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Exercise training provides cardioprotection via a reduction in reactive oxygen species in rats submitted to myocardial infarction induced by isoproterenol

MARISA J. S. FREDERICO[1], SIMONI L. JUSTO[1], GABRIELLE DA LUZ[1], SABRINA DA SILVA[1], CLEBER MEDEIROS[1], VIVIANE A. BARBOSA[1], LUCIANO A. SILVA[1], CARINA R. BOECK[2], RICARDO A. DE PINHO[1], & CLÁUDIO T. DE SOUZA[1]

[1]*Exercise Biochemistry and Physiology Laboratory, and* [2]*Laboratory of Neuroscience and National Institute for Translational Medicine, Postgraduate Program in Health Sciences, Health Sciences Unit, University of Southern Santa Catarina, Criciuma, SC, Brazil*

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Abstract

Exercise training has demonstrated cardioprotection effects. However, the exact mechanism behind this effect is not is clear. The present study evaluated the effects of 12 weeks of previous treadmill training on the levels of oxidative damage, antioxidant enzyme activity and injury in the myocardium of rats submitted to infarction induced by isoproterenol (ISO). Isoproterenol treatment (80 mg/kg given over 2 days in two equal doses) caused arrhythmias and 60% mortality within 24 h of the last injection in the control group (C + ISO) group when compared with the saline control group (saline). Creatine Kinase – MB levels were markedly increased in hearts from ISO-treated animals in the C + ISO group. Twelve weeks of treadmill training reduced superoxide production, lipid peroxidation levels and protein carbonylation in these animals, as well as increasing the activities and expressions of SOD and CAT. Previous training also reduced CK-MB levels and numbers of deaths by 40%, preventing the deleterious effects of ISO. Based on the data obtained in this study, it is suggested that 12-week treadmill training increases antioxidant enzymes, decreases oxidative damage and reduces the degree of infarction induced by ISO in the hearts of male rats.

Keywords: *Lipid peroxidation, reactive species oxygen, myocardium, physical training, infarction*

Introduction

Cardiovascular disease remains one of the leading causes of morbidity and mortality worldwide [1]. Several risk factors for the development of cardiac ischemia have been identified. These include hyperlipidemia, hypertension, smoking, diabetes mellitus [2] and oxidative injury [3]. On the other hand, several animal studies confirm that regular aerobic exercise (i.e. running or swimming) protect against cardiac ischemia [4–8].

Oxygen radicals can be generated in cardiac tissue by several mechanisms, including by the xanthine/xanthine oxidase reaction and due to the activity of NADPH oxidase and NOS [9]. Another potential source of oxygen radicals is thought to be the mitochondrial respiratory chain [10]. Increased oxidative stress can be harmful to all cellular macromolecules, such as lipids, proteins and DNA [11]. The harmful effects of ROS on cardiac tissue can be blocked by endogenous antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT) [12]. Recent evidence suggests beneficial effects of endurance training on antioxidant defense mechanisms in cardiac tissues [13].

Correspondence: Claudio Teodoro de Souza, PhD, Laboratório de Fisiologia e Bioquímica do Exercício, Programa de Pós-Graduação em Ciências da Saúde, Universidade do Extremo Sul Catarinense, 88806-000 Criciúma, SC, Brazil. Fax: +55 48 3431-2539. Email: ctsouza@unesc.net

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Lack of physical activity is a public health problem in the general population and is recognized as an independent risk factor for the development of myocardial infarction. Different studies indicate that exercise benefits patients with this disease. Consequently, exercise should be considered the cornerstone on which to base changes in lifestyle aimed at the prevention of cardiovascular disease [14]. While it is widely accepted that exercise improves tolerance against myocardial injury, the mechanism responsible for this exercise-induced cardioprotection has been related to increases in myocardial antioxidant capacity [4,5,15]. These protective mechanisms could include increased antioxidant defenses, reduced basal production of oxidants (reduced oxidative stress) and reduction of radical leak during oxidative phosphorylation [16]. In particular, exercise has been shown to increase the myocardial activity of manganese superoxide dismutase (MnSOD) [4,5,15,17]. Evidence has also linked exercise-induced increases in ventricular MnSOD activity with protection against myocardial infarction [4,5,17].

