AEROBIC SWIMMING TRAINING INCREASES THE ACTIVITY OF ANTIOXIDANT ENZYMES AND THE GLYCOGEN CONTENT IN THE SKELETAL MUSCLE OF RATS

EXERCISE AND SPORTS SCIENCES

ORIGINAL ARTICLE

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ABSTRACT

Introduction: Studies which verified the effects of physical exercise on oxidative stress biomarkers and its relation to muscle glycogen are scant. Objective: The present study verified the effects of aerobic swimming training (AST) on biomarkers of oxidative stress, glycogen content and cell growth in the skeletal muscle of rats. Methods: Eighteen male Wistar rats (60 days) were divided into two groups: Sedentary Group (SG; n = 10): sedentary rats; and Trained Group (TG; n = 8): rats subjected to AST (5.0% of body weight), 1h/day, 5x/week, during 8 weeks. The activity of antioxidant enzymes (AOE) superoxide dismutase (SOD; U/ml), catalase (CAT; µmol/min/100mg), glutathione peroxidase (GPx; nmol/min/100mg), and substances that react with thiobarbituric acid levels (TBARs; nmolMDA/mg protein) were determined in the right gastrocnemius muscle. Glycogen (mg/100mg), protein (g/100g), and DNA (g/100g) contents were evaluated in the left gastrocnemius muscle. Data were analyzed by Student t-test ($p < 0.05$). Results: AOE activity was higher in the TG group (CAT: 0.87 ± 0.04; SOD: 6.49 ± 0.45; GPX: 6.49 ± 0.52) when compared to SG group (CAT: 0.52 ± 0.03; SOD: 4.10 ± 0.37; GPx: 2.87 ± 0.35). TBARs levels was lower in TG (TG: 2.35 ± 0.45; SG: 8.90 ± 0.47). Gastrocnemius glycogen content (SG: 0.108 ± 0.013; TG: 0.320 ± 0.012) and protein/DNA ratio (SG: 24.94 ± 3.25; TG: 41.68 ± 4.02) were higher in TG group. Conclusion: Altogether, these data provide evidence that AST improved antioxidant defense, which may be associated to higher glycogen content of skeletal muscle of the animals.

Keywords: oxidative stress, glycogen, cell proliferation.

INTRODUCTION

Reactive oxygen species (ROS) are continuously made by enzymatic and non-enzymatic processes during normal metabolism, and they cause damage to lipids, proteins and cellular nucleic acids[1,2]. Some studies denote that between 2 and 5% of the oxygen we inhale changes into some kind of ROS[2,3].

The condition in which the ROS production surpasses the intracellular antioxidant capacity to eliminate them is known as oxidative stress[3]. Oxidative stress is currently widely spread as a subject, since it is closely connected with the onset of many pathologies, such as cancer, atherosclerosis and the aging process [2].

The oxidant/antioxidant imbalance may lead the unsaturated fatty acids of the phospholipids of the cellular membranes to suffer peroxidation (lipid peroxidation), resulting in significant loss of the membrane's integrity[1]. Such onset consists in one of the main effects of the oxidative damage, leading to the built of aldehydes and potentially harmful consequences [4,5], and the cell may suffer programed cellular death (apoptosis)[6]. The main defense system against oxidative stress is constituted by antioxidant enzymes, such as superoxide dismutase (SOD) (CuZn- cytosolic and extracellular SOD and mitochondrial Mn-SOD), catalase (CAT) (heme-enzyme CAT) and glutathione peroxidase (GPx; selenium-dependent or not), which decompose the superoxide, peroxide and hydroxide anion radical and seem to respond in an adaptive manner to exercise, increasing their activities in many tissues [7-9]. However, this statement is still controversial[10]. During exercise, many chemical and enzymatic reactions imply in physiological increase in the formation of ROS and nitric oxide [11], and it has been reported that this increase stimulates the transportation of glucose in the skeletal muscle through insulin-independent mechanisms[12], which could increase the content of muscle and hepatic glycogen [13,14]. On the other hand, this is not a rule, since it is known that unproportional production (non-physiological) of ROS may lead the body to oxidative stress and hence, would make it difficult for the muscle to grasp glucose, would result in the generation of inflammatory cytokines and would cause decrease of the glycogen build-up in this tissue at rest [15]. According to Winick *et al.*[16], the total number of cells of the muscle tissue may be estimated with practical aims by the determination of the total DNA content (hyperplasia), while the fiber size (hypertrophy) may be estimated by the calculation of the protein/DNA ratio. These parameters are considered performance markers and may be improved by training. There are no studies in the literature which approach the issue of the skeletal muscle growth associated with oxidative stress and muscle glycogen content.

