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Research article

Effects of pycnogenol on ischemia/reperfusion-induced inflammatory and oxidative brain injury in rats

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ABSTRACT

Background: Ischemia/reperfusion (I/R) injury results from the onset of re-circulation following a perfusion deterioration period in the tissues, resulting in more damage than that caused by perfusion deterioration. This study aimed to determine the effects of pycnogenol on I/R injury in rat brain tissues.

Methods: Eighteen albino Wistar rats were divided into three groups: I/R injury (IR, n = 6) group; I/R injury + pycnogenol (IR + P, n = 6) group; and sham group (SG, n = 6). After 30 min of transient ischemia, 24 h of reperfusion was achieved in the IR and IR + P groups. Surgical dissection, except for transient ischemia, was performed in SG. Next, histopathological and biochemical investigations were performed on brain tissues. Malondialdehyde (MDA), reduced glutathione (GSH), and glutathione peroxidase (GPO) were analyzed as oxidative stress markers; IL-1β and TNF-α were analyzed as inflammatory stress markers in biochemical tests.

Results: Histopathological examination revealed normal morphology in SG and diffuse cortex damage with edema, vasopathology, and inflammatory cell infiltration in the IR group. The IR + P group showed less cortex damage, edema, and vasopathology than the IR group. The MDA, IL-1β, and TNF-α levels were significantly higher in the IR group than those in the SG group. The values of same markers for the IR + P group were significantly lower than the IR group. The GSH and GPO levels were significantly decreased with IR damage, but PYC treatment showed significant improvement in the levels.

Conclusion: This study showed that the administration of pycnogenol ameliorated brain damage after I/R injury by reducing oxidative and inflammatory damage in the rat brain.

1. Introduction

Ischemia/reperfusion (I/R) injury is tissue damage caused by re-feeding after an ischemic or oxygen deprivation period [1–4]. The changes that occur during the absence of oxygen and other nutrients induce oxidative and inflammatory damages initiated by oxidative stress in the tissues rather than returning to the normal functions [1,4–6].

I/R injury may develop after thrombolytic therapy or mechanical endovascular thrombectomy following acute ischemic stroke, carotid endarterectomy, and vascular stenting [7–9]. I/R injury result in radiological features including edema or intracerebral hemorrhage [7]. Hemorrhagic transformation shows poor prognosis. In such cases, mortality reaches up to 63%. In addition, 80% of the survivors

Abbreviations: I/R, ischemia/reperfusion; PYC, pycnogenol; SG, SHAM group; MDA, malondialdehyde; GSH, reduced glutathione; GSSG, oxidized glutathione; GR, glutathione reductase; GPO, glutathione peroxidase; IL, interleukin; TNF, tumor necrosis factor; DTNB, dithiobis(2-Nitrobenzoic acid); TBA, thiobarbituric acid

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constitute morbidity [10].

Pycnogenol(PYC) is a herbal medicine with antioxidant properties [11–13]. The neuroprotective effects of PYC in Alzheimer's disease [14] and traumatic brain injury [15,16] have been demonstrated. Moreover, the protective effects were demonstrated in myocardial [17,18] and renal reperfusion injury [19], which may suggest that PYC may also be effective in the cerebral I/R injury. However, there are no studies in this regard. Our study seeks answers to this question.

2. Material and methods

Experimental animals were obtained from the Ataturk University Medical Experimental Application and Research Center. A total of 18 albino Wistar male rats weighing 250–265 g were randomly selected for use in the experiment. In addition, 3 groups of 6 animals each were formed in each group. The animals were housed and fed at normal room temperature (22 °C) before the experiment. The protocols and procedures were approved by the local Animal Experimentation Ethics Committee of Ataturk University. (Project approval no: 752963309-050.01.04-E.1800265252, Date: 19/09/2018).

The experimental animals were divided into 3 groups: Group with ischemia/reperfusion injury in the brain (IR), group administered with 40 mg/kg PYC with ischemia/reperfusion injury in the brain (IR + P), and the sham group (SG). PYC was provided from Pycnogenol®, Solgar, USA.

