BJPS

Brazilian Journal of
Pharmaceutical Sciences

http://dx.doi.org/10.1590/s2175-97902022e21219

Article

18β-glycyrrhetinic acid attenuates global cerebral ischemia/reperfusion-induced cardiac damage in C57BL/J6 mice

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The aim of the present study is to investigate the cardioprotective effects of 18β-glycyrrhetinic acid (18β -GA) against oxidative and histological damage caused by global cerebral ischemia/reperfusion (I/R) in C57BL/J6 mice. All male mice (n:40) were randomly divided into four groups: (1) sham-operated (Sham), (2) I/R, (3) 18β-GA, and (4) 18β -GA+I/R. Ischemia was not applied to the sham and 18β-GA groups. In the I/R group, the bilateral carotid arteries were clipped for 15 min to induce ischemia, and the mice were treated with the vehicle for 10 days. In the 18β-GA group, the mice were given 18β-GA (100 mg/kg) for 10 days following a median incision without carotid occlusion. In the 18β-GA+I/R group, the ischemic procedure performed to the I/R model was applied to the animals and afterwards they were intraperitoneally (i.p.) treated with 18β-GA (100 mg/kg) for 10 days. It was found that global cerebral I/R increased TBARS levels and decreased antioxidant parameters. The 18β-GA treatment decreased the level of TBARS and increased GSH, GPx, CAT, SOD activities. Also, the control group cardiac tissue samples were observed to have a normal histological appearance with the Hematoxylin-Eosin staining method. Histopathological damage was observed in the heart tissue samples belonging to the I/R group. The 18β-GA treatment ameliorates oxidative and histological injury in the heart tissue after global ischemia reperfusion, and may be a beneficial alternative treatment.

Keywords: Global cerebral I/R. 18β-glycyrrhetinic acid. Oxidative stress. Heart injury.

INTRODUCTION

Global cerebral ischemia leads to impaired blood flow to the brain or its certain parts thus, the brain is deprived of oxygen and glucose at levels that can lead to tissue impairment. In mature individuals, global cerebral ischemic injury occurs in some situations that cause a dramatic reduction in blood flow to the brain, such as cardio-respiratory failure, coronary artery bypass surgery and cardiac arrest (Llinas, Barbut, Caplan, 2000). Global cerebral ischemia can cause neurodegeneration in the CA1 region of the hippocampus. The mechanisms underlying this neurodegeneration are reactive oxygen species (ROS), excitotoxicity, apoptosis and inflammation (Akai, Yanagihara, 1993; Nikonenko *et al.*, 2009). This shows that global cerebral ischemia may be connected to the oxidative stress caused by excessive production of free radicals in the pathogenesis (Leak *et al.*, 2015). In this regard, the current treatment approaches for ischemia/reperfusion injury (I/R) can be ineffective (Yasuda *et al.*, 2014). It is claimed that free radicals play a vital role in the reperfusion

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progress that occurs in the continuation of ischemia. In the reperfusion process, reoxygenation provides oxygen to maintain neural viability and accompanies numerous enzymatic reactions that produce reactive oxygen species (Nita *et al*., 2001). In this respect, the beneficial effects of reperfusion are reversed by a series of oxidative physiological processes. Two contrary mechanisms could be envisaged in the course of reperfusion: an increase of free radical production due to contact of oxygenated blood with the lipid membrane diminished by ischemia, and a free radicals washing phenomenon due to blood flow restoration (Burcu *et al*., 2016). This condition affects the heart tissue sensitive to oxidative stress. The effect of reperfusion injury on the heart is characterized by various abnormalities, including the development of arrhythmia, contractile dysfunction and various defects in intracellular biochemical homeostasis (Karmazyn, 1991).

