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ORIGINAL PAPER

The effect of regular exercise on antioxidant enzyme activities and lipid peroxidation levels in both hippocampi after occluding one carotid in rat

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Abstract Regular exercise has beneficial effects on cerebrovascular diseases; however, its biochemical mechanisms are not fully known. The purpose of this study was to determine antioxidant enzyme activities and lipid peroxidation of both hippocampi after applying exercise followed by occluding one common carotid. Wistar rats were divided into four groups of control, exercise, hypoperfusion and exercise–hypoperfusion (exe-hypo). In the exercise and exe-hypo groups, the rats were forced to run on a treadmill for 1 h a day for 2 months. The right common carotid of the animals in the (exe-hypo) group was occluded after the cessation of exercise. Surgery without occlusion of the carotid was applied on the control (without exercise) and exercise groups. All animals were sacrificed 1 and 24 h after surgery. The levels of malondialdehyde (MDA) and antioxidant enzyme activities in the hippocampi were measured. A significant interaction was observed between the exercise and hypoperfusion in both hippocampi ($p < 0.05$). In comparison with the control group, there was significant elevation of catalase activity in the right and left hippocampus of the hypo group at 24 h ($p < 0.0001$). Regarding the differences between the hemispheres, there was a significant increase in MDA and decrease in catalase activity in the left hippocampus in hypoperfusion group, but the exercise in the exe-hypo group succeeded in abolishing these alterations which were caused by hypoperfusion, This study shows that exercise pre-conditioning prevents some alterations in brain oxidant–antioxidant status which are induced by cerebral hypoperfusion. Further studies are needed in order to clarify the mechanism of exercise.

Keywords Exercise · Treadmill · Hypoperfusion · Antioxidant enzyme · Lipid peroxidation

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Introduction

Recent studies suggest that physical exercise has beneficial effects on patients with cerebrovascular diseases [1–3]. It has been shown that regular physical activity reduces brain injury after acute focal cerebral ischemia [4, 5] and cerebral hypoperfusion [6] in animal models. Multiple mechanisms that underlie these effects have been reported, including enhancement of neurovascular unit integrity, increased neurotrophin expression, angiogenesis, and decreased inflammation and apoptosis [4, 7, 8]. Another proposed mechanism is the modulation of cellular oxidative status. A growing body of evidence suggests that oxidative injury plays a key role in the pathogenesis of

cerebrovascular diseases [9–13]. The nervous system exhibits unique vulnerability to the harmful effects of reactive oxygen species (ROS). Due to the active oxidative metabolism in the brain, large amounts of unsaturated fatty acids, high levels of free radicals, and low levels of antioxidants have been reported compared with other organs [13, 14].

Although exercise can induce free radical formation, which may be detrimental to cellular functions, it has been suggested that regular exercise causes fortification of the cellular antioxidant system, i.e., some authors have reported a significant increase in antioxidant enzyme activities, which increase resistance to oxidative stress, reducing cellular oxidative damage.

Nevertheless, the effects of exercise on oxidative injury or the levels of brain antioxidants remain controversial [15–18]. The literature suggests that contradictory outcomes may occur because of biases caused by the differences in the types, intensities, and duration of exercise protocols [18–20].

It has been shown that unilateral occlusion of a common carotid artery in an anesthetized rat instantly and significantly decreases the total cerebral blood flow (CBF) in both hemispheres [21]. On the other hand, the total blood flow returns to normal levels after 1 day, and this return is faster in the unoccluded side. This hypoperfusion model may emulate some human disorders, such as carotid artery disease including common carotid artery occlusion (CCAO).

A predominant cause of CCAO is atherosclerosis, and the less common causes include dissection of the aortic arch and CCA, postirradiation arteriopathy, aortic arch aneurysm, cardiac embolism, fibromuscular dysplasia, hypercoagulability, and craniocervical traumatism [22–24].