All surgical procedures were performed under sterile conditions in appropriate laboratory conditions. PYC was administered by gavage at a dose of 40 mg/kg one hour prior to anesthesia to the IR + P group. The IR and SG groups received the same volume of distilled water. Anesthesia was achieved with 50 mg/kg ketamine hydrochloride intraperitoneally. Cerebral I/R injury method was performed as described in previous report [20]. The procedure was performed under microdissection using a midline incision. The trachea was noticed, the paratracheal muscles were dissected, and the common carotid arteries (CCA) were reached. Subsequently, bilateral CCA was temporarily occluded for 30 min with microclips. The rats in the SG group underwent all procedures except for the microclip application. At the end of 24 h after reperfusion, the animals were sacrificed by high-dose anesthesia. The cerebral tissues were examined histopathologically. Biochemical analysis of malondialdehyde, reduced glutathione, glutathione peroxidase, tumor necrosis factor-alpha, and interleukin-1β levels were performed on cerebral tissues.

2.1. Histopathological examination

All tissue samples were first identified in a 10% formaldehyde solution for light microscopic assessment. Following the identification process, the tissue samples were washed under tap water in cassettes for 24 h. The samples were then treated with conventional grade of alcohol (70%, 80%, 90%, and 100%) to remove the water within the tissues. The tissues were then passed through xylol and embedded in paraffin. Four-to-five micron sections were cut from the paraffin blocks and hematoxylin–eosin staining was administered. Their photos were taken following the Olympus DP2-SAL Firmware Program (Olympus® Inc. Tokyo, Japan) assessment. Histopathological assessment was performed by the pathologist blind for the study groups.

2.2. Malondialdehyde (MDA) analysis in tissues

The method described by Ohkawa et al. was used in MDA analysis [21]. Spectrophotometric analysis of absorbance of the pink-colored mixture composed by thiobarbituric acid(TBA) and MDA was performed. The tissue homogenate sample (0.1 mL) was mixed with a dilution including 1.5 mL of 8 g/L TBA, 1.5 mL of 200 g/L acetic acid, 0.2 ml of 80 g/L sodium dodecyl sulfate, and 0.3 mL distilled water. The admixture was incubated for 1 h at 95 °C. After cool down, 5 mL of n-butanol:pyridine (15:1) was supplemented. Subsequently, The mixture was swirled for 1 min and centrifuged at 4000 rpm for 30 min. The absorbance of the supernatant was evaluated at 532 nm. The standard curve was acquired with 1,1,3,3-tetramethoxypropane.

2.3. Reduced glutathione (GSH) analysis in tissues

Glutathione analysis was performed by the Sedlak and Lindsay's method [22]. Dithiobis(2-Nitrobenzoic acid) (DTNB) disulfide is chromogenic in the medium, and DTNB is reduced with ease by sulfhydryls. The yellow color generated through the reduction is measured by spectrophotometry at 412 nm. For analysis, a mixture with 80 µL 625 U/L glutathione reductase, 2.8 mL 1 mM DTNB, 3.75 mL 1 mM NADPH, and 5.85 mL 100 mM Na-phosphate buffer was formed. Before analysis, deproteinization was achieved with mixture of 0.1 mL meta-phosphoric acid and 0.1 mL tissue homogenate that centrifuged at 2000 rpm for 2 min. The 0.15 mL previously formed solution was mixed with 50 µL of supernatant. The standard curve was acquired with GSSG.

2.4. Glutathione peroxidase (GPO) analysis in tissues

The activity of the GPO enzyme was measured using Lawrence and Burk's method [23]. A mixture was formed by adding potassium phosphate buffer, EDTA, GSH, NADPH, NaN_3, and GR to the tissue sample, followed by incubation. The supernatant obtained after homogenization was used to determine the GPO enzyme activity. Spectrometric measurements after addition of H_2O_2 to the supernatant were considered as a result of enzyme activity.

2.5. IL-1β and TNF-α analysis in tissues

The measurements were made following the manufacturer's recommendations. In the tissues, the IL-1β and TNF-α concentrations were evaluated with Rat-specific sandwich enzyme-linked immunosorbent assay Rat Interleukin-1β ELISA Kit (Cat no: YHB0616Ra, Shanghai LZ) and Rat Tumor Necrosis Factor α ELISA kits (Cat no: YHB1098Ra, Shanghai LZ). In these kits, the rat IL-1β and TNF-α specific monoclonal antibodies are located on microplates. The mixture of tissue homogenate, biotinylated monoclonal antibody, and streptavidin-HRP was placed into the kits and then incubated. After washing, chromogen reagent A and chromogen reagent B were added, and the incubation was repeated. Later, the stop solution was added to mixture. After the process, the plates were read by spectrophotometrically via microplate reader(Bio-Tek, USA). The values obtained were accepted as the measurement results for IL-1β and TNF-α.

