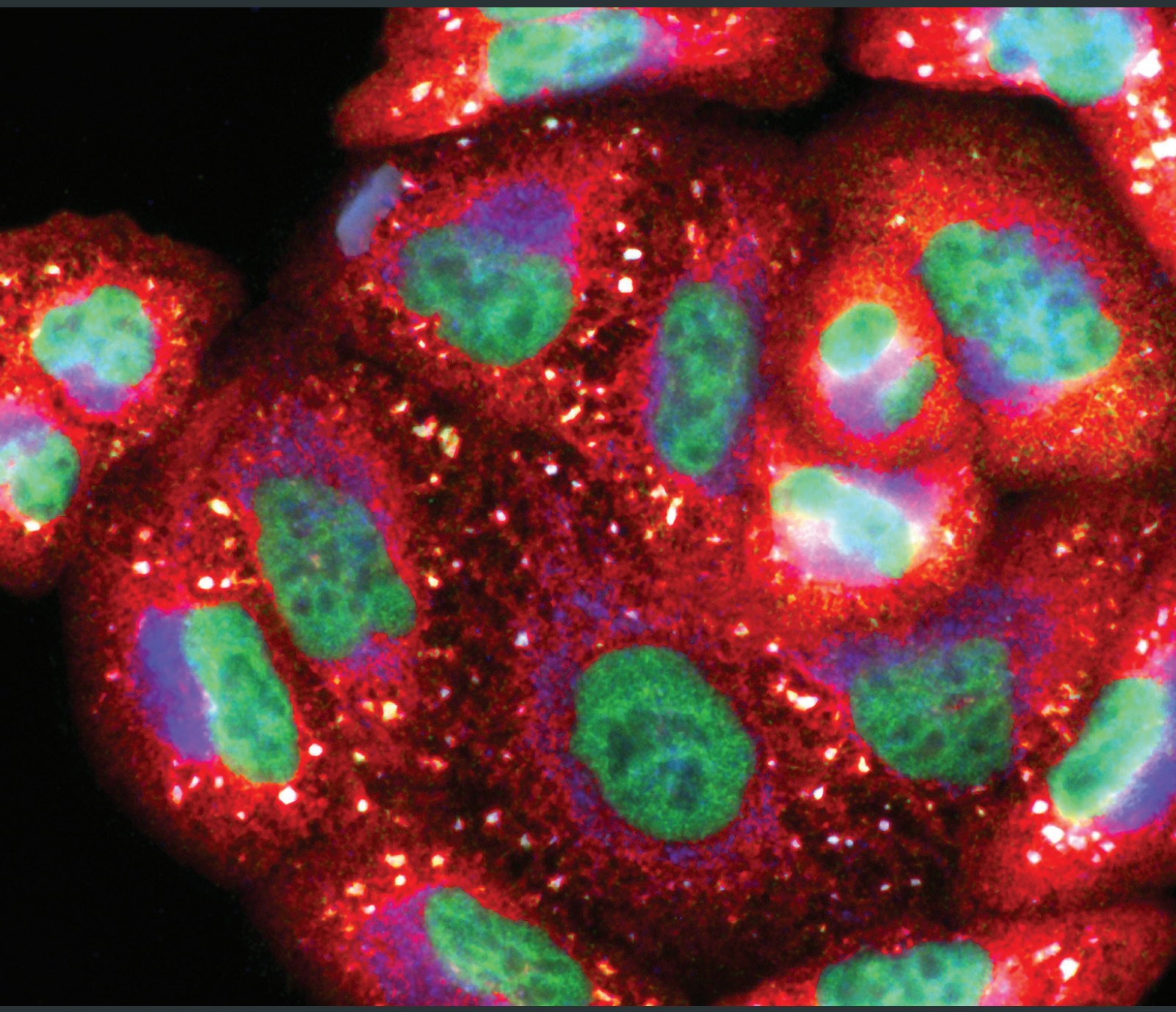
Oxidative Medicine and Cellular Longevity

Oxidant Antioxidants and Adaptive Responses to Exercise

Guest Editors: Paola Venditti, Mari Carmen Gomez-Cabrera, Yong Zhang, and Zsolt Radak



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Editorial Board

Contents

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Editorial

Oxidant Antioxidants and Adaptive Responses to Exercise

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The extensive damage produced by unaccustomed (acute) exercise and the health benefits of regular physical activity are well-known phenomena as well as the role played in them by reactive oxygen species (ROS).