Carotid occlusion is also a necessary therapeutic procedure for the treatment of complex aneurysms, including giant, traumatic, and intracavernous aneurysms [22, 25]. In cases of CCAO, perfusion of the ipsilateral cerebral hemisphere is provided through the collateral circulation. The flow is intracranially maintained through the circle of Willis via the anterior and posterior communicating arteries [24].

Our study was designed to examine the effects of preconditioning exercise on antioxidant status within the right and left hippocampus after the occlusion of the right common carotid. The applied exercise has been previously shown to be neuroprotective [26, 27]. Nevertheless, the effects on the antioxidant status within the brain have not yet been assayed. We selected the hippocampi for this study because this region of the brain is most vulnerable to oxidative stress [28] and plays an important role in learning and memory.

In this study, we attempted to clarify the interaction of hypoperfusion and exercise on antioxidant enzyme activities in both hippocampi. According to the mentioned studies, we assume that (1) after occluding the right common carotid, antioxidant activity increases in both hippocampi in response to the reduction in CBF, (2) this enhancement is expected to be more persistent in the right hippocampus, and (3) exercise increases antioxidant enhancement in the hippocampus after inducing cerebral hypoperfusion. To the best of our knowledge, this is the first study to analyze both hippocampi pro-oxidant/antioxidant responses to unilateral carotid occlusion and preconditioning exercise.

Materials and methods

Animals

Male Wistar rats (weight 250–300 g) were accommodated four per cage and kept on a 12–12 h light–dark cycle in an air-conditioned room and maintained at (23 ± 1 °C) for at least 10 days before any experimental procedure. Food and water were provided ad libitum. Experimental procedures were conducted in accordance with the Guidelines for Reporting Animal Research [29]. The Ethic Committee for Animal Experiments at Lorestan University of Medical Sciences approved the study.

The animals were divided into four groups: the control group, the hypoperfusion group, the exercise group, and the exercise–hypoperfusion (exe-hypo) group ($n = 12$ for each experimental group).

Treadmill running

The rats in the exercise and exe-hypo groups were made to run at a speed of 17 m/min for 1 h a day, 5 days a week, for 11 weeks at 0° of inclination. A modified human treadmill was applied. A shock grid was made into each channel to enforce the animals to run. On the first day, the animals were left on the treadmill with no operation while the shocks were on. This was performed to acquaint the animals with the experimental set-up. Then, from the second day, the treadmill was switched on and its speed increased to 17 m/min gradually and the duration increased from 10 to 60 min for 10 days. From the 10th day, after warm-up, the speed and duration were maintained constant at 17 m/min. The running was performed between 9:00 and 12:00 a.m. The animals which were reluctant to run were excluded from the study. The nonrunner groups were put on the treadmill without running for the same duration as the runners.

Surgery

After the cessation of the exercise, the rats were anesthetized with chloral hydrate (400 mg/kg, i.p.). The right

common carotid arteries of the rats were carefully separated from their sheaths and cervical sympathetic and vagal nerves through a ventral cervical opening. The right common carotid arteries were permanently ligated with 4–0 type surgical silk in the hypoperfusion and exe-hypo groups, whereas they were not ligated in the sham-operated (control and exercise) groups. Lidocaine (1 %) was applied as a local anesthetic. The operation was performed on a heating pad (Harvard Apparatus, USA) to maintain body temperature at 37.0 ± 0.5 °C.

Determination of biochemical factors

Every experimental group was divided into two subgroups. One group after 1 h ($n = 6$), and the other after 24 h ($n = 6$) were sacrificed after surgery. Then, the brains of all the animals were separated on an ice-cold surface and kept frozen (−70 °C) until further processing. On the day of experiment, after the hippocampi were thawed (+4 °C) and separated, they were weighed, and homogenized three times for 5 s at 25,000 rpm with the use of ULTRA-Turrax T25 homogenizer in 10 volumes of ice-cold homogenization buffer (30 mmol/l KH2PO4, 5 mmol/l EDTA, 0.2 M tris–HCl buffer and 0.3 mmol/l PMSF (phenylmethylsulphonylfluoride), pH 7.4).