2.6. Statistical analysis

The results were presented for continuous variables as mean ± standard deviation. Each group consisted 6 animals. For comparison of groups Kruskal Wallis test was used with Dunn's test as a post-hoc test. The statistical level of significance for all tests was considered 0.05. Statistical analysis was performed using the IBM SPSS ver. 19 package program (IBM Corp. Released 2010. IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corp.)

3. Results

3.1. Histopathological findings

According to the microscopic evaluations of cerebral cortex tissues, the structure and morphology of neurons, glial cells, and blood vessels were normal in the SG (Fig. 1a and b). In the IR group, microscopic examinations revealed diffuse cerebral cortex damage, including pericellular edema, increased microglial cell existence suggestive of apoptosis in the cortex neuronal/glial cells, dilated and congested blood

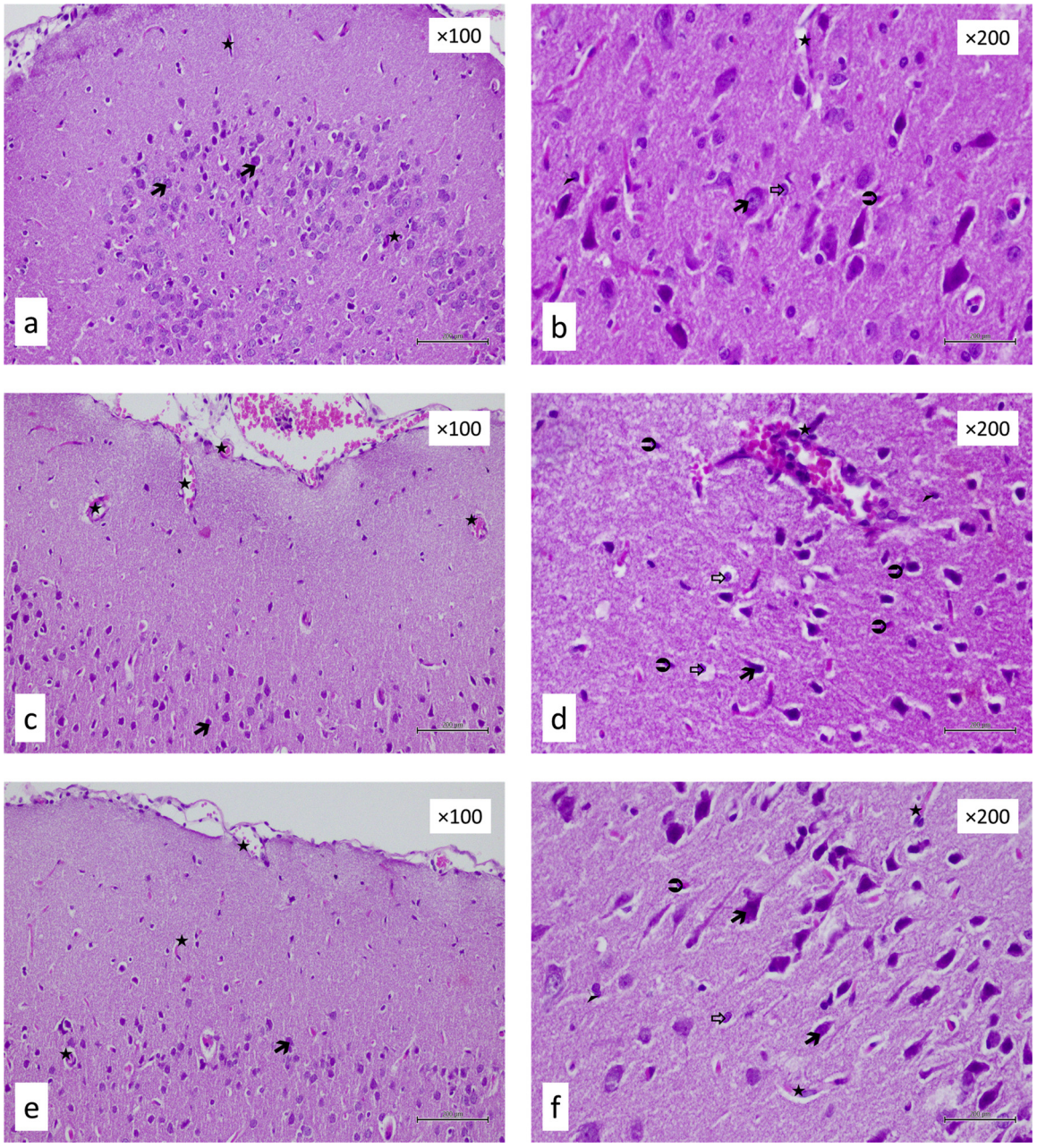

Fig. 1. (a) Hematoxylin–eosin staining in cerebral cortex tissue in the control group; ➔: neuron, ★: blood vessel, x100. (b) Hematoxylin–eosin staining in cerebral cortex tissue in the control group; ➔: neuron, ⇨: astrocyte, ➢: oligodendrocyte, ➲: microglia, ★: blood vessel, x200. (c) Hematoxylin–eosin staining in cerebral cortex tissue in the ischemia/reperfusion group; ➔: neuron, ★: congested and dilated blood vessel, x100. (d) Hematoxylin–eosin staining in cerebral cortex tissue in the ischemia/reperfusion group; ➔: pericellular edema in neuron, pericellular edema in ⇨: astrocyte, ➢: oligodendrocyte, ➲: increased microglial cells, ★: congested and dilated blood vessel, x200. (e) Hematoxylin–eosin staining in cerebral cortex tissue in the ischemia/reperfusion + taxifolin group; ➔: neuron, ★: decreased congestion in blood vessel, x100. (f) Hematoxylin–eosin staining in cerebral cortex tissue in the ischemia/reperfusion + taxifolin group; mild pericellular edema in ➔ : neuron, ⇨: astrocyte and ➢: oligodendrocyte, ➲: microglial cell, ★: decreased congestion in blood vessel, x200.

