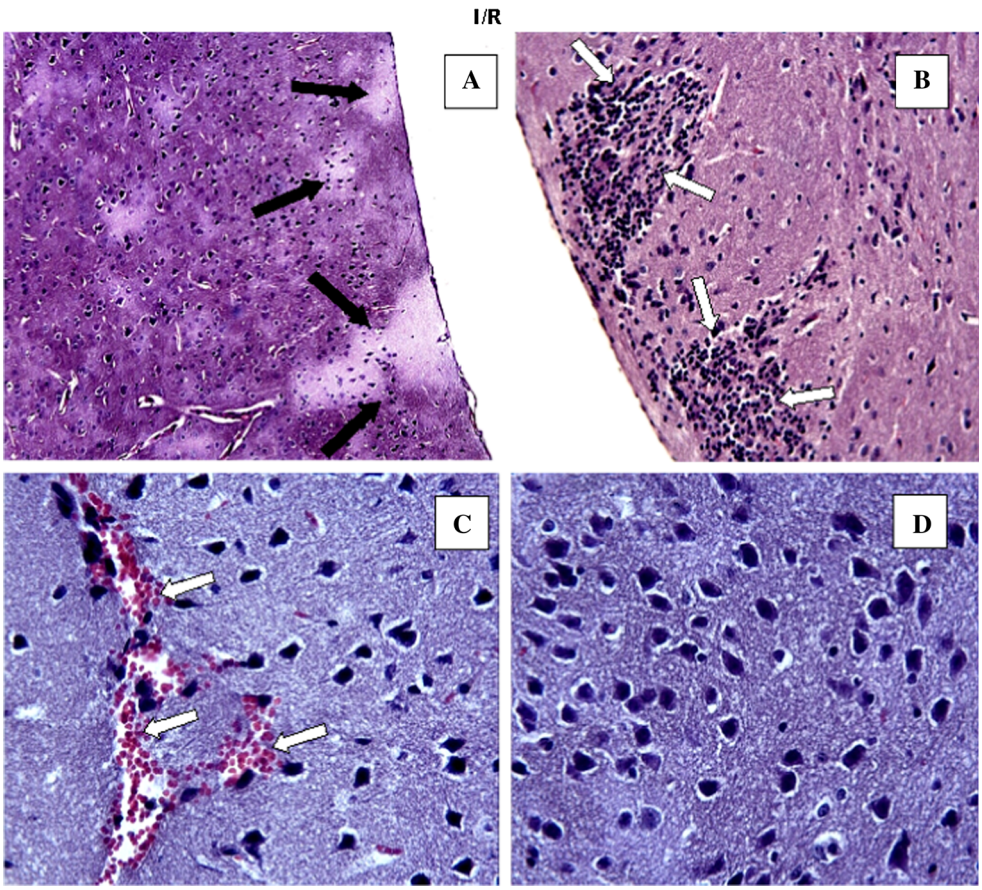

Fig. 2 Areas of focal ischemia (*black arrows*) (**a**), mononuclear cell infiltration (*white arrows*) (**b**), hemorrhage (*white arrows*) (**c**), cytoplasmic shrinkage and extensively *dark* picnotic neuronal nuclei (**d**) were detected in the I/R group. **a** H–E; ×10. **b** H–E; ×20. **c**, **d** H–E; ×40

the brain were reduced in the IR + MYR group (Fig. 4d), as compared to the I/R group.

Discussion

Bilateral common carotid artery occlusion in the C57BL/J6 mouse model is commonly used as a global cerebral I/R model since the pathophysiology closely resembles global ischemic stroke. Using this experimental model, we have determined that MYR treatment (200 mg/kg) has a neuroprotective effect against global cerebral I/R-induced damage due to a significant decrease in oxidative stress and histological damage in the brain. The mechanism of neuroprotection is primarily associated with inhibition of neuronal apoptosis and a reduction in lipid peroxidation, in