Half of the homogenate was centrifuged at 10,000*g*, 4 °C for 15 min, and used for the total antioxidant analysis and protein concentration measurement. Total antioxidant was measured by Cayman's Antioxidant Assay Kit according to the manufacturer's instruction. The protein concentration was determined by the method of Lowry et al. [30] with the use of bovine serum albumin as standard.

The other half was divided into 3 independent microtubes containing different buffers. The buffer of the first tube was (HEPES (20 mM), EDTA (1 mM), Manitol (20 mM) and Sucrose (70 mM)) for SOD measurement. The buffer of the second tube was potassium phosphate (50 mM) and MEDTA (1 M) for catalase measurement, and the third tube contained RIPA buffer for thiobarbituric acid reactive substances (TBARS) measurement. All the buffers had pH = 7. The homogenate was centrifuged at 8,000*g* for 5 min at 4 °C to separate the nuclear debris, and the supernatant was collected. The supernatants were diluted by potassium phosphate buffer pH 7.4 to the final concentration of 1 mg Prot./ml and were subjected for the assay of free radical metabolizing enzymes including catalase, SOD and TBARS (all Cayman, USA) according to the manufacturer's instruction.

Total antioxidant assay

The combined antioxidant activities of aqueous and lipid soluble antioxidants were assessed. The assay [31] relies on the ability of antioxidants in the sample to inhibit the oxidation of ABTS (2, 2′-Azino-di-[3-ethylbenzthiazoline sulphonate]) to ABTS$^{®}$·+ by metmyoglobin. The amount of ABTS$^{®}$·+ produced was monitored by reading the absorbance at 405 nm (μmol/mg protein). Trolox, which is a water-soluble vitamin E analog, was applied as a standard or control antioxidant.

TBARS determination

Lipid peroxidation was determined by the method of Chatterjee et al. [32] by estimating TBARS.

A 100-μl aliquot of homogenate was added to a reaction mixture containing 200 μl 8.1 % (w/v) lauryl sulphate, 1.5 ml 20 % (v/v) acetic acid, 1.5 ml 0.8 (w.v) thiobarbituric acid, and 700 μl distilled water. The samples were then boiled for 1 h at 95 °C and centrifuged at 3,000*g* for 10 min. The absorbance of the supernatant was measured spectrophotometrically at 650 nm. 1, 1, 3, 3-tetraethoxy propane, which is a form of MDA, was used as standard in this assay. Lipid peroxidation was expressed as MDA μmol/mg protien.

Catalase activity assay

Catalase activity was spectrophotometrically measured in homogenates [33] following the manufacturer's protocol. This kit used the CAT peroxidatic function to determine enzyme activity. The method was based on the enzyme reaction with methanol in an optimal concentration of H_2O_2. The formaldehyde produced was measured colorimetrically using 4-amino-3-hydrazino-5-mercapto-1, 2, 4-triazole (purpald) as the chromogen. Purpald specifically formed a bicyclic heterocycle with aldehydes, changing from colorless to a purple color upon oxidation. This method used a formaldehyde solution as standard. The absorbance of the standard and samples was read at 540 nm with a plate reader (Biotek Instruments, Vermont, USA). Catalase activity was expressed in nmol/min/mg of protein.

SOD activity assay

Total SOD activity (cytosolic, mitochondrial and extracellular) was determined according to Paoletti and Mocali method [34] following the manufacturer's instructions. The kit, in short, utilized a tetrazolium salt to detect superoxide radicals generated by xanthine oxidase and hypoxanthine. The amount of enzyme that is needed to exhibit 50 % dismutation of the superoxide radicals is defined as one unit of SOD. The results are expressed as units per mg of protein (U/mg of protein).

Statistical analysis

Two-way analysis of variance (ANOVA) was used to assess the interaction between exercise (two levels) and hypoperfusion (two levels) at 1 or 24 h after occlusion in the right or left hippocampus and to determine the significant main effects and interactions between main factors. Comparisons between the right and left hippocampus within groups were made with paired t-tests. Post hoc comparisons were made using Tukey's test. A value of $p < 0.05$ was considered to be significant. To present the data, mean ± standard error of the mean (SEM) was applied.