Roots and rhizomes of the licorice plant (*Glycyrrhiza)*, one of the medicinal plants used for many years contain medicinally active components. The active components originated from *Glycyrrhiza* species include saponins, flavonoids, coumarins, and stilbenoids (Asli, Hosseinzadeh, 2008). Glycyrrhizic acid is the main component of licorice root. Glycyrrhetinic acid (GA), which is composed of the hydrolysis of glycyrrhizic acid as the result of the glucuronidase reaction, has two separate components; 18α-glycyrrhetinic acid (18α-GA) and 18β -glycyrrhetinic acid (18β-GA) (Wang *et al*., 1991). 18β-GA, one of the bioactive constituents of licorice, has been recently indicated to be utilized for anti-cancer, antioxidant, nephroprotective, hepatoprotective and anti-inflammatory effects (Mahmoud, Al Dera, 2015; Abd El-Twab *et al*., 2016).

The aim of this study is to examine the protective effects of the 18β-GA treatment that alleviates the oxidative and histological heart injury occurring after global cerebral I/R in C57BL/J6 mice.

MATERIAL AND METHODS

Chemicals

All chemicals, reagents and 18β-GA used for experimental assays were obtained from Sigma Chemical Co. (St. Louis, MO), and were of analytical grade or the highest grade available.

Animals and Treatment

The present study was approved by the Ethics Committee on Animal Research of Pamukkale University (protocol number: PAUHDEK2021/10), and carried out in accordance with The Guidelines for Animal Research from the National Institutes of Health (NIH). The animals placed in cages were held under standard laboratory conditions during the experiment. C57BL/J6 male mice (clean grade) weighing 18–22 g (2-3 month old) were housed in sterilized polypropylene cages and given an ad libitum diet of standard commercial food pellets and water.

A total of 40 animals was randomly divided into four groups (n = 10): sham-operated group (Sham), I/R, 18β-GA group and 18β-GA+I/R group. The groups formed in this study are as follows;

1) In the Sham group, the mice were administered with 0.1% carboxymethyl cellulose (CMC) solution alone as vehicle.

2) In the IR group, the mice were administered with 0.1% CMC solution alone as vehicle.

3) In the 18β-GA group, the mice were treated with 18β-GA 100 mg/kg for 10 consecutive days. 18β-GA was dissolved in CMC and administered intraperitoneally (i.p.) (Ciftci, Oztanir, Cetin, 2014).

4) In the 18β-GA+I/R group, the mice were treated with 18β-GA 100 mg/kg for 10 consecutive days.

At the end of the treatment period, all mice were euthanized under anesthesia. The heart tissue samples were collected and separated for biochemical analyses and histological examination.

Surgical Procedures

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 ischemic procedure was performed according to the methods of Yonekura *et al*. (2004). After making a midline cervical incision, the bilateral common carotid arteries of animals in the

I/R and 18β-GA+I/R groups were isolated and blocked for 15 min using two vascular mini clips. The same surgical procedure was applied to the Sham and 18β-GA groups except that the carotid arteries were not clipped. Following the surgery, all mice were placed in a thermal room until they recovered from anesthesia.

Biochemical analyses

The tissues were homogenized for the examination of biochemical parameters. The thiobarbituric acid reactive substance (TBARS) level of the heart tissue homogenates was determined by the thiobarbituric acid reaction using the method described by Yagi (1998). The glutathione (GSH) level of heart tissues was detected at 412 nm according to the Sedlak and Lindsay (1968) method and the results were expressed as nmol/mL. The determination of superoxide dismutase (SOD) enzyme activity was performed by the method described by Sun, Oberley and Li (1988). The SOD enzyme activity was determined using a photochemical method based on the inhibition of nitro blue tetrazolium (NBT) reduction by xanthine/xanthine oxidase enzyme activity O^{-2}. The heart tissues were measured at 560 nm in a spectrophotometer. The catalase (CAT) level of the tissues was determined by H_2O_2 consumption according to the Aebi method. (Aebi, 1984). The glutathione peroxides (GPx) level was measured spectrophotometrically using the method of Paglia and Valentine (Paglia, Valentine, 1967). The protein levels were determined according to the method of Lowry *et al*. (1951). Bovine serum albumin was used as a standard in the analyzes.