Thus, the aim of the present study was to verify the swimming aerobic training effects on the biomarkers of oxidative stress and its influence on the glycogen content and the skeletal muscle growth in rats.

MATERIALS AND METHODS

Animals

Male Wistar rats, with 60 days of age at the beginning of the experiment, obtained from the Central Animal Facility of the Federal University of Mato Grosso and which had free access to water and food (commercial chow for rodents – NUVLAB®) were used. The animals were placed in collective polyethylene cages (four to five animals per cage) (37.0 x 31.0 x 16.0 cm) and were kept in a room with controlled temperature (25 ± 1ºC) and humidity (45% to 55%) with a 12-hour light/dark cycle. All the adopted procedures followed the Brazilian Resolutions specific to bioethics concerning experimentation with animals (Law # 6.638, from May 8, 1979 and Decree # 26.645 from July 10, 1934). The present study was approved by the Ethics Committee in Research with Animals from the Federal University of Mato Grosso, Cuiabá, Brazil (Protocol # 23108.020868/09-4).

The animals were divided in two groups:

• Sedentary Group, n = 10 (SG): kept sedentary during the entire experimental period.

• Trained Group, n = 8 (TG): submitted to swimming physical training. The animals' weight was checked once a week (Sartorius®, accuracy of 10 g).

Adaptation to the water environment

Adaptation consisted in keeping the animals in contact with shallow water at the temperature of 31 ± 1°C[17], (Initial® thermometer) during one week, five days per week, during 30 minutes[18]. On the second week, the rats were submitted to the same protocol but this time in deep water, in order to establish the correct mechanics for the swimming pattern of each animal. The adaptation had the aim to reduce the animal stress to the physical exercise performed in water.

Physical training

On the third and fourth weeks, the animals were submitted to the training with lead loads (small fabric bags [cotton] with elastic and Velcro®) attached to their thorax already with 5% of their body weight, following this timetable: (table 1)

Table 1. Physical training timetable which the animals were submitted to.

Day	Training time (min)
1	10
2	15
3	20
4	25
5	30
6	35
7	40
8	45
9	50
10	55

The training protocol used, previously established in the literature[18,19], was characterized for being of moderate intensity. After the adaptation period, the TG animals were submitted to the swimming exercise in collective tanks (100 cm x 80 cm x 80 cm) with water at 31 ± 1°C, for eight weeks, five days per week, one hour a day, bearing load equal to 5% of their body weight[18,19]. The animals were weighed at the beginning of each week for training overload adequation.

The rats performed physical training in polyethylene cylinders (PVC) to avoid direct contact, making it easy hence the correct swimming mechanics.

Euthanasia

The animals from both experimental groups were euthanized (CO_2 inhaling) and submitted to cervical dislocation 48 hours after the last exercise session. Subsequently, aliquots of the right and left gastrocnemius muscle (~250mg) were collected for analyses.