Results

The interaction of exercise and hypoperfusion on antioxidant enzyme activities and MDA level and their main effect

Right hippocampus

Exercise caused a greater increase in catalase activity in the hippocampus of the exe-hypo group compared with that in the hypoperfusion group at 1 h. [(exercise × hypoperfusion) interaction, $F(1,20) = 4.72$, $p < 0.05$ at 1 h and $F(1,20) = 4.84$, $p < 0.05$ at 24 h]. The effect at 24 h after occlusion was contrary, i.e., exercise decreased the catalase activity, which was increased by hypoperfusion.

ANOVA revealed a significant main effect of hypoperfusion on catalase activity at 1 h [$F(1,20) = 5.33$, $p < 0.05$] and at 24 h [$F(1,20) = 21.95$, $p < 0.0001$] in the right hippocampus.

Left hippocampus

Exercise caused a greater increase in catalase activity in the hippocampus of the exe-hypo group compared with that in the hypoperfusion group at 1 h [(exercise × hypoperfusion) interaction, $F(1,20) = 4.83$, $p < 0.05$].

A significant main effect of hypoperfusion on catalase activity [$F(1,20) = 4.57$, $p < 0.05$] at 24 h was demonstrated in the left hippocampus, although this effect at 1 h was not significant.

The interaction of exercise with hypoperfusion on SOD activity, total antioxidant capacity, and MDA levels in both hippocampi was not significant.

Differences of antioxidant enzyme activity and lipid peroxidation of the groups in comparison to the control group

The hippocampus antioxidant enzyme activities and MDA level of the groups are shown in Table 1 and Figs. 1, 2, 3, and 4. No significant alteration was observed between the groups and the control group at 1 h, but a significant increase in catalase activity in the right and left hippocampus of the hypo group was observed when compared to the control at 24 h ($p < 0.0001$) (Fig. 2). No significant changes in SOD, total antioxidant and MDA level in the groups were noted as compared to the control group (Table 1; Figs. 3, 4).

Differences in antioxidant enzyme activities and lipid peroxidation between the right and left hippocampus of each group

According to Fig. 1, catalase activity significantly increased in the right hippocampus compared with that in the left hippocampus in the hypo and exe-hypo groups at 1 h ($p < 0.01$).

Although the total antioxidant capacity in the right side increased, it was not significant (Table 1). MDA level in the left hippocampus of the hypo group significantly increased when compared to the right hippocampus at 1 h ($p = 0.02$) (Fig. 3).

As shown in Fig. 2, the significant differences in catalase activity between the two hippocampi in the hypo and exe-hypo groups at 24 h were abolished. In addition, the difference in lipid peroxidation of the two hippocampi in the hypo group at 24 h was no longer significant (Fig. 4).

Discussion

Regular exercise reduces the incidence of a variety of age-related diseases [3, 8, 35]. The literature indicates that physical exercise initiates cellular and molecular pathways that are involved in neuroprotection. The reduction in oxidative cellular damage is among the proposed mechanisms of neuronal protection. However, few studies have focused on pro-oxidant/antioxidant alterations in the brain as a result of both exercise and a hypoxic/ischemic insult [6, 18]. Accordingly, the purpose of this study was to examine the effect of physical activity on antioxidant enzyme activity and MDA levels of both hippocampi, after the occlusion of only one common carotid artery. We also aimed to evaluate the adjustment of the two hippocampi to the induced hypoperfusion. To our knowledge, this is the first study that sheds light on such changes in the hippocampi.