vessels (Fig. 1c and d). In IR + P group, marked amelioration of cerebral cortex damage was noticed. In this experimental group, the sections showed mild pericellular edema, normal intensity of microglial cells, and decreased congestion in the blood vessels (Fig. 1e and f).

3.2. Biochemical results

The results of all markers are shown in Table 1 and Figs. 2 and 3.

3.3. Oxidative stress markers

When the IR group was compared with SG, a significant increase ($p < 0.001$) in MDA, an oxidative stress marker, was noted. In the IR + P group treated with PYC before I/R injury, the MDA levels were significantly decreased as compared to the IR group($p = 0.002$).

When the activity of GPO enzyme, an enzymatic endogenous antioxidant, was evaluated, a significant decrease was noted in the IR group activity in comparison to that in SG ($p < 0.001$). Similarly, the level of GSH, a non-enzymatic antioxidant, was significantly lower in the IR group as compared to that in the SG group ($p < 0.001$). On the other hand, comparison of both the IR + P and IR groups, both GSH ($p < 0.001$) and GPO ($p < 0.001$) values were elevated in favor of the IR + P group.

Table 1
Effects of pycnogenol on oxidative and inflammatory biochemical parameters in the cerebral tissue of experimental groups.

Parameters	SHAM–Group (mean ± SD)	IR Group (mean ± SD)	IR + P Group (mean ± SD)
MDA (nmol/g tissue)	2.91 ± 0.59	5.70 ± 0.26	4.27 ± 0.77
GSH (nmol/mg protein)	4.81 ± 0.51	2.20 ± 0.51	3.94 ± 0.41
GPO (U/mg protein)	4.60 ± 0.34	1.98 ± 0.45	3.38 ± 0.34
TNF-α (pg/ml)	1.29 ± 0.11	5.76 ± 0.39	2.61 ± 0.39
IL-1β (pg/ml)	0.84 ± 0.16	5.51 ± 0.44	2.55 ± 0.53

IR: ischemia/reperfusion, IR + P: ischemia/reperfusion + pycnogenol, MDA: malondialdehyde, GSH: reduced glutathione, GPO:glutathione peroxidase, TNF- α: tumor necrosis factor-alpha, IL-1β: interleukin-1 beta, SD: Standard deviation.