ROS can produce myocardial contractile dysfunction and structural damage [18]. There is growing evidence to indicate that ROS are increased during heart failure and may contribute to disease progression and worsening [11,19]. Hill and Singal [20] showed that antioxidant enzyme activities are decreased and that TBARS are increased in the failing myocardium due to myocardium infarction. On the other hand, it has been shown that exercise reduces the production of ROS and cardioprotection when performed after induced infarction. However, the effects of the performance of previous exercise training on the production of ROS, oxidative damage and the heart after infarction has not been investigated. Thus, the present study investigated whether a previous 12-week treadmill training programme is capable of increasing antioxidant enzymes, decreasing oxidative damage, reducing the degree of infarction and, finally, survival rates in ISO-induced infarction rats. From our results, it may be speculated that the beneficial effects of previous exercise training are mediated, at least in part, through the prevention of ROS-induced damage.

Materials and methods

Animals

Forty male Wistar rats, weighing 250–300 g and aged 2 months, were obtained from our breeding colony (UNESC) and housed in a temperature-controlled room (24°C) with a 12:12-h reverse light-dark cycle (07:00–19:00 h dark: 19:00–07:00h light). Tap water and standard rodent laboratory diet were supplied *ad libitum*. The animals were looked after in accordance with the guiding principles in the care and use of animals [21] and protocols were approved by the Ethics Committee (protocol number – 306) of the University of Southern Santa Catarina, Brazil. The animals were randomly assigned to four groups: Control (C) ($n = 10$), Control plus ISO (C + ISO) ($n = 10$), Exercise Training (ET) ($n = 10$), Exercise Training plus ISO (ET + ISO) ($n = 10$). After myocardial infarction, the percentage of death in the C + ISO group was 60% ($n = 4$), while death in the ET + ISO group occurred in 20% of animals ($n = 8$). One day after myocardial infarction (24 h after the second dose of ISO), blood was collected from the tail for determination of CK-MB. Afterwards, the rats were anaesthetized with 50 mg/kg intraperitoneal pentobarbital sodium, killed by decapitation and hearts were surgically removed; samples were aliquoted and stored at −70°C for western blot analysis. For biochemical analysis, samples were processed immediately.

Exercise protocol

The groups of rats that were exercised were habituated on a nine-channel motor-drive treadmill with a speed of 10 m/min for 10 min/day during 1 week to reduce their stress to the new environment. The rats did not receive any stimulus to run. The exercise groups performed an incremental running programme to obtain progressive levels of intensity (Table I) during 12 weeks for 5 days/week. The untrained animals were placed on the switched-off treadmill during the same 12 weeks as the exercise-trained groups.

Myocardial infarction model

After 12 weeks of training exercise, the ET + ISO group received an injection/day (s.c.) of 80 mg/kg/day ISO hydrochloride (Sigma-Aldrich, Buchs, Switzerland) for 2 days. The C + ISO group was also induced by injection of 80 mg/kg/day ISO hydrochloride. Control rats received subcutaneous (sc) physiological saline (0.5 ml).

Table I. Incremental running protocol.

Week	Belt speed (m/min)	Daily duration (min)	Inclination (degrees)
1	10	10	0
2	10	15	0
3	10	20	0
4	10	25	0
5	10	30	0
6	10	35	0
7	10	40	0
8	10	45	0
9	10	50	0
10	10	50	0
11	10	50	0
12	10	50	0

Biochemical assays

Creatine Kinase–fraction MB. Serum creatine kinase fraction MB were assessed by colorimetric assays (LaborLab, São Paulo, Brazil).