Histological analysis

For light microscopic evaluation, the heart tissue samples were fixed in 10% formalin. The samples were processed by routine tissue techniques, and were embedded in paraffin. The paraffin-embedded specimens were cut into 5 mm thick sections, mounted on slides and stained with Hematoxylen-Eosin (H-E). The sections obtained were examined under a Leica DFC280 light microscope by Leica Q Win and Image Analysis System (Leica Micros Imaging Solutions Ltd.; Cambridge, U.K).

Statistical analysis

SPSS 13.0 software package (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses of all biochemical results. The data were subjected to one-way analysis of variance (ANOVA) and post hoc Tukey's Honestly Significant Differences test. The degree of significance was set at $p \leq 0.01$.

We examined the sections for intense mononuclear cell infiltration, eosinophilic stained and pyknotic nuclei cells, necrosis, vascular congestion, vacuolisation and hemorrhage. The histopathologic damage score was calculated by using these findings. The statistical analyses of histological results were performed with SPSS 13.0 (SPSS Inc., Chicago, Ill., USA) and MedCalc 11.0 (Belgium) statistical software. All data are expressed as arithmetic mean ± SE. For comparisons between groups the Kruskal-Wallis and Connover tests were used. Exact *p* values were given where available, and $p < 0.0001$ was accepted as statistically significant. histopathological scores were given in Table I.

TABLE I - Comparison of the effect of 18*β*-GA on histopathological damage caused by I/R in heart tissue (Mean ± SE)

GROUPS	HISTOPATHOLOGIC DAMAGE (Mean±SE)
SHAM	0,48 ± 0,08[a]
I/R	2,38 ± 0,09[b]
18β-GA	0,67 ± 0,09[a]
18β-GA+I/R	1,53 ± 0,10[c]

The mean differences the values bearing different superscript letters within the same column are statistically significant. (p≤0,0001). SE: Standart Error

RESULTS AND DISCUSSION

Biochemical Results

The antioxidant (SOD, CAT, GPx and GSH) and oxidant (TBARS) levels of the heart tissue are presented in Table II. The I/R group had significantly increased TBARS

levels, which are the indicator of lipid peroxidation than the Sham group had, whereas the GSH level and SOD, GPx, and CAT activities were significantly decreased in the I/R group than in the Sham group. It was shown that brain I/R caused a significant increase in lipid peroxidations in the heart tissue. There was no significant difference between the 18β-GA and Sham group. GSH, SOD, GPx and CAT activities were significantly increased in the heart tissues of the I/R + 18β-GA groups compared to the I/R group. In general, the results indicated that 18β-GA significantly reduced lipid peroxidations and increased antioxidant parameters in the heart tissue.

TABLE II - The levels of TBARS, GSH, CAT, GPx and SOD in heart tissue of C57BL/J6 mice. Values are presented as means ± SD. The relationships between groups and results of biochemical markers are assessed by one-way ANOVA test (post hoc Tukey test)

GROUPS	TBARS (nmol/g tissue)	Reduced GSH (nmol/ml tissue)	CAT (k U/mg protein)	SOD (U/mg protein)	GPx (U/mg protein)
SHAM	6,35 ± 0,54[a]	60,1 ± 4,18[a]	0,0075 ± 0,0002[a]	83,33 ± 5,21[a]	25,7 ± 2,01[a]
I/R	10,21 ± 0,65[b]	43,5 ± 3,81[b]	0,0038 ± 0,0003[b]	48,75 ± 3,52[b]	15,5 ± 1,98[b]
18β-GA	6,85 ± 0,59[a]	63,8 ± 5,93[a]	0,0073 ± 0,0004[a]	85,28 ± 4,63[a]	24,1 ± 2,14[a]
18β-GA+I/R	7,02 ± 0,72[a]	57,2 ± 5,61[a]	0,0059 ± 0,0005[c]	68,45 ± 3,62[c]	19,8 ± 2,20[c]

Means bearing different superscripts within the same column are significantly different ($p < 0.01$).

