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EXERCISE SESSION PROMOTES ANTIOXIDANT CHANGES IN BRAZILIAN SOCCER PLAYERS

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Abstract. Daily physical exercise routine of professional athletes improves physiological functions and performance. However, it also promotes alterations in the antioxidant capacity to combat the augmented oxygen consumption and, therefore, augmented reactive oxygen species (ROS) generation. The main purpose of this study was to evaluate the effects of sport training session on the antioxidant defenses and indicators of oxidative stress in blood of soccer players. Non-enzymatic antioxidant defenses: reduced and total glutathione (GSH and TG), vitamin C (Vit C), and vitamin E (Vit E); enzymatic antioxidant defenses: catalase (CAT), superoxide dismutase (SOD), glutathione S-transferase (GST), glutathione reductase (GR), and glutathione peroxidase (GPx); indicators of oxidative stress: contents of thiobarbituric acid-reactive substances (TBARS) and oxidized glutathione (GSSG); and also contents of glucose, triacylglycerol, and total plasma protein were analyzed before and after one intense training session (six hours), in 13 Brazilian professional soccer players. Red cell GSH contents, CAT and GST activities showed significantly decreased values, whereas GR activity was increased after training. TBARS contents of red cells were lower after training, but no significant differences were observed in plasma. Also, Vit C concentrations in plasma increased after training, whereas Vit E concentrations decreased. The results provide evidence of antioxidant changes after the training session in soccer players. ***(Biol.Sport 23:255-265, 2006)***

Key words: Reactive oxygen species - Antioxidants - Sport - Soccer

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Introduction

A training routine of professional soccer players demands high physical stress, which is an important element to enhance performance, technique and tactic during competitions [34,35]. Despite the health benefits, exercise can induce, in different levels, tissue oxidative stress, especially in tissues such as muscle, liver and blood [2,23,32], and under normal physiological conditions mitochondrial electron transport chain is a major source of reactive oxygen species (ROS) [23].

Several studies dealing with oxidative stress and exercise have shown that ROS concentrations can increase during exhaustive exercise both in humans and laboratory animals [12,14,23,24,25,35,39,40]. Chronic augmented ROS generation associated with strenuous aerobic performance can also promotes many kinds of injuries, illness, and probably, premature aging of the athletes [24,33].

The main purpose of this study was to test the hypothesis that exercise training session promotes oxidative stress in professional soccer players, through blood samples collected before and after sport training sessions. According to many researches, it has been postulated that the generation of free radicals is higher during exercise as a result of increase in mitochondrial oxygen consumption and electron transport flux, inducing lipid peroxidation [24,34]. The mitochondria from skeletal muscle under intense exercise are responsible for most of oxygen consumption of humans and other animals [8]. Considering that ROS generation is fairly parallel to oxygen utilization in mammals and other vertebrates [8], an unbalance between ROS and antioxidants is expected to occur in tissues devoid of an adequate antioxidant protection.

Materials and Methods

Materials and experimental protocol: Thirteen young men (average age 18.9±1.53 years; average weight 67.79±5.33 kg; and average height 176.1±4.46 cm), top professional soccer players belonging to the club *Avaí Futebol Clube* (top division of the State of Santa Catarina, located in Florianópolis, south Brazil) participated in the present study. The study was carried out through blood samples (3-5 ml) that were collected just before (early morning) and immediately after (late afternoon) a daily training session. The coach and his technical team supervised the training session, which was divided into 3 h of physical exercises and 3 h of technical and tactical exercises. In the morning, players were submitted to a following program: (1) 30-min callisthenic warm-up; (2) 2-h combination of various aerobic exercises, with intensity corresponding to 15 (hard heavy),

according to the Borg scale [7]; and (3) 30-min cool-down including stretching and massage, while in the afternoon was alternated to technical and tactical exercises. Diet habits (quality and quantity) were controlled by the team's medical staff, and its amount regarding antioxidant contents were similar among the players (fruit, vegetables, etc.). All of the subjects were devoid of familial or personal histories of diseases, and they were not using drugs, cigarettes, or alcohol. The experimental protocols were in accordance to the Helsinki Declaration and to guidelines of the local ethical committee (approved by CEUA process number 029/99). Informed consent was obtained from all participants.