Superoxide anion assay. Superoxide anion was measured by a previously described McCord and Fridovch's [22] method and expressed in nmol/min/mg protein. Superoxide was determined according to the rate of oxidation of adrenaline, determined by absorbance at 780 nm.

Thiobarbituric acid reactive species (TBARS). The formation of TBARS during an acid-heating reaction [23] was used as an index of lipid peroxidation. Briefly, the liver samples were mixed with 1 ml 10% trichloroacetic acid and 1 ml 0.67% thiobarbituric acid and were then heated in boiling water for 30 min. TBARS were determined by the absorbance at 535 nm using 1,1,3,3-tetramethoxypropane (Sigma Chemical, St Louis, MO, USA) as an external standard. Results were expressed as malondialdehyde equivalents per milligram of protein.

Protein carbonyls. The oxidative damage to protein was determined by measuring the carbonyl groups, based on the reaction with dinitrophenylhydrazine (DNPH) [24]. Proteins of liver sample were then precipitated by the addition of 20% trichloroacetic acid and reacted with DNPH. Samples were then redissolved in 6M-guanidine hydrochloride and carbonyl contents were determined from the absorbance at 370 nm, using a molar absorption coefficient of 22 000 M^{-1}.

Catalase (CAT) and superoxide dismutase (SOD) activities. In order to determine CAT activity, tissue samples were sonicated in 50 mM phosphate buffer and the resulting suspension was centrifuged at 3000 g for 10 min. The supernatant was used for enzyme assay. CAT activity was measured by the rate of decrease in hydrogen peroxide (10 mM) absorbance at 240 nm [25]. SOD activity was assayed by measuring the inhibition of adrenaline self-oxidation absorbance at 480 nm [26].

Protein determination. The amounts of protein in the assays for catalase, SOD, carbonyl and TBARS were assayed by Lowry et al.'s [27] method.

Protein analysis by immunoblotting

As soon as anaesthesia was assured by the loss of pedal and corneal reflexes, the abdominal cavity was opened. Myocardial tissue was then excised. The tissue were pooled, minced coarsely and homogenized immediately in extraction buffer (mM) (1% Triton-X 100, 100 Tris, pH 7.4, containing 100 sodium pyrophosphate, 100 sodium fluoride, 10 EDTA, 10 sodium vanadate, 2 PMSF and 0.1 mg of aprotinin/ml) at 4°C with a Polytron PTA 20S generator (Brinkmann Instruments model PT 10/35) operated at maximum speed for 30 s. The extracts were centrifuged at 11 000 rpm and 4°C in a Beckman 70.1 Ti rotor (Palo Alto, CA) for 40 min to remove insoluble material and the supernatants of these tissues were used for protein quantification, using the Bradford [28] method. Proteins were denatured by boiling in Laemmli [29] sample buffer containing 100 mM DTT, run on SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked, probed and developed, as described previously [30]. In direct immunoblot experiments, 0.2 mg of protein extracts obtained from each tissue were separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted with anti-SOD or anti-CAT antibodies. Chemiluminescent detection was performed with horseradish peroxidase-conjugate secondary antibodies. Visualization of protein bands was performed by exposure of membranes to RX-films.

Statistical analysis

Biochemical data were expressed as means and mean standard errors and analysed statistically by one-way variance analysis (ANOVA), followed by the Tukey *post-hoc* test. The results of blot analysis were expressed as the means $\pm$ SEM of densitometric units. Differences between the groups were evaluated using one-way analysis of variance (ANOVA). When ANOVA indicated significance, a Tukey *post hoc* test was performed. For mortality, statistical analysis of the unilateral comparison mean survival rates *t*-test was used. A probability of less than 0.05 was considered to be significant. The software used for analysis of the data was the Statistical Package for the Social Sciences (SPSS) version 12.0 for Windows.