As shown in Table 1 and the figures, treadmill exercise did not result in a significant change in the activities of antioxidant enzymes and MDA levels in the hippocampi of the exercise group after 1 and 24 h. Our findings are in line with those of previous reports [18, 36, 37], suggesting that

Table 1 Effect of exercise on SOD activities and total antioxidant capacity in the left and right hippocampus after occluding the right common carotid

Variables	Time (h)	Control		Hypoperfusion		Exercise		Exe-hypo	
		R	L	R	L	R	L	R	L
Total antioxidant (μmol/protein)	1	0.106 ± 0.028	0.057 ± 0.004	0.098 ± 0.004	0.092 ± 0.023	0.121 ± 0.016	0.089 ± 0.028	0.075 ± 0.013	0.060 ± 0.014
	24	0.091 ± 0.019	0.101 ± 0.027	0.105 ± 0.017	0.078 ± 0.025	0.093 ± 0.011	0.113 ± 0.070	0.080 ± 0.009	0.108 ± 0.015
SOD (μ/mg protein)	1	9.355 ± 1.126	8.462 ± 0.680	11.702 ± 0.832	9.621 ± 0.651	9.378 ± 0.833	10.183 ± 0.622	11.17 ± 0.920	10.758 ± 1.366
	24	11.128 ± 0.405	9.175 ± 0.268	9.712 ± 1.201	8.141 ± 0.452	8.424 ± 1.436	8.741 ± 0.670	10.860 ± 1.661	10.619 ± 1.597

The values are expressed as mean ± SEM

* $p < 0.05$ compared with the other hippocampus in the same group

** $p < 0.05$ compared with the control group (*R* right and *L* left hippocampus)

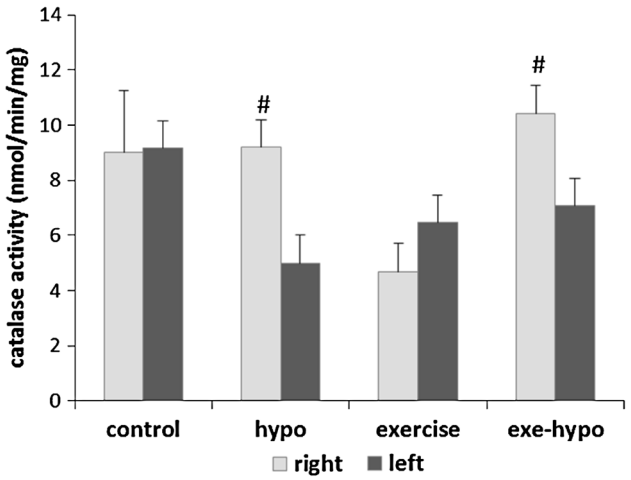

Fig. 1 The effect of cerebral hypoperfusion and exercise on catalase activity in the left and right hippocampus at 1 h after occluding the right common carotid, $^{\#}p < 0.01$ the difference between right and left hippocampus (*hypo* hypoperfusion)

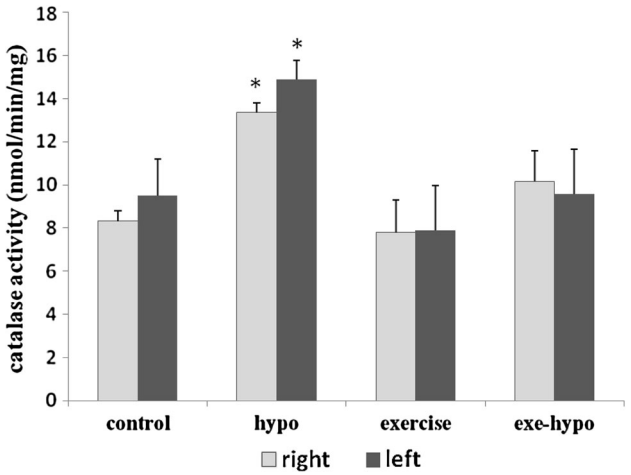

Fig. 2 The effect of cerebral hypoperfusion and exercise on catalase activities in the left and right hippocampus at 24 h after occluding the right common carotid. $^{*}p < 0.0001$, compared to the control (*hypo* hypoperfusion)

exercise does not result in an oxidative load on the hippocampus.