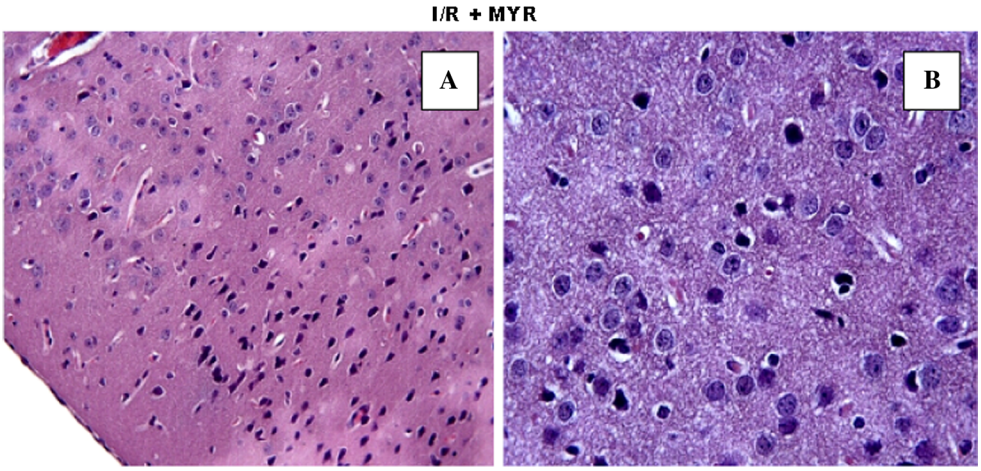

Fig. 3 Decreased areas of focal ischemia, hemorrhage, mononuclear cell infiltration, cytoplasmic shrinkage and extensively *dark* picnotic neurons nuclei in the I/R + MYR group. **a** H–E; ×20. **b** H–E; ×40

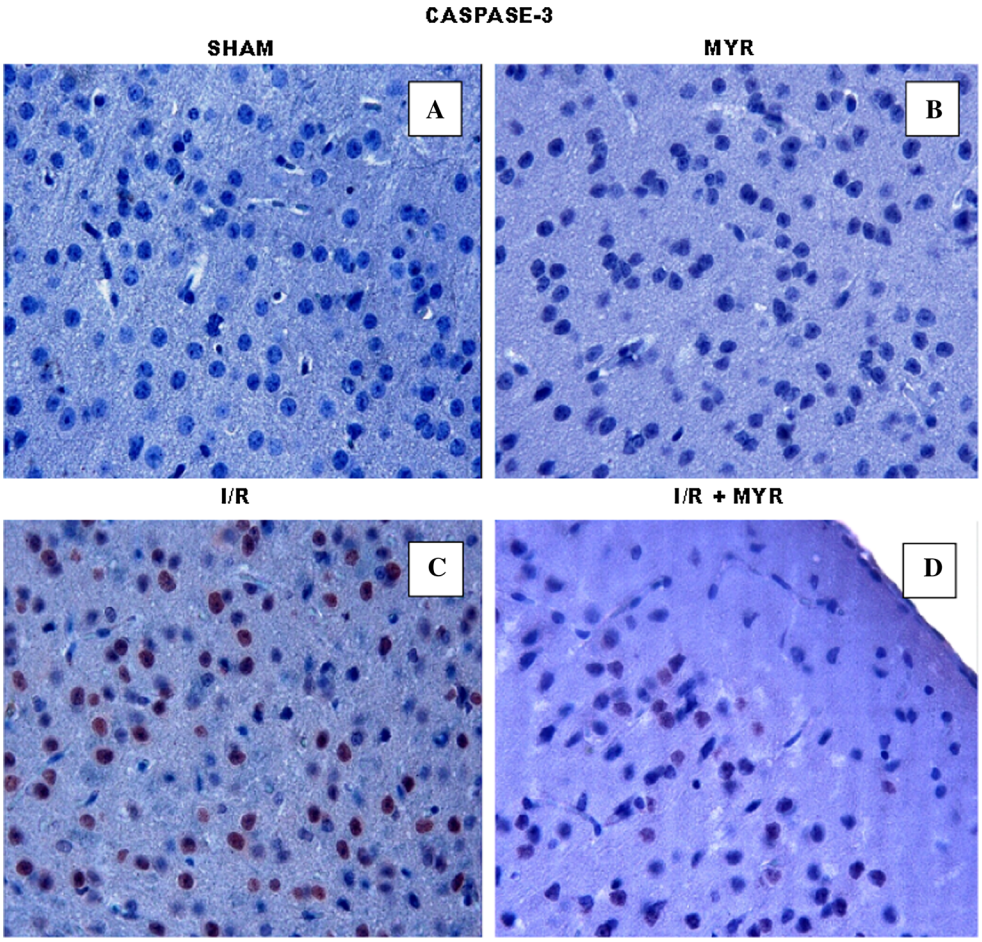

Fig. 4 Caspase 3 staining of tissue sections. There are no caspase 3-positive cells in the SH (**a**) and MYR (**b**) groups. Caspase 3-positive staining is greater in the I/R group (**c**) compared with the I/R + MYR group (**d**). Caspase 3; ×40

addition to a significant induction of the enzymatic and non-enzymatic antioxidant defense systems.