Results

Exercise reduces mortality and levels of CK-MB induced by isoproterenol

The administration of isoproterenol produces high rates of mortality [31]. Interestingly, in the present study, the sample was sufficient to prove with 95% confidence that exercise increased the rate of survival of animals that received isoproterenol (8 ± 1.26), when compared to the C + ISO (4 ± 1.55) group (20% vs 60% of mortality, respectively; $p < 0.001$ by *t*-test), demonstrating the beneficial effect of previous exercise training in this model (Table II).

We next measured the effect of physical exercise on levels of clinical markers of cardiac infarction after submission to ISO-induced infarction. As expected,

Table II. Comparison of mean survival rates.

Groups	C	ET	C+ISO	ET+ISO
Mean±SEM	10±0	10±0	4±1.55	8±1.26*

*$p < 0.001$ for ET+ISO vs C+ISO group, $n = 10$ for all groups. *t*-test was performed.

the C + ISO group presented dramatically higher levels of CK-MB (2.3-fold), when compared with the C group. On the other hand, previous training (ET + ISO group) reduced (1.5-fold) the extent of infarction in rats induced by ISO (df = 3; $F = 18.172$; $p = 0.0001$) (Figure 1).

Effect of training exercise on myocardial superoxide production and oxidative damage in rats submitted to ISO-induced infarction

Figure 2A demonstrates that infarction produced a significant increase in superoxide production (C + ISO), as compared to the control group (1.5-fold). Exercise training altered these values and prevented superoxide production in rats submitted to ISO-induced infarction (reduction of 2.3-fold) (df = 3; $F = 8.68$; $p = 0.0001$).

Oxidative damage to lipids was assessed by malondialdeyde (MDA) formation. MDA levels were lower in the ET group when compared to the respective controls. ISO-induced infarction in non-trained animals (C + ISO) presented 2.6-fold of increase in MDA, when compared with controls. In contrast, previous exercise training (ET + ISO) reduced MDA levels (1.8-fold) (df = 3; $F = 26.104$; $p = 0.0001$) (Figure 2B).

To verify the oxidative damage in proteins, we assessed the carbonyl groups based on a reaction with dinitrophenylhydrazine. Our findings demonstrate an increase in protein carbonylation in the control group after ISO-induced infarction, when compared with controls (2.0-fold). However, previous training reduced protein carbonylation levels to 1.9-fold (df = 3; $F = 9.077$; $p = 0.0001$) (Figure 2C).

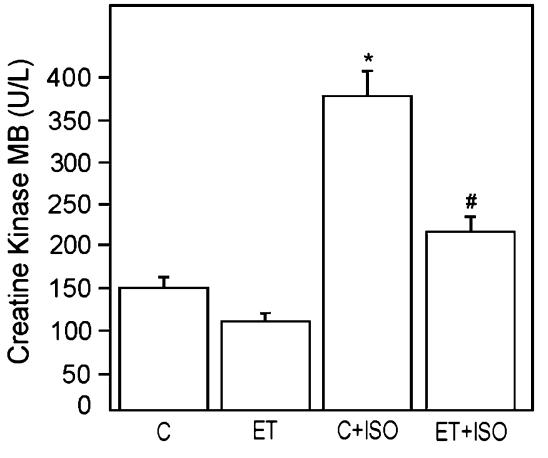

Figure 1. CK-MB levels in a model of isoproterenol-induced infarction in rats submitted to physical training. CK-MB levels were evaluated in ISO-induced infarction rats submitted to exercise training. Results are expressed as U/L. Bars represent means ± SEM of $n = 8$ rats for C, ET, ET+ISO and $n = 4$ for C+ISO. *$p < 0.001$, C+ISO group vs C and #$p < 0.05$, ET+ISO vs C+ISO group.

Antioxidant enzyme activities and expressions in the cardiac tissue of rats submitted to 12-week exercise training protocols

SOD is an important enzyme that protects against free radicals. SOD activity was observed to increase after the 12-week training programmes in the ET group (2.2-fold), when compared with the control group. Importantly, in the previously-trained group and increase of 2.7-fold in SOD was observed when compared with the C + ISO group (df = 3; $F = 49.818$; $p = 0.0001$) (Figure 3A). CAT is an antioxidant enzyme responsible for converting hydrogen peroxide into water. CAT activity was increased in the ET and ET + ISO groups, when compared with control (2.0-fold) and C + ISO groups, respectively (3.0-fold) (df = 3; $F = 48.42$; $p = 0.0001$) (Figure 3B).