Sample preparation: Blood samples were obtained from the antecubital vein in chilled tubes containing heparin as anticoagulant in overnight fasting subjects. Acid extracts were immediately obtained for glutathione assays and the remaining volumes were immediately centrifuged (5000 g for 5 min), and aliquots of plasma and erythrocytes were frozen in liquid nitrogen. Hemolysates were obtained in Triton X-100, 0.12M NaCl, 30 mM NaPO4, pH 7.4 (1:4, blood: buffer volume). For enzymatic assays, blood was centrifuged at 3000 g for 3 min at 5°C, washed twice with cold saline solution and then centrifuged again (5000 g for 5 min), and hemolysis was carried out by freezing/thaw procedure. After this, samples were centrifuged at 15000 g for 10 min and the supernatants were stored in liquid N2. Beside hemolysates, aliquots of acid extracts and plasma were stored in liquid nitrogen for 2-4 days and examined separately for each assay. Enzymatic evaluations were carried out in the supernatants (glutathione S-transferase, GST; glutathione reductase, GR; glutathione peroxidase, GPx; catalase, CAT; and superoxidade dismutase, SOD) and also in plasma (GST and CAT). The contents of thiobarbituric acid-reactive substances (TBARS) were examined in plasma and also in whole blood, and the contents of reduced glutathione (GSH), total glutathione (TG), and oxidized glutathione (GSSG) in whole blood extracts, whilst the contents of Vitamin C (Vit C) and Vitamin E (Vit E), as well as the contents of glucose, triacylglycerol, and total protein were examined in plasma. All TBARS and GSH contents were examined in fresh blood samples immediately after blood collection. Frozen samples were not used because even when reacted with BHT they showed artifactual lipid autoxidation, and, therefore, enhanced TBARS levels, and also because GSH is rapidly oxidized at relatively low temperatures [11].

Enzymatic and non-enzymatic antioxidants: SOD activity was measured at 550 nm according to the method of cytochrome c reduction [17]. CAT activity was determined by measuring the decrease in a freshly prepared solution of hydrogen peroxide (10 mM) concentration at 240 nm [1]. GPx was measured at 340 nm through the glutathione/NADPH/glutathione reductase system, by the dismutation

of *tert*-butylhydroperoxide [16]. GR was measured at 340 nm through the oxidation rate of NADPH, in a reaction medium containing 0.1 M NaPO4 buffer, pH 7.0 containing 0.1% DPTA (diethylenetriaminepentacetic acid) and 1 mM GSSG [10]. GST was measured at 340 nm according to Habig [20], using CDNB (1-chloro-2,4-dinitrobenzene) as substrate and 0.15 M GSH concentration. All activities were expressed in milliliters of whole blood.

TBARS assay: Determination of TBARS was used to assay endogenous lipid oxidation according to Ohkawa [32] and Bird and Draper [6]. Aliquots of 0.2 mM butylhydroxytoluene (BHT) were added to blood samples to avoid further artifactual lipid oxidation. Acid extracts were obtained by the addition of the homogenate to 12% trichloroacetic acid (1:4 v/v), followed by centrifugation. Supernatants were centrifuged at 5000 g for 3 min, added to 0.67% (w/v) 2-thiobarbituric acid, maintained in boiling water for 60 min, cooled at 5°C for 30 min, and then measured spectrophotometrically at 535 nm. Absorbance was expressed as nmol TBARS/ml (E535 = 153 mM-1 cm-1).

Glutathione assay: GSH was measured according to Beutler [5], using the Elmann's reagent (2-dithionitrobenzoic acid, DTNB). Acid extracts were obtained by the addition of 12% trichloroacetic acid (1:4 v/v), followed by centrifugation (5000 g for 5 min at 5°C). Supernatants from the acid extracts were added to 0.25 mM DTNB in 0.1 M Na-PO4, pH 8.0, and the formation of thiolate anion was determined at 412 nm. TG was also measured at 412 nm according to the method of Tietze [41], and GSSG was calculated in equivalents of GSH.

Vitamin C and vitamin E assays: The Vit C (ascorbic acid) in plasma was analyzed through high performance liquid chromatograph (HPLC), using Supelcosil LC-18 column, reverse phase, as stationary phase, and methaphosphoric acid 0.8 % as mobile phase, electrochemichal detection, mainly according to Motchink and collaborators [31]. The Vit E in plasma (α-tocoferol) was also analyzed by HPLC, using a C-8 column as stationary phase, and methanol-water (97.5 : 2.5 ml, V/V), with LiClO4 20 mM as mobile phase, also according to Motchink and collaborators [31].

Other biochemical assays: Plasma concentrations of glucose, triacylglycerol and total protein were measured through corresponding kits (Diagnostica, MG, Brazil).