The present issue reports some interesting studies showing that the Janus faced effects of exercise-induced ROS in skeletal muscle.

Most studies dealing with ROS contribution to acute exercise-induced tissue damage determine the levels of markers of oxidative damage to specific substances but they do not take into account total redox status of an individual before and after exercise. In their research article D. Stagos et al. used markers measuring plasma static (sORP) and capacity (cORP) of oxidation-reduction potential (ORP) and more common redox markers for evaluating the effect of eccentric exercise. They showed that only in individuals exhibiting decrease in cORP after exercise, there were increases in lipid peroxidation, suggesting that cORP decrease after exercise can be indicative of oxidative stress induction.

Acute exercise-induced damage can be prevented by supplementation with single antioxidants. On the other hand, several studies suggest that a diet containing a mixture of different antioxidants is more effective in prevention of diseases dependent on high ROS production. In their paper, S. Carfagna et al. used a diet supplemented with *G. sulphuraria,* an alga exhibiting high antioxidant properties, mainly due to high content GSH and phycobilin proteins, known for their capacity to protect membranes from oxidative attack, for animals subjected to acute exercise. It was observed that *G. sulphuraria* prevented the oxidative stress in various tissues and preserved mitochondrial functionality, thus showing that dietetic integration with a source rich in antioxidants offers high protection against exercise-induced oxidative stress.

Mitochondrial respiratory chain is considered a major source of ROS during exercise, even though underlying mechanisms remain to be completely elucidated. In their work P. Wang et al. showed that, during exercise, there was a time-dependent increase in mitochondrial ROS production, which was correlated with upregulation of $p66^{shc}$ (a protein involved in H_2O_2 generation) and FOXO3a (a factor activating the transcription of mitochondrial superoxide dismutase and catalase). The authors suggest that the ROS production stimulated by $p66^{shc}$ is counteracted by catalase transcription by FOXO3a.

In human athletes, S. Mrakic-Sposta et al. evaluated the effects of a high-intensity discontinuous training period on ROS production and reducing capacity (assessed in capillary blood by EPR and redox sensor, resp.) after acute exercise. They showed that acute exercise induced a ROS increase dependent on exercise intensity, which was reduced by training confirming that this offers protection against oxidative stress induced by acute exercise.

Regular physical activity is associated with many health benefits including reduced risk for disorders such as cardiovascular disease, cancer, and diabetes and in the promotion of healthy aging. The training-induced cardiovascular protection has been investigated by A. Pósa et al., which showed that the training preservation against cardiac injury

might be associated with the decreased activity of matrix metalloproteinases 2.

In another paper, A. Pósa et al. showed that physical exercise, associated with caloric restriction, is able to prevent the metabolic syndrome induced by high calorie diet.

Despite diversity, it is our believe that the articles comprised in this special issue could represent an important contribution to improve the knowledge of the mechanisms that are verified during physical activity and training and that make the exercise an ideal strategy in the prevention and treatment of many pathological conditions.