To determine the effect of 12-week training programmes on antioxidant enzyme expression after ISO-induced infarction, we performed Western blot analyses to evaluate SOD and CAT expression. Results show that previous training also increased SOD expression, as compared to the sedentary control group (1.7-fold) and the ISO-induced infarction group (1.9-fold) (df = 3; $F = 49.24$; $p = 0.0001$) (Figure 3C). Increased CAT expression was also observed in the ET and ET + ISO groups, when compared with the control (2.9-fold) and C + ISO (2.1-fold) (df = 3; $F = 120.93$; $p = 0.0001$) groups, respectively (Figure 3D).

Discussion

Cardiovascular diseases, including myocardial infarction, are a leading cause of death worldwide; recent studies have shown that oxidative damage appears to be involved in infarction pathophysiology and that it may be decisive in the extent of the event [32,33]. Reactive oxygen species (ROS) may attack any type of molecule, but their main target appears to be polyunsaturated fatty acids, which is the precursor of lipid peroxide formation [34]. Increased lipid peroxidation is thought to be a consequence of oxidative stress, in which the dynamic balance between pro-oxidant and antioxidant mechanisms is impaired [16]. Elevation of lipid peroxides in ISO-induced infarction could be attributed to the accumulation of lipids in the heart and the irreversible damage to the myocardial membranes.

On the other hand, the cardioprotective effects of exercise training are well known and undisputed;

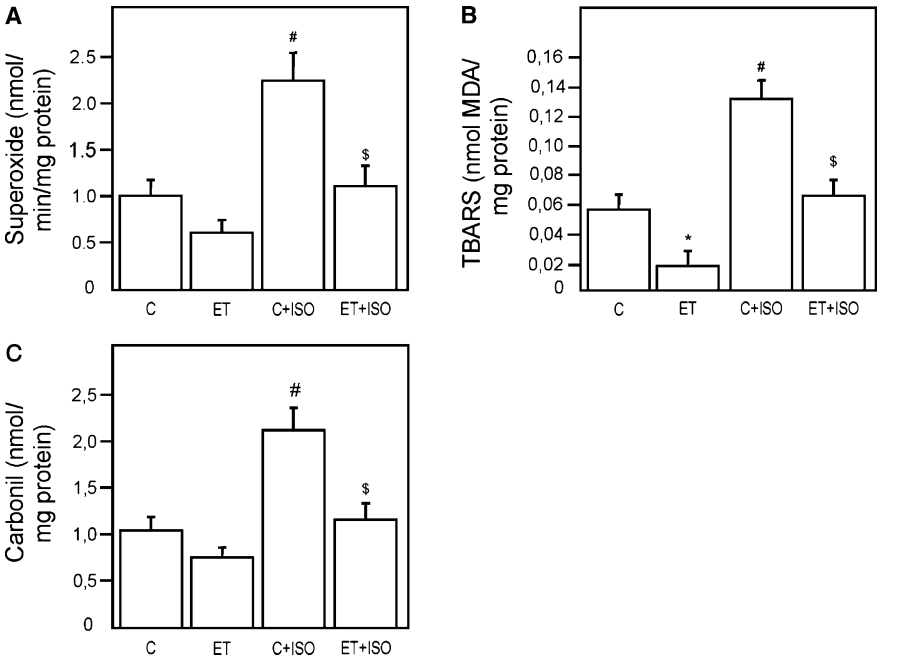

Figure 2. Production of superoxide and oxidative damage in rats with isoproterenol-induced myocardial infarction submitted (or not) to physical training. Superoxide production (A), Thiobarbituric acid-reactive substances (TBARS) (B), Carbonylation levels (C) in myocardium after ISO-induced lesion. Superoxide production is expressed as nmol/min/mg protein (A). TBARS and Carbonyl damage are expressed as nmol/mg protein (B) and (C), respectively. Bars represent means ± SEM of $n = 8$ rats for C, ET, ET + ISO and $n = 4$ for C + ISO. $^{\star}p < 0.05$, ET vs C group; $^{\#}p < 0.01$, C + ISO vs C + group and $^{\$}p < 0.05$, ET + ISO vs C + ISO group.