Histological Results

In the sham (Figure 1A) and 18β-GA (Figure 1B) groups, the heart tissue showed a normal histological appearance (Figure 1). Intense mononuclear cell infiltration (white asteriks) (Figure 2A), eosinophilic stained and pyknotic nuclei cells (black asteriks) (Figure 2B), necrosis (arrows) (Figure 2C), vascular congestion (blue asteriks) (Figure 2D), vacuolisation (thin black arrows) (Figure 2E) and hemorrhage (white arrows) (Figure 2F) were observed in the IR group (Figure 2). 18β-GA supplementation significantly reduced the I/R-induced histopathological changes. Heart damage was significantly decreased in this group. Little vascular congestion (black arrow) (Figure 3A) and hemorrhage (thin black arrow) (Figure 3B) were observed in the 18β-GA+I/R group (Figure 3).

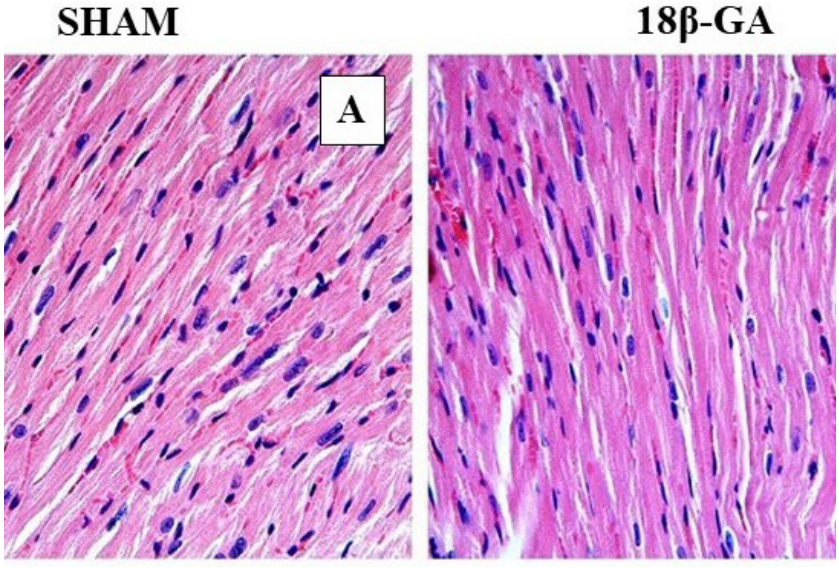

FIGURE 1 - Histological appearance of the heart tissues in the sham and 18*β*-GA groups (A):H-E; X40, (B): H-E; X40.