Biomarkers for oxidative stress

In order to verify the antioxidant status, the catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) enzymatic activities were determined in the right gastrocnemius muscle of the animals. As an indicator of the lipid peroxidation (damage to the muscle membrane), the concentration of substances which react with the thiobarbituric acid (TBARS) was evaluated in the same tissue. The methods for determination of these variables are described as follows:

CATALASE (CAT)

In order to determine CAT activity, the muscle tissue samples were placed in cold Eppendorf® tubes containing 1 ml of phosphate buffer 0.05 N (composition, in g/L:. KH_2PO_4, 1.34 and $NaHPO_4$ $2H_2O$, 7.1), sonicated and centrifuged at 10,000 rpm during five minutes. The supernatant was separated and stored at -20°C for analysis with commercial kits (Cayman Chemical®, Michigan, USA).

Superoxide dismutase (SOD) and glutathione peroxidase (GPx)

SOD and CAT activity was determined with the muscle tissue samples being immediately washed with PBS (pH 7.4) containing heparin (0.16 mg/ml), in order to remove the blood cells. Immediately after, the samples were homogenized (in ice), in 1 ml of HEPES buffer (20 mM, pH 7.2) containing (in mM): 1 EGTA, Manitol 210 and sucrose 70, as well as centrifuged during 15 minutes at 10,000 rpm (4ºC). The supernatant was separated and stored at -20°C for subsequent analysis of total SOD (cytoplasmic and mitochondrial) and GPx with commercial kits (Cayman Chemical®, Michigan, USA).

Biomarkers of lipids peroxidation: TBARs concentration

In order to determine the TBARs concentrations, the muscle tissue samples were placed in cold Eppendorf® tubes containing 1.5 ml of phosphate buffer 0.05 N (composition in g/L: KH_2PO_4, 1.34 and $NaHPO_4$ $2H_2O$, 7.1), homogeneized in Polytron® and centrifuged during five minutes at 10,000 rpm. After that, the supernatant was separated and stored at -20°C for analysis with commercial kits (Cayman Chemical®, Michigan, USA).

Muscle glycogen

The muscle glycogen concentration was determined by colorimetric method with phenol and sulphuric acid in the left gastrocnemius muscle of the animals[20].

Skeletal muscle cell growth

In order to measure the skeletal muscle growth, the DNA contents, as index of the cell number and the protein/DNA ratio, and as index of cell growth, in the left gastrocnemius muscle of the animals were determined[16]. The protein amounts were determined by folin-phenol method[21], while the DNA rates in the muscle were evaluated by diphenylamine method[22].

Statistical procedure

The data were analyzed with a statistical package BioEstat® 5.0 (Brazil) and expressed in mean ± standard deviation. Normality was verified with the Kolmogorov-Smirnov test. The Mann-Whitney test was used to verify the difference between means and the *Effect Size* (ES) was additionally verified with the GPower® 3 software[23]. Significance level was pre-set at 5%.

RESULTS

The mean value of the CAT activity (umol/min.mg protein) was lower ($p < 0.0004$; ES: 11.3) in the SG (0.52 ± 0.03) when compared with the TG (0.87 ± 0.04) (figure 1). Such results demonstrate increase of 69% in the activity of this enzyme in the TG compared with the SG.

Mean concentration of the SOD activity (U/ml) was lower ($p < 0.0004$; ES: 3.8) in the SG (4.10 ± 0.37) when compared with the TG (6.49 ± 0.45) (figure 2). Thus, it was observed increase of 58% in the activity of this enzyme in the TG compared with the SG.

Concerning the GPx activity (nmol/min/100 mg), it was possible to observe that the SG presented mean concentration (2.87 ± 0.35) lower ($p < 0.0004$; ES: 8.7) than the TG (6.49 ± 0.52) (figure 3), representing increase of 126% in the TG compared with the SG.

The TBARs concentrations are presented in figure 4. The TBARs mean value (nmolMDA/mg protein) found in the TG (2.35 ± 0.45) was lower ($p < 0.0004$; ES: 14.6) than in the SG (8.90 ± 0.47); such values correspond to values 74% lower for the animals which exercised. The rats from the TG presented heavier body weight at the end of the experiment (table 2). Moreover, increase in the glycogen content in the gastrocnemius of the trained animals was observed. The protein/DNA ratio also increased in the TG (higher cell growth), confirming the efficiency of the physical training. No difference has been found in the muscle DNA values (number of cells) (table 2).