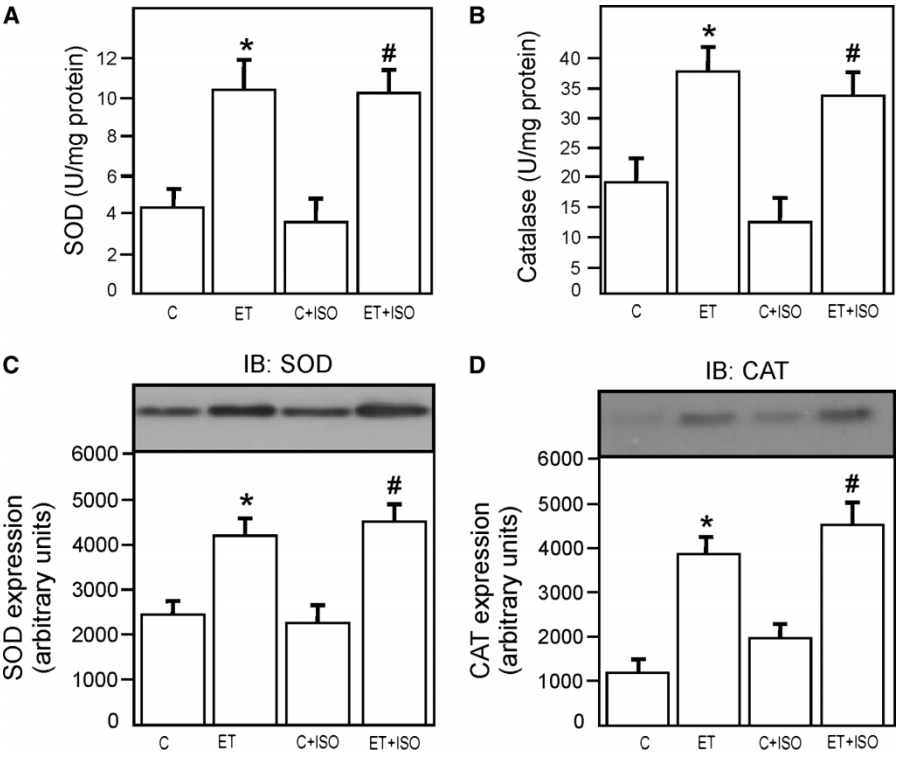

Figure 3. Activity and expression of antioxidant enzymes in isoproterenol-induced infarction rats submitted to physical training. Superoxide dismutase (SOD) (A) and catalase (CAT) (B) in ISO-induced myocardial rats submitted to physical training. SOD (C) and CAT (D) expression in heart of ISO-induced infarction rats submitted to physical training. Heart extracts from rats were prepared, as described in Methods. SOD and CAT activities are expressed as U/mg protein, A and B respectively. SOD and CAT expression are expressed as arbitrary units in C and D, respectively. Bars represent means ± SEM of $n = 8$ rats for C, ET, ET + ISO and $n = 4$ for C + ISO. $^{\star}p < 0.05$, ET vs control group and $^{\#}p < 0.05$, ET + ISO vs C + ISO group.

recent studies have clearly shown that training at > 60% $\dot{V}O_2$max increases myocardial tolerance to ischemia-reperfusion [35], improves cardiac performance and ameliorates the cell defense capacity against oxidative stress [8,36]. However, the effects of the performance of previous exercise training on the production of ROS, oxidative damage and heart after myocardial infarction have not been investigated. Thus, the present study shows that a previous 12-week treadmill training programme is capable of increasing antioxidant enzymes, decreasing oxidative damage, reducing the degree of infarction and, finally, increasing survival rates in ISO-induced infarction rats. Importantly, we demonstrated that previous exercise training induced higher activity and expression of antioxidant enzymes, led to lower ROS production and is associated with a decreased degree of infarction and, importantly, higher survival rates in ISO-induced infarction rats. We may postulate that this may occur, at least in part, due to the change status in cellular oxidative stress that is induced by 12 weeks of exercise training.