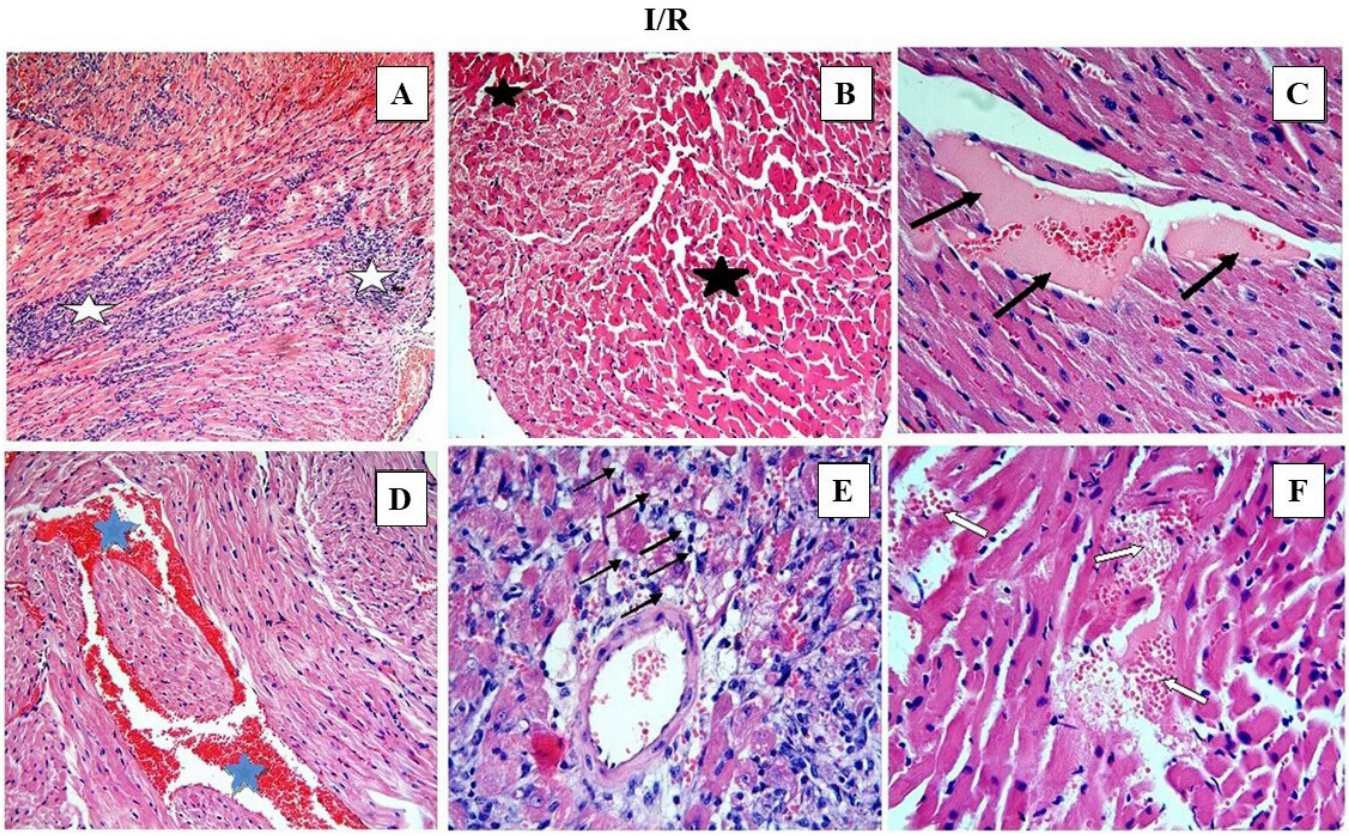

FIGURE 2 - Intense mononuclear cell infiltration (white asteriks) (A), eosinophilic stained and pyknotic nuclei cells (black asteriks) (B), necrosis (arrows) (C), vascular congestion (blue asteriks) (D), vacuolisation (thin black arrows) (E), hemorrhage (white arrows) (F) observed in the I/R group. A:H-E; X10, B, D:H-E; X20, C, E, F: H-E; X40.