Oxygen consumption of the brain is known to be constant during exercise. Therefore, it is unlikely that chronic and moderate exercise poses an oxidative stress to the brain [38]. Husain and Somani [39] showed that acute exercise significantly increases lipid peroxidation in the medulla, striatum, and cerebral cortex but not in the hypothalamus and cerebellum. In the present study, rats were acutely run on a treadmill at 100 % VO_{2max}. The angle of inclination and speed of the belt were gradually increased.

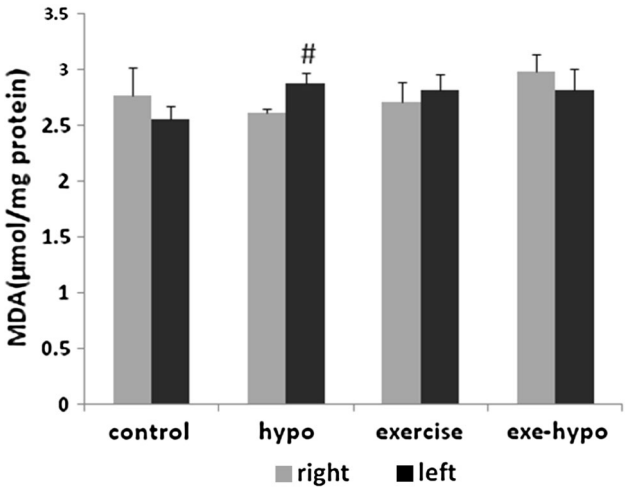

Fig. 3 The effect of cerebral hypoperfusion and exercise on MDA level in left and right hippocampus at 1 h after occluding the right common carotid. $^{\#}p < 0.05$, the difference between right and left hippocampus (*hypo* hypoperfusion)

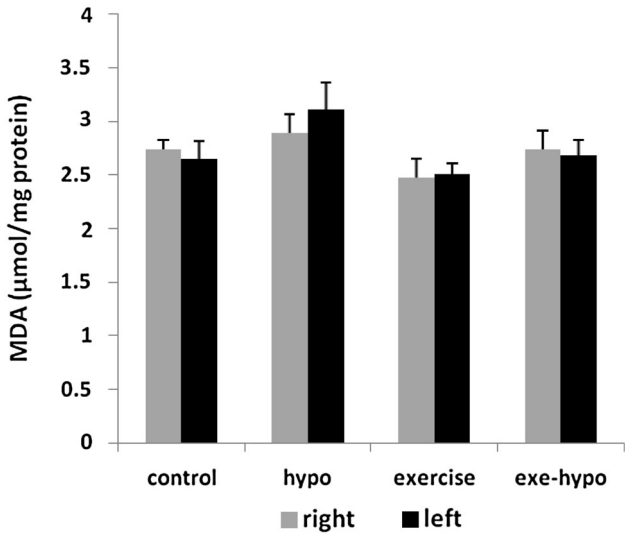

Fig. 4 The effects of cerebral hypoperfusion and exercise on MDA level in left and right hippocampus at 24 h after occluding the right common carotid (*hypo* hypoperfusion)

Devi et al. [40] found increased SOD activity in the hippocampus and cerebral cortex after a 4-month swimming exercise compared to controls. Somani et al. [41], on the other hand, did not find any change in hippocampus SOD activity after a 7.5-week exercise. Aksu et al. [42] reported that Glutamate may be reduced in the hippocampus because of the lowering effect of mild regular exercise on basal corticosterone levels, which might have resulted in reduced formation of superoxide radicals. The decrease in superoxide radicals may describe the decrease in activity of SOD [42]. The differences in type, duration, and severity of the exercise may affect hippocampal SOD activity differently.

The brain contains powerful collateral systems, e.g., the circle of Willis, which can compensate for the interruption of the blood supply via redistribution of flow from unoccluded vessels. The circle of Willis, as the most efficient collateral system in the brain, serves as a connection among the major supplying arteries [38, 43].

According to chronic experiments, total CBF returns to normal 1 day after unilateral carotid occlusion, although the hemispheric blood flow is slightly asymmetric, i.e., the blood flow in the unoccluded side returns to normal faster [21]. An increase in the flow through the unoccluded carotid artery and the development of anastomotic vessels with the vertebral arteries can be considered as the compensatory mechanisms that form the basis of normalization of hemispheric blood flow during chronic unilateral carotid occlusion [21].