Superoxide anions are formed in excess during infarction [37], as clearly observed in our experimental model. Reactive oxygen species lead to increased lipid peroxidation formation, causing cellular damage. Previous studies have shown that lipid peroxidation, in Fisher 344 rats, can be decreased in heart tissue by an 8-week treadmill training programme [38]. In the current study, previous exercise training significantly decreased the levels of myocardial TBARS in ISO-induced infarction rats.

Protein carbonyl derivatives, a marker of oxidative injury, have been reported to increase after ischemic insult to the heart [39]. Until now, the relationship between previous exercise, myocardial infarction and levels of carbonylation had not been described. In the present study, previous training exercises decreased myocardial protein carbonyl content in an experimental rat model of infarction. Thus, the mechanism by which exercise provides protection against ISO-induced infarction could to be linked to oxidative modification of cardiac proteins.

Studies have reported that over-expression of myocardial antioxidants or the introduction of a mitochondrial-targeted antioxidant minimizes myocardial infarction-induced injury [40]. In addition, dietary supplementation with antioxidants has been shown to lower myocardial oxidative injury and the magnitude of infarction [40]. However, the activity and expression of antioxidant enzymes, induced by 12 weeks of prior exercise, before myocardial infarction in rats has not been reported. Thus, we evaluated activity and expression of antioxidant enzymes. Recent studies have reported an increased SOD activity in male Wistar rats [41], in Fisher 344 rats [42] and in female Sprague-Dawley rats [43] after acute exercise. In addition, increased SOD activity has been found following treadmill training for 6.5 weeks in male [44], 8 weeks in male [38] and 10 weeks in female [15,42] Fisher 344 rats. Increased total and mitochondrial SOD activity has been demonstrated in male Wistar rats following 24-week treadmill training [7] and in female Sprague-Dawley rats that were treadmill-trained for 10 weeks [43] and in female mice that were treadmill-trained for 8 weeks [45]. Swimming training for 4 weeks also increases SOD activity in the left and right ventricles of male rats [6]. The present study shows an increased activity of SOD in the trained group and this increase persisted in ISO-induced infarction rats, demonstrating one of the antioxidant defense mechanisms that may be affected by exercise training in the myocardium during infarction. In addition, we observed that the expression of SOD is increased in the same groups in which the activity of SOD increased, suggesting that exercise training enhances both the activity and expression of this enzyme and leads to a defense mechanism against cellular production of ROS.

Increased CAT activity was observed in Fisher 344 rats trained for 8 weeks [38] or 10 weeks [42], Swiss male mice after swim training for 21 weeks [46] and also in Sprague-Dawley rats after 1 h of swimming [47]. Our findings show the relationship between previous exercise training, activity and expression of catalase and myocardial infarction, since we observed increased CAT expression and activity in infarcted animals.

Previous exercise training resulted in an overall decrease in ROS and protein and lipid oxidation. These effects may also be associated with increased cardiac remodelling [48], a decrease in inflammation [49] and cellular apoptosis [50], approaches that were not evaluated in this study. Therefore, future studies with other approaches should be carried out to ascertain the beneficial effects of previous exercise training in individuals that are susceptible to myocardial infarction.

Moreover, strategies to modulate oxidative stress through adaptations of the antioxidant system, such as exercising with appropriate intensity, frequency and duration, may provide prevention and reduction in the level of infarction in predisposed individuals.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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