3.4. Proinflammatory stress markers

When the levels of IL-1β and TNF-α from protein cytokines were examined in terms of inflammatory response, a significant increase in the IR group compared to the SG group for both the mediators ($p < 0.001$) was noticed. At the same time, the IR + P group was significantly lower than the IR group for both the mediators ($p < 0.001$).

4. Discussion

PYC is a specific concoction of bioflavonoids refined from the bark of the French maritime pine (Pinus pinaster Aiton) [11,24]. Flavonoids, oligomeric procyanidins, and polyphenols are the main components of PYC [25]. Besides, PYC is an efficient antioxidant [12,13]. The antioxidant activity is the result of its pharmacological effects on increasing the synthesis of antioxidant enzymes, providing restoration of vitamins C and E as well as acting as free radical scavenger [11]. In addition, it was advocated that PYC has beneficial effects in various medical conditions, such as asthma, venous insufficiencies, diabetes, hypertension, melasma, migraine, osteoarthritis, high cholesterol, retinopathy, and systemic lupus erythematosus [11,24].

The neuroprotective effect of diverse antioxidants in cerebral I/R injury has been demonstrated. α-lipoic acid, an endogenous short-chain fatty acid and oxygen radicals scavenger, has been shown to protective against cerebral I/R injury by reducing of oxidative stress and caspase-dependent apoptosis [26]. Oleuropein, an antioxidant agent that is obtained from olive leaves, has been shown to protective toward the apoptosis and infarct volume in cerebral I/R injury [27]. In addition, several antioxidants are suggested to attenuate damage after intracerebral hemorrhage (ICH). Edaravone, a novel antioxidant, has been shown to decrease ICH-induced brain edema, neurological deficits and oxidative injury [28]. Sulforaphane, obtained from cruciferous vegetables, has been shown to stimulates microglia function and hematoma resorption by activating the antioxidative defense components [29].

Reperfusion of ischemic tissues causes microvascular dysfunction. Vasodilatation of arterioles was seen with endothelial deterioration [1,30], resulting in fluid accumulation, leukocyte migration, and extravasation of plasma proteins [1]. The activation of endothelial cells increases the production of oxygen radicals and produces less nitric oxide. The resulting oxidative stress causes the secretion of inflammatory mediators [4]. This process results in neuronal damage and edema, leading to worse damage than initiated with ischemia [31]. In our study, when the I/R injury was created, edema, blood vessel dilatation, and microglial cell infiltration were observed with significant cortical damage.PYC treatment decreased edema and neuronal cell damage and improved vasopathology. In a study investigating the effect of PYC on I/R injury on kidney tissue, it was shown that edema, inflammatory cell infiltration, and glomerular damage were ameliorated by PYC treatment [19]. Similar to these results, we observed significant amelioration of cerebral cortex damage in the I/R + P group.

TNF-α is a protein cytokine that has several biological functions, especially effective in inflammatory and immune reactions. This cytokine is effective in the development of inflammatory responses by acting on acute phase protein secretion, endothelial cell permeability, surface adhesion molecules, and other inflammatory mediators [32,33]. These changes induce leukocyte infiltration and neuronal damage in the tissues during cerebral ischemia. Studies have shown that TNF-α causes increase in the astrocyte counts and astrocyte-induced cytotoxicity, resulting in oligodendrocyte damage [34,35]. A study showed that TNF-α increased to peak after 12 h after focal ischemia/reperfusion injury in the brain, reaching a plateau value within 5 days [36]. In our study, histopathological data showed inflammatory processes and tissue damages in the cerebral cortex tissues in the IR group. It was noted that the TNF-α values were significantly increased in the IR group as compared to that in the SG group in tissue samples in this area. With PYC application, the TNF-αvalues were significantly reduced and histopathological results showed that tissue damage was significantly avoided. The study that investigated the effect of PYC on IR injury in kidney tissue found that TNF-α values increased significantly after injury. In the same study, it was found that PYC administration caused regression of values as in our study [19]. In another study regarding the neuroprotective effect of PYC on traumatic brain injury, it was observed that the post-injury TNF-α values were significantly increased and the values were decreased with treatment [15].