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Research Article

Cardioprotective Effects of Voluntary Exercise in a Rat Model: Role of Matrix Metalloproteinase-2

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Background. Regular exercise at moderate intensity reduces cardiovascular risks. Matrix metalloproteinases (MMPs) play a major role in cardiac remodeling, facilitating physiological adaptation to exercise. The aim of this study was to examine the influence of voluntary physical exercise on the MMP-2 enzyme activity and to investigate the cardiac performance by measurement of angina susceptibility of the heart, the basal blood pressure, the surviving aorta ring contraction, and the cardiac infarct size after I/R-induced injury. *Methods*. Male Wistar rats were divided into control and exercising groups. After a 6-week period, the serum level of MMP-2, basal blood pressure, cardiac angina susceptibility (the ST segment depression provoked by epinephrine and 30 s later phentolamine), AVP-induced heart perfusion and aorta ring contraction, infarct size following 30 min ischemia and 120 min reperfusion, and coronary effluent MMP-2 activity were measured. *Results*. Voluntary wheel-running exercise decreased both the sera (64 kDa and 72 kDa) and the coronary effluent (64 kDa) MMP-2 level, reduced the development of ST depression, improved the isolated heart perfusion, and decreased the ratio of infarct size. *Conclusion*. 6 weeks of voluntary exercise training preserved the heart against cardiac injury. This protective mechanism might be associated with the decreased activity of MMP-2.

1. Introduction

Regular physical exercise has been shown to reduce many cardiovascular risk factors and is associated with a number of cardiovascular benefits. The reduced levels of cardiovascular mortality and cardiac ischemic events in patients who participate in regular exercise training are mainly due to the control of cardiovascular risk factors (e.g., high blood pressure and obesity) [1, 2].

Although regular exercise training has been confirmed as a pragmatic and sustainable countermeasure for cardioprotection, the precise underlying mechanisms for these beneficial effects remain to be defined.

One group of enzymes that is important in mediating the destructive effects of cardiovascular disease is the family of matrix metalloproteinases (MMPs). The MMPs are a large family of calcium-dependent, zinc-containing endopeptidases that have the ability to remodel the extracellular matrix in both physiological and pathological processes. Of this diverse family of enzymes, MMP-2 (also known as gelatinase A) is found in nearly all cell types and plays a key role in the cardiovascular system, ranging from heart development to ischemia/reperfusion (I/R) injury [3]. The release of MMP-2 increases with increasing duration of ischemia and correlates negatively with functional recovery. Over the past few decades, many approaches to the relationship between MMP-2 activity and infarct size have been studied [4]. These investigations have revealed that the inhibition of MMP-2 protects the heart from cardiac dysfunction [5]. However, the

activation of MMP-2 during an ischemic insult is associated with a larger infarct size of the heart [6]. Myocardial ischemia followed by reperfusion results in I/R injury characterized by a decreased myocardial function and tissue necrosis which is a marker of a long-term left ventricular function in patients who have suffered an acute myocardial infarction [7]. The purpose of this study was to assess the effects of 6 weeks of voluntary physical exercise on MMP-2 activity and release into the coronary effluent and to investigate the cardiac performance. We additionally measured the angina susceptibility of the heart, the basal blood pressure, and surviving aorta ring contraction and determined the cardiac infarct size after I/R-induced injury.

2. Materials and Methods

2.1. Animals and Experimental Design. All experimental procedures were performed in accordance with the standards of the European Community guidelines on the care and use of laboratory animals and had been approved by the local Institutional Ethics Committee at the University of Szeged.

Male Wistar rats ($n = 70$, weighing 200–230 g; Toxi-Coop Zrt., Hungary) were randomly assigned to control and exercising groups. The exercising animals were placed individually into cages fitted with a running wheel (Acellabor Ltd., Budapest, Hungary) and were allowed free access to the wheel for 24 h per day for 6 weeks [8]. The exercising protocol, defined as a voluntary wheel-running model, was selected in an effort to isolate the effects of exercising from the additional stress associated with forced exercise protocols. During the exercising period, the average running distance was 3.91 ± 1.27 km/day/animal. Control rats were placed in standard holding cages without a running wheel for the same period. All animals were housed in a temperature-controlled facility (23°C) maintained on a 12:12 h light-dark cycle with food and water provided *ad libitum*.