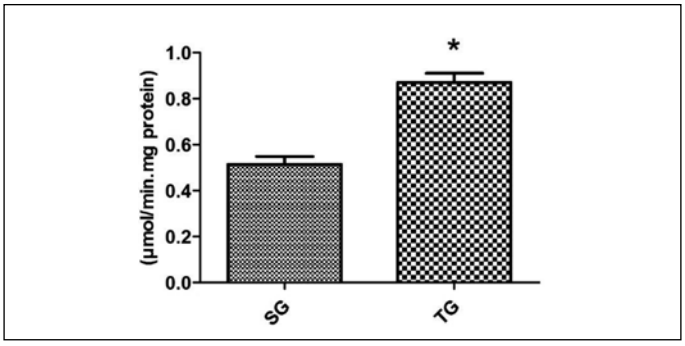

Figure 1. Activity of the catalase enzyme (CAT) in the gastrocnemius muscle of the animals at the end of the experiment. Results expressed as mean ± standard deviation. Trained (TG; n = 8) > Sedentary (SG; n = 10).

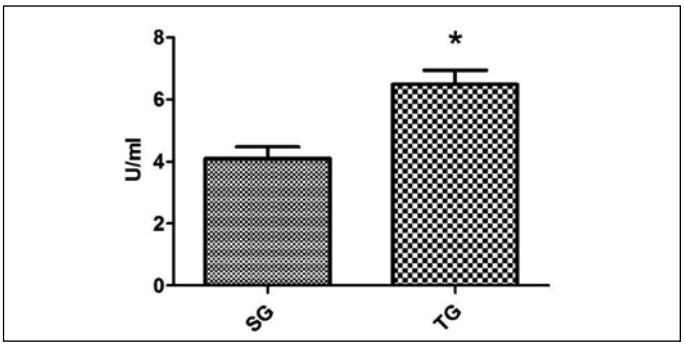

Figure 2. Activity of the superoxide dismutase enzyme (SOD) in the gastrocnemius muscle of the animals at the end of the experiment. Results expressed as mean ± standard deviation. Trained (TG; n = 8) > Sedentary (SG; n = 10).

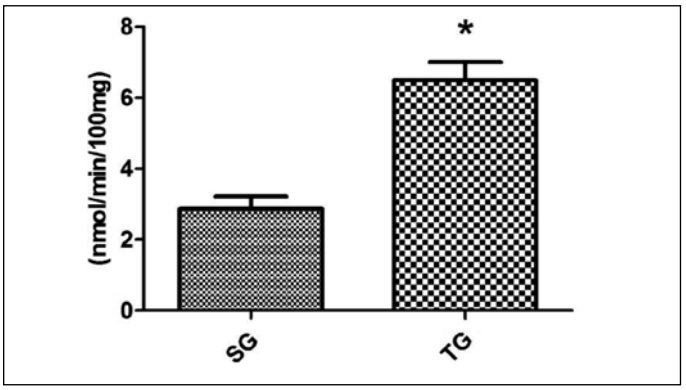

Figure 3. Activity of the glutathione peroxidase enzyme (GPx) in the gastrocnemius muscle of the animals at the end of the experiment. Results expressed as mean ± standard deviation. Trained (TG; n = 8) > Sedentary (SG; n = 10).

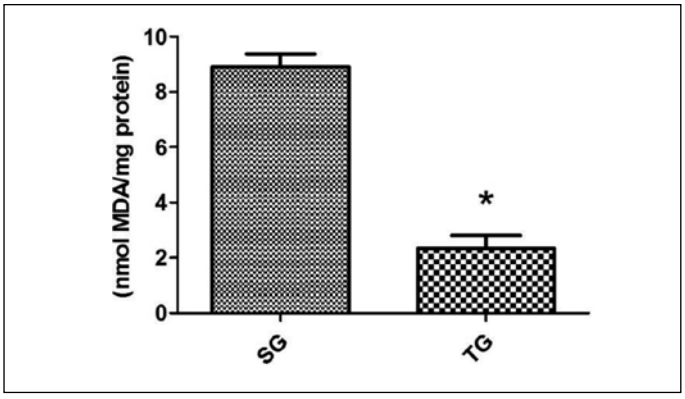

Figure 4. Substances which react to the thiobarbituric acid (TBARs) in the gastrocnemius muscle of the animals at the end of the experiment. Results expressed as mean ± standard deviation. Trained (TG; n = 8) < Sedentary (SG; n = 10).