IL-1 is a proinflammatory cytokine involved in several pathologies [37]. IL-1 is effective in the expression of multiple mediators, inflammatory cell infiltration, and tissue damage [38]. IL-1 α and IL-1 β are the sub-members of IL-1. They bind to the same receptor. In a study, I/R injury was created on brain tissue, and IL-1 was found to play a major role in the damage. In the same study, IL-1β was shown to be more effective than IL-1 α in damages [39]. In a study that applied I/R injury in renal tissues, the IL-1 β levels were found to reach the maximum level in 24 h [40]. IL-1β converting enzyme provides the formation of mature form of IL-1 β. It has been shown to reduce the ischemic brain injury in the case of blocking the IL-1β converting enzyme [41,42]. In our study, IL-1 β levels were found to increase when I/R injury was created. With the application of pycnogenol, it was observed that the IL-1β levels decrease with the amelioration of tissue damage and inflammatory response. The results of Sehirli et al. were consistent with our results. It was observed that IL-1 β values increased when I/R damage created and decreased after PYC administration [19]. In a randomized controlled trial of severe osteoarthritis, PYC therapy was found to significantly decrease the IL-1 values by anti-inflammatory effect [43].

Oxidative stress is known to have great efficacy during ischemia/reperfusion injury [44]. Lipid molecules are among the most affected biomolecules from oxidative stress. MDA is an oxidative stress product that occurs during polyunsaturated fatty acid peroxidation [45,46]. MDA is known to cause degradation of DNA and protein structures due to its mutagenic properties [47]. Previous studies have shown increased levels of MDA when the I/R injury is induced in brain tissues [48]. In our study, the MDA values were significantly increased when the I/R injury was generated. When the PYC treatment was applied, MDA values were decreased. Likewise, there are similar results of the effect of PYC on lipid peroxidation products in I/R injury in the literature. Studies on cardiac [17] and kidney tissues [19] demonstrate that PYC