Our results showed that the only significant difference between the hypoperfusion and control groups was catalase activity, which showed an increase in the right and left hippocampus of the hypoperfusion group after 24 h (Fig. 2). It is possible that a compensatory rise in catalase activity occurs in response to the increased concentration of H_2O_2, one of the primary ROSs, which can directly increase endonuclease activity in neurons, leading to DNA fragmentation and cell death [1–3, 12, 44]. Other antioxidant factors did not change significantly compared with the control group. Although MDA levels in both hippocampi of the hypoperfusion group increased after 24 h compared with the control group, this elevation was not significant (Fig. 4). Because the degree of induced hypoperfusion was in the moderate range, this result was not unexpected.

This model of hypoperfusion resembles ischemia preconditioning [45, 46], where the antioxidant enzyme activity that converts hydrogen peroxide to water and oxygen increases. When the four-vessel occlusion was applied for 5 min to induce brain preconditioning in rats, catalase activity increased significantly at all time points (with a peak at 24 h) [47]. In Table 1, we noticed that the superoxide dismutase (SOD) activity was not significantly altered. A possible explanation is the difference in kinetics between SOD and catalase [46]. The choice of time points for sample collection and measurements will significantly affect the results. This is because diverse events affecting ROS generation can occur during distinct periods over an extended time span, depending on the preconditioning stimulus under study. Ischemic preconditioning in rats results in sustained increased levels of SOD when induced by the occlusion of four vessels for 5 min [47], but not when induced by MCAO for 3 min [48]. With regard to the hemispheres, a significant increase in catalase activity was

observed in the right hippocampus of the hypoperfusion group after 1 h (Fig. 1). Furthermore, the left hippocampus in the hypoperfusion group showed a significant elevation of MDA (Fig. 3). Nevertheless, this asymmetry disappeared after 24 h, which can be ascribed to blood flow redistribution and compensation [21].

As indicated in the results, exercise alone did not significantly augment the antioxidant enzyme activities, but it further increased the enhancement of catalase activity in response to hypoperfusion at 1 h after occlusion. Exercise improves antioxidant gene expression including catalase in a variety of tissues. Investigating the antioxidant gene expression can partly clarify the mechanism [49–51].

As shown in Figs. 2 and 3, exercise in the exe-hypo group succeeded in abolishing some differences that are induced by hypoperfusion in the hypoperfusion group compared with the control group. These differences include an elevation in catalase activity in the right hippocampus after 24 h and hemispheric differences in MDA levels after 1 h. Physical activity can result in an increase in blood vessel density in different parts of the brain including the hippocampus and cortex [8]. Endress et al. [52] demonstrated that physical activity upregulates the expression of endothelial nitric oxide synthase, thereby increasing nitric oxide-dependent vasodilation, which leads to an increase in regional CBF in mice, reducing the cerebral infarct size.

Better blood flow perfusion, increased neurovascular integrity, and the consequent improvement of cellular metabolism, which are induced by exercise, can partly explain our results in the exe-hypo group [8, 53].

Conclusion

In conclusion, we observed an enhancement of catalase activity at 1 h after occlusion because of significant interactions between exercise and hypoperfusion in both hippocampi. Our results also indicate that exercise minimizes some alterations in antioxidant enzyme activity and MDA levels that are induced by occlusion of the right common carotid artery. In other words, exercise preconditioning enhances the adaptability of the brain with regard to hypoperfusion. This effect is probably the result of improvement of the vascular bed with increased capacity for necessary CBF and nutrient delivery to neurons; this beneficial change ensures greater tolerance to hypoperfusion and concomitant compensation.

To fill gaps in our knowledge regarding these phenomena, further studies on the assessment of other antioxidant enzyme activities, such as glutathione peroxidase, and antioxidant gene expression, are required to elucidate the effects of exercise on the antioxidant status in rat brain after hypoperfusion.

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