Table 2. Body weight gain and glycogen, protein, DNA and protein/DNA ratio in the gastrocnemius muscle of the animals.

Variables	Sedentary group	Exercised group	p-value	Effect size
Weight gain (g)	70.6 ± 13.5	85.4 ± 25.0	0.04	5.3
Muscle glycogen (mg/100mg)	0.108 ± 0.013	0.320 ± 0.012	0.0004	21.0
Muscle protein (g/100g)	2.09 ± 0.20	3.52 ± 0.36	0.0004	4.4
Muscular DNA (g/100g)	0.084 ± 0.006	0.085 ± 0.002	0.63	0.0
Protein/DNA ratio	24.94 ± 3.24	41.67 ± 4.02	0.0004	4.6

DISCUSSION

In order to fight the oxidative stress, our body makes use of highly regulated chemical mechanisms[2]. In the present investigation, the effect of swimming training over the activities of the antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and a biomarker of lipid peroxidation (TBARs) was verified; moreover, the muscle concentrations of glycogen, protein and DNA were also checked.

Although exercise is a known cause for ROSs[3], the stimulus caused seems to have been a compensation. In the present study, training was efficient in increasing the activity of the antioxidant enzymes, as well as reducing the magnitude of the lipid peroxidation process, a biomarker of oxidative stress. These results evidence the importance of physical exercise in the promotion of positive physiological adaptations concerning the mechanisms of antioxidant defense.

The CAT enzyme, found in the peroxisomes, play a specific role to metabolize the hydrogen peroxide, a highly toxic substance to the cell[2]. In this study, significant increase of the activity of the activities of this enzyme was observed. On the other hand, in a study with humans by Schneider *et al.*[24], no difference in the catalase activity was found, regardless of the training intensity or the group evaluated. Araújo *et al.*[25],in a study with rats exercised on treadmill and supplemented with creatine, they verified reduction in catalase activity in the liver of animals which performed physical exercise. The justification for this disagreement in results seems to be at least partially associated with the studied body, the exercise protocol applied and the use of an ergogenic substance. The SOD enzyme has three isoforms present in the human body: SOD1 is present in the cytoplasm, SOD2 in the mitochondria and SOD3 in the extracellular fluid. This enzyme catalyzes the dismutation (oxidation and reduction redox reaction) of the superoxide in oxygen and hydrogen peroxide[2,8,9]. In the present study, activity values of this enzyme (we analyzed total SOD) significantly different between the two evaluated groups, where the trained group presented higher mean values than the group kept sedentary during the experiment were found. The reports by Schneider *et al.*[24] did not show SOD significant differences before and after the application of the exercise protocol in the trained group. The investigation by Araújo *et al.*[4], who verified the activity of this enzyme in the hepatic tissue, did not find significant difference between the groups in their experiment, which were trained at the anaerobic threshold or 25% above it. The GPx enzyme, found in the mitochondria, has the role to reduce hydroperoxides to alcohols and hydrogen peroxide to water. In our study, we found significant differences of this enzyme's activity between the two evaluated groups, being the mean values higher in the trained group when compared to the sedentary one. In another investigation[24], the authors only identified significant difference of the glutathione peroxidase in some training intensities, evidencing again that the protocol-dependence issue may be the explanation for the disagreement between the results obtained by us compared with other studies. As a limitation to the study, we did not perform the ROSs direct biochemical determination; however, an important and reliable marker of oxidative stress was measured: TBARs. The TBARs concentrations were significantly different when the two studied groups were compared. The sedentary group presented TBARs mean concentrations higher compared with the trained group, which indicates reduction of the lipid peroxidation (biomarker of oxidative stress, injury and/or rupture in the membrane of the muscle fiber) in the muscle of the trained animals. Corroborating our findings, Araújo *et al.*[25] verified higher TBARs concentrations in the hepatic tissue of non-exercised rats when compared with the trained group. Contrary to these findings, Koçtürk *et al.*[26] verified increase in the TBARs concentrations in their study, which was developed with rats and used an acute exercise protocol on treadmill until exhaustion of the animals. Increase in the TBARs concentrations was verified up to six hours after the end of the exercise. In the latter case, the difference in experimental protocol possibly had an important influence on the results, especially concerning the variables analyzed and the type of exercise applied. There is evidence that the physiological increase in the production of radical species may favor the transport of glucose to inner part of the muscle fiber through a pathway mediated by the contraction of the skeletal muscle, and can cause increase in the glycogen concentrations in this tissue[13,14]. In our study, the muscle glycogen concentrations were increased in the exercised group. Such fact may be associated with the increase of glycogenesis, greater glucose transport through insulin-independent mechanism[27] and also increase of the activity of the glucose transporters, type GLUT-4[28]. In order to confirm this hypothesis, the GLUT-4should be determined, a fact which did not occur in the present study.