Chemicals: All other reagents were purchased from Sigma Chemical Co. (Ohio, USA).

Statistical treatment: The statistical analyses were conducted using the SPSS (11.0) for Windows statistical software package (SPSS, Chicago, Ill., USA), and the results were processed by “student’s paired t test”. The accepted level of

significance was set at $P < 0.05$ for all statistical tests. All biochemical parameters were measured in duplicate, and values were expressed as mean ± SEM.

Results

Tables 1 and 2 show values of the parameters before and after training measured in blood and plasma, of soccer players who participated in the training session.

Analysis of blood: Significant decrease in GSH values measured in whole blood were obtained in samples from after training compared to those from before training session (Table 1). No differences were found in the in contents of TG and GSSG, but significant increases in GSSG:GSH ratio was observed after exercise (Table 1). Also, erythrocytic GST showed significant decreased activity, while GR activity increased, and the activity of CAT in erythrocytes decreased after the training session (Table 1). Erythrocytic GPx and SOD, and TBARS measured in whole blood, did not show significant changes (Table 1).

Table 1
Whole blood levels of GSH, TG, GSSG and TBARS and erythrocytic enzymatic antioxidants in professional soccer players pre and post a training session

Parameter	Pre training	Post training
GSH (mM)	0.82±0.11	0.70±0.16*
TG (mM)	1.18±0.41	1.11±0.33
GSSG (mM)	0.44±0.38	0.58±0.52
GSSG:GSH	0.54±0.15	0.83±0.17*
GST (μmol min^{-1} ml^{-1})	12.64±3.95	5.66±2.73***
GR (μmol min^{-1} ml^{-1})	1.78±1.06	8.18±3.47***
GPx (μmol min^{-1} ml^{-1})	4.08±2.12	5.02±1.88
CAT (mmol min^{-1} ml^{-1})	68.05±7.5	39.79±10.9***
SOD (U ml^{-1})	178.6±47.6	180.5±47.2
TBARS (μmol ml^{-1})	95.84±17.68	97.84±17.61

*p<0.05; ***p<0.001

Analysis of plasma: Triacylglycerol, total protein, and glucose concentrations did not show significant changes (Table 2). However, concentrations of Vit C increased significantly after training, while concentrations of Vit E decreased

(Table 2). The plasma activity of GST decreased significantly, but the activity of CAT and the concentrations of TBARS did not present significant changes (Table 2).

Table 2
Plasma levels of various antioxidants and oxidative stress markers, and biochemical parameters in professional soccer players pre and post a training session

Parameter	Pre-training	Post-training
Vitamin C (μM)	13.62±3.16	20.66±4.23***
Vitamin E (μM)	21.73±5.25	16.94±5.09***
GST (μmol min^{-1} ml^{-1})	8.56±3.46	3.61±1.73**
CAT (nmol min^{-1} ml^{-1})	28.11±3.18	27.09±1.52
TBARS (nmol ml^{-1})	2.88±0.87	3.06±1.22
Glucose (mg100 ml^{-1})	98.1±6.6	86.1±2.6
Triacylglycerol (mg100 ml^{-1})	56.6±5.0	52.3±3.6
Total protein (mg100 ml^{-1})	7.5±0.5	6.9±0.1

**p<0.01; ***p<0.001

Discussion

The present study did not find significant differences in plasma levels of glucose, triacylglycerol, and total protein before and after the training session. In contrast, a previous study of athletes participating in the Hawaii Ironman triathlon showed decreased values in these parameters after exercise [18]. The decreased values observed in the previous study were probably due to the exercise intensity of a triathlon competition compared to the relatively mild exercise carried out in the present study. In addition, no significant changes were also observed in TBARS levels in plasma, GSSG and TG concentrations in whole blood, in CAT activity in plasma, as well as in erythrocytic GPx activity. Also, erythrocytic SOD activity showed no significant differences after the training session in the present study. However, SOD isoform (EC SOD) from plasma and from the extracellular matrix of tissues may be a better enzymatic biomarker related to exercise compared to the isoforms placed in the cytosol and mitochondrial matrix [33].