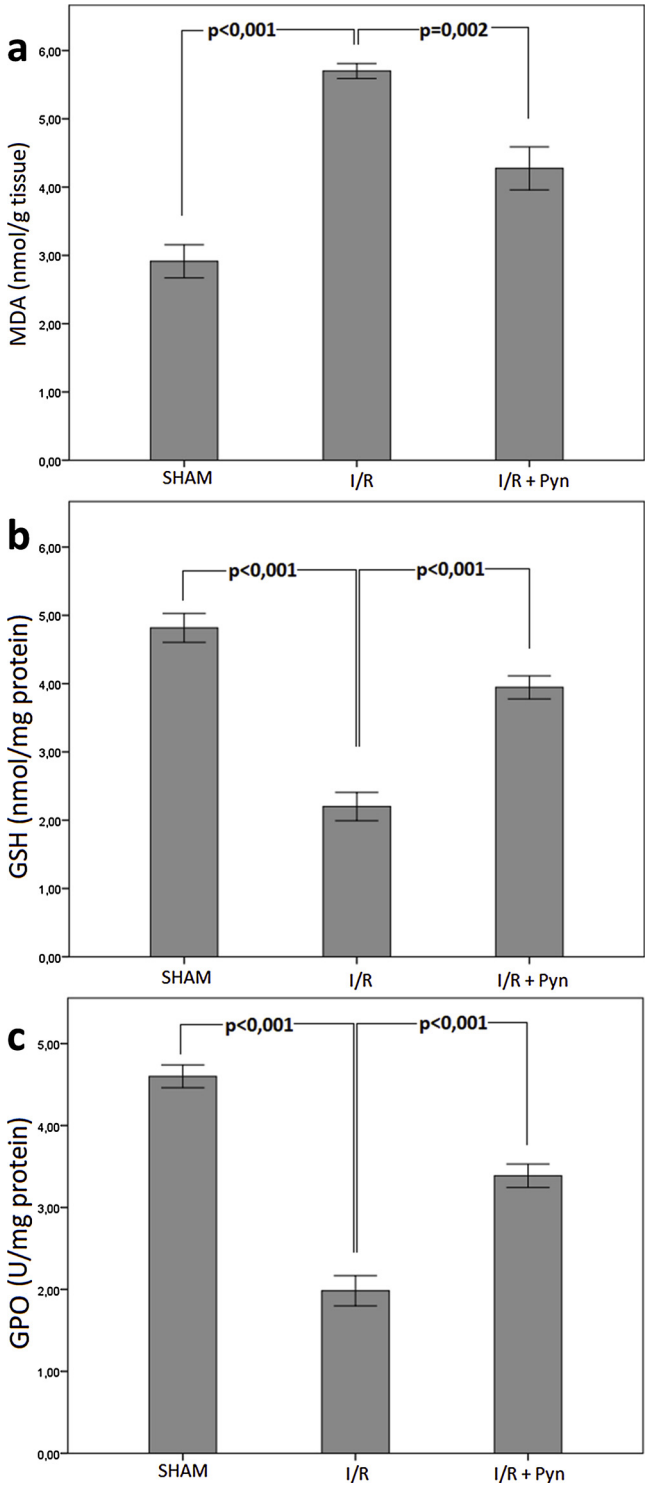

Fig. 2. (a) Malondialdehyde (MDA), (b) reduced glutathione (GSH) and (c) glutathione peroxidase (GPO) levels in the brain tissue of I/R, I/R + P and sham groups. Each experimental group consists of six rats. The statistical differences between the SG and I/R group and between the I/R and the I/R + P groups are shown on the figure.

treatment decreases the MDA levels. In an another study, in which the neuroprotective effect of PYC was investigated by glucose-oxygen restriction and re-oxidation on rat astrocyte cells, the MDA levels were found to decrease significantly with treatment [49].

In case of oxidative stress, free oxidant radicals appear in the cell [50]. These molecules lead to changes in the cell, such as protein damage, deterioration of the DNA structure, leading to a process that results in apoptosis and tissue damage [51]. Glutathione plays an important role in the elimination of these radicals. Glutathione is an endogenous tripeptide. It is present in 2 different forms as reduced glutathione (GSH) and oxidized glutathione (GSSG) [52]. When free radicals increase in the cell, the reduction of GSSG to GSH is catalyzed by the glutathione reductase enzyme in order to reduce these radicals. While the GSH/GSSG ratio is above 100 in the resting state, this ratio can decrease to between 1 and 10 under oxidative stress situations [53,54]. A high rate of free radicals was also noted in cerebral I/R injury, resulting in edema and vascular pathology [55]. This process causes depletion of GSH depots. Glutathione depletion in neuronal tissues is both a result of oxidative stress and a cause of increased oxidative stress-related damage [56], which may suggest a restoration of GSH, leading to improvement in I/R injury. In addition, in a study of I/R injury on rabbit heart, the administration of GSH showed improvement in cardiac contractile functions and oxidative damage [57]. In our study, the GSH values decreased significantly in I/R injury, but PYC treatment resulted in improved GSH values. Our results also support this idea. Owing to the antioxidant effect of pycnogenol, the improvement in histopathological results and GSH elevation were observed together. Other studies investigating the effect of PYC on I/R injury have shown similar results. Renal I/R injury study [19], and cardiac I/R injury study [17] show that the GSH levels were markedly decreased with I/R injury, but the levels were improved with PYC treatment. In an another study in which the neuroprotective effect of PYC was investigated by experimental seizures on mice, the treatment provided a significant improvement in the GSH values [58].

Glutathione peroxidase (GPO) is a selenium-dependent antioxidant enzyme that catalyzes the reduction of hydrogen peroxide and lipid peroxides to water and lipid alcohols by oxidation of GSH to GSSG [52]. Studies have shown that this enzyme has an effect on the damage level of I/R injury. A study revealed that the infarct area and apoptosis were significantly increased with I/R injury in knockout applied mice in GPO gene locus [59]. Another study in which the focal cerebral I/R injury was performed on transgenic mice showed that the infarct volume and edema were less in transgenic mice with GPO overexpression than in non-transgenic samples [60]. Our study was consistent with this literature. I/R injury caused a decrease in the enzyme values, with more severe damage and edema in the brain tissues. PYC treatment resulted in the improvement in neuronal damage and levels of enzyme. In addition, the effect of PYC was investigated in ethanol-induced cerebellar granule cells, and it was observed that the treatment significantly improved the GPO activity [61]. Moreover, research on the effect of PYC on cardiac I/R injury has reported results on the GPO levels consistent with those in our study. It was observed that the GPO values decreased with I/R injury and improved when the PYC treatment added [17].

5. Conclusions

In the present study, when the ischemia and reperfusion were applied on rat brain tissues, it was shown that brain damage occurred by oxidative and inflammatory stress formation, and biochemical investigations revealed a significant alteration in the oxidative and inflammatory stress markers. Histopathology also reveals findings consistent with that of brain injury in I/R injury-induced rats. Our findings also showed that PYC produces a neuroprotective effect by decreasing the oxidative and inflammatory stress markers when administered before the formation of I/R injury. Histopathological examinations also support the protective effect of PYC against brain damage.