Thus, the findings of the present study concerned with the biomarkers of oxidative stress (increase of the activity of antioxidant enzymes and decrease of lipid peroxidation), associated with the increase in the content of muscle glycogen of the animal from the trained group, suggest that the physical training applied was possibly able to positively regulate the antioxidant machinery for the accumulation of this important energetic substrate. Therefore, physiological increase may have been observed in the ROSs production aassociated with the concomitant increase of the glycogen contents in the tissues of these animals, corroborating hence the studies by Higaki *et al.*[13] and Roberts *et al.*[14].

Conversely, the animals kept sedentary presented besides high TBARs concentrations, lower concentrations of muscle glycogen; such fact indicates, at least partially, that the sedentary condition promotes the supraphysiological production of ROSs and harms the accumulation of glycogen in the mentioned tissue. In order to confirm this hypothesis, it would be necessary to directly measure the ROSs or even the expression and/or activity of the glycogen sintase enzyme; however, in the present study such variables have not been determined.

Blair *et al.*[29] investigated the molecular mechanisms involved in the reduction of insulin sensitivity in response to the oxidative stress. These authors[29] verified that the increase of the hydrogen peroxide concentrations in the skeletal muscle causes inhibition of insulin pathways of glucose transport, which on its turn, may cause decrease of the synthesis of muscle glycogen. These findings support the hypothesis that the animals from the sedentary group of our study may have experienced, due to sedentarism, supraphysiological increase of ROSs, which hypothetically harmed the transport of glucose to the inside of the muscle fibers, leading hence to lower concentrations of glycogen when compared with the animals from

the trained group. Moreover, in our study, it was verified that the rats submitted to aerobic exercise presented higher mean value of the protein/DNA ratio in the assessed muscle (gastrocnemius), evidencing cell growth in that tissue, which may have helped both to control the ROS production and increase the content of muscle glycogen of the trained rats. Concerning the total amount of muscle DNA, no significant difference has been found between groups. Future studies are needed to better clarify the physical training effects on the oxidative stress mechanism and its influence on the glycogen content and skeletal muscle growth in rats.

CONCLUSION

The antioxidant defense system was improved in the gastrocnemius muscle of the animals submitted to aerobic training when compared with sedentary rats. These results indicate that regular physical exercise is an important strategy to fight the supraphysiological production of ROSs, since it increased the antioxidant enzymes activity and reduced the lipid peroxidation in the skeletal muscle. Such onset may have influenced, at least partially, on the increase o the glycogen content as well as the skeletal muscle growth of the animals.

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