However, the GSH levels found in whole blood were significantly decreased, similarly to other results obtained in humans after relatively short exercise

programs [12,13,19,27]. Consistently, erythrocytic GR activity significantly increased after exercise. The same result was found in humans submitted to weightlifting training [42]. The increase in GR activity might be related to a compensatory response to recover GSSG and guarantee adequate GSH levels in the cells [21]. High GSSG contents and, therefore, low GSH levels, are dangerous to cells, and both indices characterize a condition of oxidative stress [26], and are considered very important indicators of exercise-induced oxidative stress [39]. The pivotal role of glutathione in modulating exercise-induced oxidative stress was already demonstrated in humans after intense performance [27,34]. Glutathione is ubiquitous and found in high concentrations in animal cells and is, together with protein-SH groups, the main determinants of total cellular antioxidant capacity [4,15]. Interestingly, it was recently demonstrated that protein-bound sulphydril groups present in blood, mostly hemoglobin cysteine residues, through a thiolation-dethiolation reversible process, could also participate as an important antioxidant under moderate physical exercise-induced oxidative stress [23], an antioxidant property of hemoglobin proposed many years ago by Reischl [36].

Strenuous and endurance exercises generally promote increase in lipid peroxidation in humans [2,11,14,22,29] and this effect can be enhanced under hypoxic conditions in acute exercise [3]. In the present study no differences were found in TBARS levels both in the red cells and in plasma. This result can be a consequence of antioxidant depletion such as glutathione and Vit E, as found in the present study. In such a way their consumption would prevent further increases in tissue damage by ROS generation under exercise. Similar finding was described in young subjects after cycling for 40 min, showing an inverse correlation between blood GHS and TBARS levels [19].

CAT and GST activities in erythrocytes were significantly decreased after training, and the same result was obtained in plasma GST activity. The GST decrease is probably related to GSH depletion verified in blood after the training sessions, considering that GSH is an important cofactor for the normal GST activity. GST is part of the so-called Phase II of the biotransformation process of toxic compounds including endogenous hydroperoxides derived from the lipoperoxidation process, and is responsible for the conjugation and excretion of such compounds derived from the Phase I process, which involves the intervention of a super-family of different isoforms of cytochromes [21].

Plasma Vit C contents showed increased concentrations after exercise, whereas Vit E concentrations showed decreased values. The latter finding indicates that Vit E was probably used to counteract ROS generation and lipoperoxidation associated with intense exercise [21,34]. The benefits of Vit E supplementation in various

kinds of exercises are well documented in the literature [36,37,39], and it seems that some discrepancies reported about this effect are attributable to aspects involved in the assessment of oxidative stress [36]. The increase in Vit C concentrations seems to be mostly a consequence of the ingestion of orange juice of all subjects during training intervals, a common practice among local soccer players, an ancient Brazilian tradition that suggests, among others, prevention against oxidative stress. A further contribution for the plasma Vit C increase is the redistribution and rise of this hydrosoluble antioxidant upon exercise [21].

The plasma concentrations of Vit E and Vit C found in the present study were low in comparison with values reported for soccer players from Argentina (≅26μM and ≅98μM, respectively [9]; ≅30μM and ≅60μM [35], respectively), and more similar to the values found in sedentary young subjects (≅ 40μM and 23μM, respectively [9]). The relatively low Vit E and Vit C contents showed by the athletes examined in the present study suggest the existence of a chronic low antioxidant status of the athletes previously to the training sessions, and under these circumstances a better athletic performance will probably be jeopardized [19,34].

Upon extended periods of time the human organism seems to be able to recover from an oxidative stress elicited by routine exercise and even to maintain an improved antioxidant status compared to sedentary people [9,24,25]. The soccer players submitted to a relatively low intensity exercise seem to counteract the deleterious effect of ROS generation, if the maintenance of TBARS levels, the augmented enzymatic activity of glutathione reductase to compensate the depletion of GSH, and also the consumption of plasma contents of Vit E are taken into consideration. On the other hand, under chronic intense aerobic exercise such as in marathon practice, or after exhaustive or eccentric exercise performed by humans [24] the organism can be severely stressed by oxidative events. The apparent improved antioxidant protection of top young athletes compared to sedentary subjects [9,14] including aged people [25], not necessarily avoid long term injuries due to intense exercises accumulated during years and reflected in the elderly [25,26,34]. Therefore, regular and mild physical exercise is recommended and preferable compared to strenuous performances and over-training [14,24,25].

From the results obtained in the present study it is suggested that an appropriate training protocol together with a balanced diet rich in nutritional antioxidants [38] or a prudent antioxidant supplementation coupled to individual and specific needs [18,30], would compensate the deleterious effects related to ROS overgeneration in professional soccer players. Taken into consideration the data available in the

concerned literature, these recommendations seem to apply for other kinds of exercise and sport practice [14,18,22,24,34,39,40].

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