To the best of our knowledge, this study is the first study to investigate the effect of PYC on I/R injury in brain tissues. The neuroprotective effect of PYC on I/R injury was demonstrated on rat brains in this study. We expect that the present study will provide further insight in clinical studies on the amelioration of cerebral I/R injury.

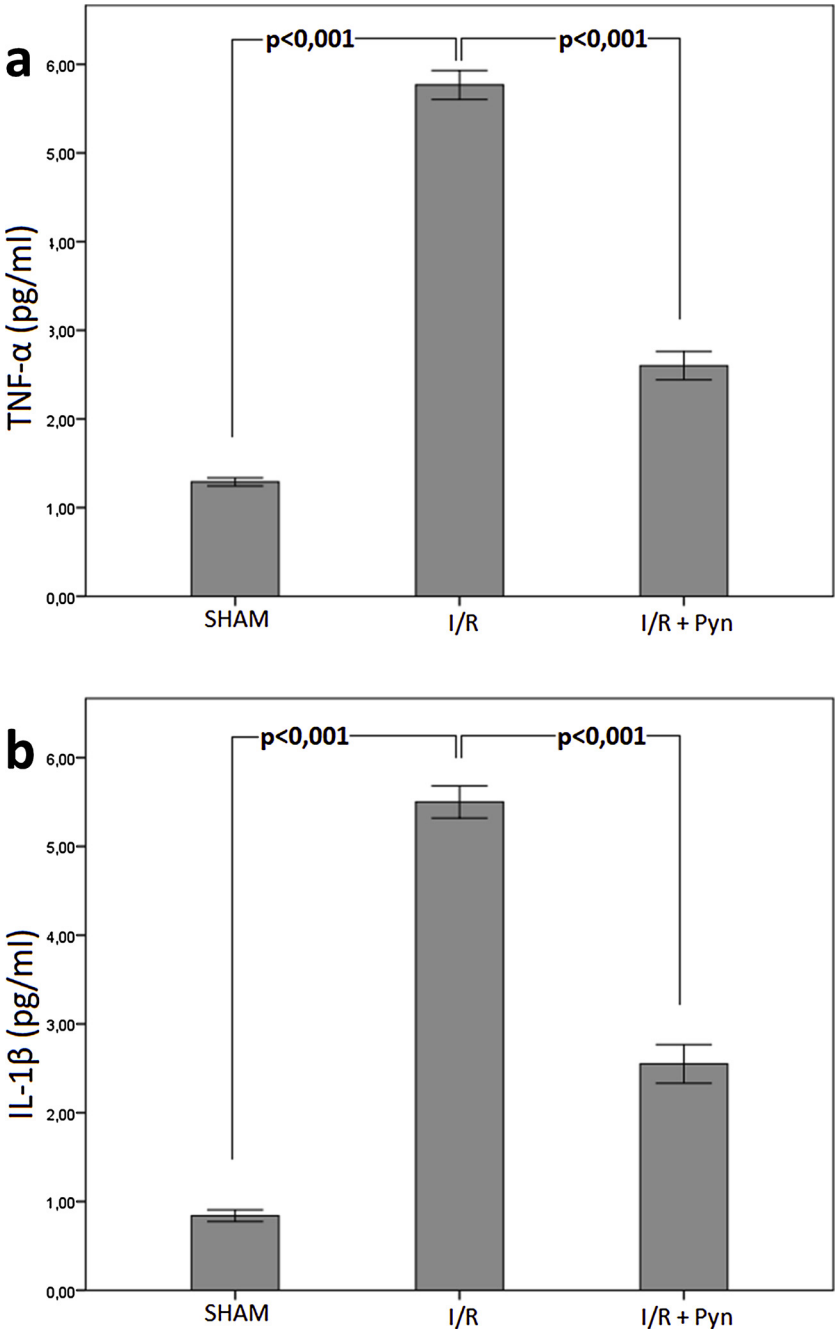

Fig. 3. (a) TNF-α and (b) IL-1β levels in the brain tissue of I/R, I/R + P and sham groups. Each experimental group consists of six rats. The statistical differences between the SG and I/R group and between the I/R and the I/R + P groups are shown on the figure.

Declarations of interest

None.

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