In ischemic stroke injury, oxidative stress has an important role, due to induced lipid peroxidation following I/R. Lipid peroxidation is one of the primary pathological hallmarks of irreversible cell death in neurons. It produces and releases ROS, which are highly reactive chemicals that attack lipids, proteins and nucleic acids, and down-

regulates the antioxidant defense systems including SOD, CAT, GPx and GSH [16–18]. The exact mechanism of I/R injury remains unclear, but it is 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 [4, 5]. This damage may partially occur during the ischemic process and reperfusion phase. Our previous study [6, 7] determined that the primary damage occurs during the reperfusion process, suggesting that managing oxidative stress may prevent neuronal damage in the brain. In this study, we determined that global cerebral I/R induced lipid peroxidation via elevated TBARS levels. Furthermore, the antioxidant defense system was suppressed by I/R via decreased enzymatic activities of SOD, CAT, and GPx and in the expression levels of GSH, a specific ROS scavenger. Recent studies [19, 20] have confirmed our findings. For example, Malik et al. [19] showed that global cerebral ischemia induced by occluding both common carotid arteries for 10 min in mice led to a significant increase in TBARS levels and induced neuronal injury. Oztanir et al. [6] determined that global cerebral I/R in C57BL/J6 mice caused an imbalance in brain tissue, which led to significant oxidative damage in the brain.

Importantly, the current study demonstrated that MYR treatment caused a significant attenuation of IR-induced TBARS elevation and significant increases in SOD, CAT, and GPx activities and GSH levels in ischemic mice. Thus, MYR treatment reduces oxidative damage and reverses the brain damage caused by I/R. This is the first study examining the relationship between MYR treatment and pathological changes caused by I/R and has clearly shown that MYR may be effective in reducing oxidative damage caused by I/R injury in the brain. There are few studies describing the pharmacological activity of MYR and these studies showed that MYR has important analgesic, anti-inflammatory, anti-ulcer activity and antioxidant properties [9–12]. The studies by Ciftci et al. [11, 12] showed that MYR significantly decreased lipid peroxidations caused by TCDD, indicating that MYR had immunomodulating effects via changes in CD4 and CD161 lymphocytes. Similarly, Bonamin et al. [10] determined that MYR prevented peptic ulcer disease in rats. These findings agree with the present study and confirm our results demonstrating MYR as a strong antioxidant agent. Therefore, MYR may protect brain tissue via an oxidant/antioxidant mechanism and prevent damage and lipid degeneration in neurons.

Previous studies [6, 7, 13] of the histopathological structure during global I/R damage in the brain reported that significant structural changes occurred in the brain during the I/R process. Similarly, in the current study we determined that significant histopathological and immunohistological changes occurred in the I/R group, compared with the SH and other experimental groups. The primary damage included diffuse focal ischemic areas in the cerebral cortex as well as secondary issues, such as mononuclear cell infiltration, hemorrhage, cytoplasmic shrinkage, and the presence of extensively dark picnotic nuclei in cortical neurons. Additionally, I/R caused an increase in the number of caspase-3 stained cells, which are indicative of the apoptotic state of neurons. Many studies [20–22] have confirmed our histological results. For example, Oztanir et al. [6] reported that global cerebral I/R led to structural damage in the brain and neuronal cell death, which correlated with caspase-3 immunoreactivity in the brain. Moreover, Yonekura et al. [13] determined that 14 min of global cerebral ischemia leads to injury in all brain regions of C57BL/6 J mice. Besides, our previous study [7] clearly demonstrated that I/R caused apoptosis in neurons and this effect may be due to oxidative changes in brain tissue. Additionally, other studies [15, 21] indicated that significant histological alterations in brain correlated with lipid peroxidations in neurons. However, MYR treatment reversed the negative histological changes caused by I/R, including a notably significant decrease in the number of caspase-3 stained cells. Also, MYR administration reduced the incidence of histological structure defects in brain such as mononuclear cell infiltration, hemorrhage, cytoplasmic shrinkage, and the presence of extensively dark picnotic nuclei. Moreover ischemic areas of brain was seen so little in MYR treatment group. To our knowledge, there is no study describing the effects of MYR on brain histological structure and this is the first study describing the potent beneficial effects of MYR against I/R injury in brain. A correlation between the oxidative status of the brain and I/R-induced histopathological changes indicates that these beneficial effects may be due to the antioxidant and radical scavenger properties of MYR.

Conclusion

The current study described the beneficial effects of MYR against I/R injury in the brain and clearly indicated that MYR treatment ameliorates the neurodegenerative effects caused by global cerebral I/R in C57BL/J6 mice. This effect of MYR is associated with its antioxidant properties and is correlated with a decrease in oxidative stress. Therefore, we conclude that MYR 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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