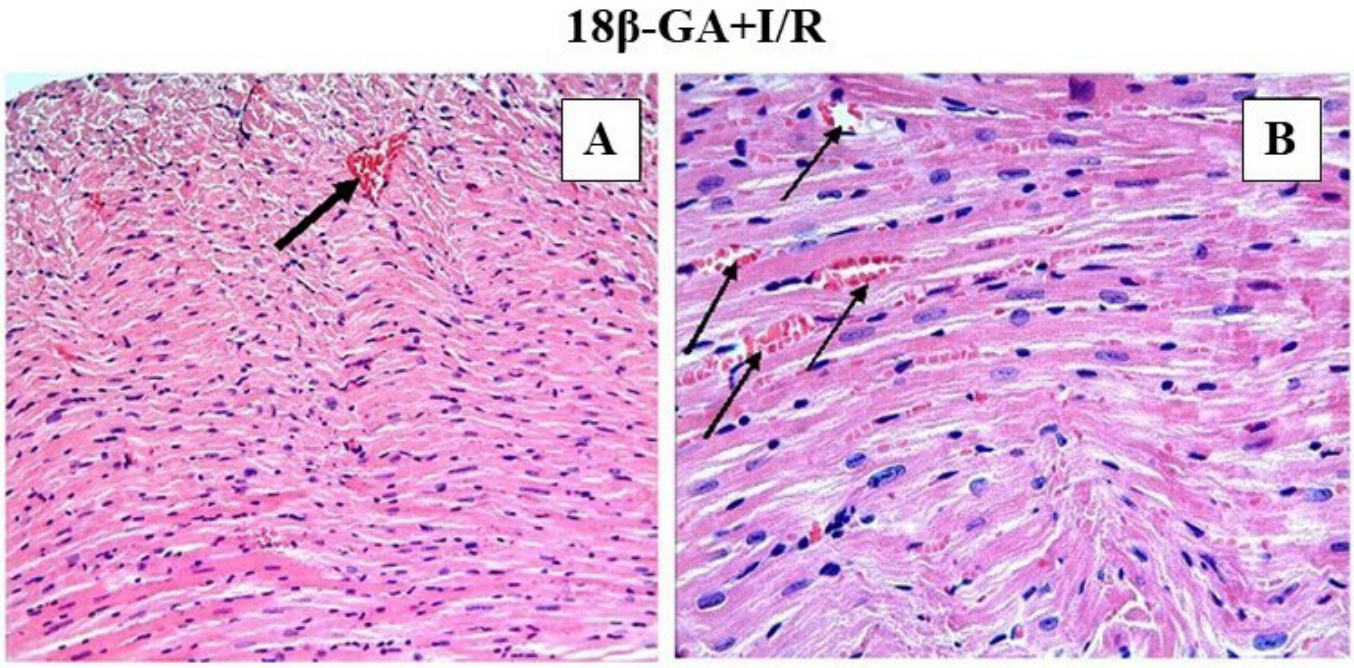

FIGURE 3 -Little vascular congestion (black arrow) (A), hemorrhage (thin black arrow) (B) observed in 18*β*-GA+I/R group. A: H-E; X20, B: H-E; X40.

DISCUSSION

Restoration of blood flow after global cerebral ischemia causes permanent tissue damage. The damages in different tissues and organs have been reported after I/R induction. Damage in distant organs such as kidney, lung and intestines after global cerebral I/R were examined. In the present study, the ameliorating effects of 18β-GA on the oxidative and histopathological damage were investigated in the heart tissue after global cerebral I/R induced on mice. It has been shown that lipid peroxidation in the heart tissue significantly increased after I/R. The 18β-GA treatment reduced lipid peroxidation in the tissues.

After creating the I/R model, it was shown that 18β-GA had protective effects on the heart. The experimental I/R model is used to reveal damage in different tissues and to develop amelioration models. The experimental studies have focused on reducing oxidative damage that occurs after I/R. The protective effects of different antioxidants have been investigated in recent studies (Dai, Cheng, Li, 2015; Yaman *et al*., 2007). It is considered that 18β-GA is a strong antioxidant molecule that induces the enzymatic and non-enzymatic antioxidant defense system in heart tissue.

Ischemic brain injury involves complex and highly integrated mechanisms such as oxidative stress, inflammation and apoptosis. (Danton, Dietrich, 2003; Lejay *et al*., 2016). The oxidative stress plays a key role in the pathogenesis of I/R injury (Sanderson *et al*., 2013). The mitochondrial transport system is the most important source of ROS. In addition to I/R mitochondrial damage, re-oxygenation by reperfusion induces the formation of ROS radicals such as superoxide and hydrogen peroxide (Ambrosio *et al*., 1991). ROS have been considered to be excessively produced during reperfusion following cerebral ischemia. ROS formation creates high TBARS levels that will cause damage to the cell membrane. However, the mechanism of I/R is multi-factorial and involves different biological mechanisms, such as immune activation, ion accumulation and toxic ROS production. Increased ROS levels lead to oxidative stress. Increased oxidative stress level and the inflammatory reaction in reperfused post-ischemic tissue can be so widespread that exposure of a single organ to ischemia and reperfusion may subsequently cause inflammatory activation in distant non-ischemic organs, eventually leading to multiple organ dysfunction (Park *et al*., 2011; Nishikata, Kato, Hiraiwa, 2014). Ischemia and limited oxygen damage the endothelial tissues covering the blood vessels, and heart tissues are severely affected by this condition (Ogawa *et al*., 1992).

Free radical formation that occurs after I/R causes oxidative stress and subsequent brain damage. The protective effects of protocatechuic acid, formed as a result of the metabolization of antioxidants in green tea, on the experimental I/R model have been demonstrated. The improvements observed in antioxidant enzyme activity are consistent with the results of our study. It has been shown that it 18β-GA can prevent tissue damage by affecting ROS formation (Kho *et al*., 2018). In the current study, the administration of 18β-GA for 10 days had beneficial effects on the antioxidant defense system elements, GPx, CAT, SOD and GSH. It was reported in the literature that 18β-GA treatment administrated for ten days following experimental I/R significantly decreased TBARS levels in heart tissues (Pinelli *et al*., 2009). Besides, there seemed to be a close relationship between TBARS and cardiac necrosis markers.

Cerebral I/R is a multi-factorial disease in which oxidative stress, inflammation and apoptosis play a role. Consistent with previous studies, a significant decrease in the heart tissue GSH level was observed after I/R (Ciftci, Oztanir, Cetin, 2014). Reduction in the total glutathione level is one of the symptoms of oxidative stress following I/R damage. Also, the exacerbated TBARS levels detected after I/R exposure are in agreement with earlier studies (Yang *et al*., 2013; Ciftci, Oztanir, Cetin, 2014). An imbalance between TBARS levels and antioxidant defense systems is anticipated to play a vital role in I/R injury. Similiar studies have shown the biochemical and histopathological beneficial effects of chrysin with experimental I/R on the C57BL/J6 mice model (Durak *et al.,* 2016).

The present study is the first to investigate the the relationship between global cerebral I/R and 18β-GA on the heart. Biochemical parameters and histopathological changes were compared between the I/R group and the other

groups. Also, the I/R group showed distinctive appearance of cardiotoxicity with various degrees of focal damages including eosinophilic stained and pyknotic nuclei cells, vacuolisation, hemorrhage, mononuclear cell infiltration, congestion, and necrosis. Previous studies indicated that 18β-GA administration decreased histopathological changes that occurred with I/R injury (Ciftci, Oztanir, Cetin, 2014; Durak *et al*., 2016).

18ß-GA has been demonstrated to possess several beneficial pharmacological activities, which include anti-inflammatory, antiviral, anti-tumorigenic, anti-ulcerative, and antioxidant effects, in vitro and in vivo (Kalaiarasi, Pugalendi, 2011). The protective effects of increased antioxidant response against I/R damage have been shown in previous studies (Patel *et al*., 2014). In the current study, the toxic effects caused by global cerebral ischemia were reduced with the 18β-GA treatment. 18β-GA significantly increased the activities of antioxidant enzymes (SOD, CAT, GSH, GPx) that were decreased in the experimental I/R model. The histopathological damage caused by ischemia has also improved. The results of our study revealed that 18β-GA with its antioxidant properties reduced the oxidative stress and histopathological changes caused by I/R.

CONCLUSION

The present study indicated that 15 min of cerebral I/R in the mice results in adverse effects associated with increases in oxidative stress and histopathological changes in the heart tissue. In summary, the treatment of cerebral I/R injury with 18β-GA was effective in reducing heart damage. The adverse progress in tissue oxidative stress markers and histopathological changes detected in this study point out that the 18β-GA treatment may be a convenient approach to prevent I/R injury. The beneficial effects of 18β -GA are considered intimately linked with the removal of lipid peroxidation and improvement of antioxidant enzyme activities.

ACKNOWLEDGEMENT

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors. The authors declare that they have no conflict of interest.

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Received for publication on 23rd March 2021
Accepted for publication on 04th July 2021