2.2. Measurement of MMP-2 Activity. Metalloproteinase activity was detected by gelatin zymography. Sodium dodecyl sulfate-polyacrylamide gels (8%) were copolymerized with 20 mg/mL gelatin. A constant amount of protein was loaded in each well under nonreducing conditions and was separated by electrophoresis. After electrophoresis, the gels were washed with 2.5% Triton X-100 and incubated overnight in incubation buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, and 0.05% NaN_3) at 37°C. After staining with 0.05% Coomassie Brilliant Blue in a mixture of methanol/acetic acid/water and being destained with aqueous 4% methanol/8% acetic acid, enzyme activity was detected as colorless bands against the blue-stained background. A protein ladder (Spectra Multicolor Broad Range Protein Ladder, Thermo Scientific) was used to identify the individual enzymes (MMP-2, 72 kDa and 64 kDa isoforms) for molecular weights. Zymograms were scanned digitally and the band intensities were quantified by using Quantity One software (Bio-Rad, Hercules, CA, USA).

2.3. Measurement of Basal Blood Pressure. The animals were anaesthetized with 30.0% urethane (0.50 mL/100 g, i.p.) and the mean arterial blood pressure was measured in the right carotid artery, expressed as mmHg. The right carotid artery was separated from the vagus nerve and clamped with a bulldog clamp for cannulation. Then the carotid artery cannulation was connected to the HAEMOSYS computerized complex hemodynamic analysis system (Experimetria UK, London) to record the mean blood pressure. After 10–15-minute stabilization period, the baseline recording has carried out over a 10-minute period to examine the mean blood pressure. The core temperature of the rats was maintained at 37°C with a homeothermic control unit (Harvard Instrument, UK).

2.4. Experimental Angina Provoked by Epinephrine Plus Phentolamine. The animals were anesthetized with 30.0% urethane (0.50 mL/100 g, i.p.) and the standard limb lead II of the surface electrocardiogram (ECG) was recorded by the HAEMOSYS system and expressed as mV. The change in ST segment was measured and used as the index of angina severity. The mean ECG voltage 13 ms after the peak of the S wave was defined as the value of the ST segment, as described previously [9]. In the epinephrine plus phentolamine model, single doses of epinephrine (10.0 μg/kg) and 30 s later the α-adrenoreceptor antagonist phentolamine (15.0 mg/kg) were administered into the tail vein of the rat. Each agent was dissolved in 0.20 mL of physiological saline and injected over 2 s. To measure the difference in the amplitude of ST segment, the ECG recording has been carried out from the challenge of epinephrine over a 10-minute period.

2.5. Measurement of Arginine Vasopressin- (AVP-) Induced Heart Perfusion according to Langendorff. After cervical dislocation, the hearts were rapidly excised and then perfused according to Langendorff at a constant pressure of 75 mmHg with Krebs-Henseleit buffer (118.0 mM NaCl, 4.70 mM KCl, 2.50 mM $CaCl_2$, 1.18 mM $MgSO_4$, 25.0 mM $NaHCO_3$, 1.18 mM KH_2PO_4, and 5.50 mM glucose), gassed with a mixture of 95% O_2/5% CO_2 at 37°C. Following the cannulation, the heart perfusion in response to AVP was measured. The AVP quantity (0.01, 0.1, and 1.0 μg) was added in Krebs-Henseleit buffer and then 0.5 mL of AVP-Krebs solution was injected into the Langendorff apparatus by way of a valve attached to the cannula. We administrated the perfusion changes every 30 s for 5 min. Each dose of AVP was injected after a 15-minute stabilization period. Data are expressed as a percentage of the decrease relative to the basal value.

2.6. Measurement of Surviving Aorta Contraction. The rats were killed by cervical dislocation, and the abdominal aortas were removed and placed in chilled Krebs-Henseleit solution (118.0 mM NaCl, 4.70 mM KCl, 2.50 mM $CaCl_2$, 1.18 mM $MgSO_4$, 25.0 mM $NaHCO_3$, 1.18 mM KH_2PO_4, and 5.50 mM glucose) which was gassed with 95% O_2 and 5% CO_2. The aorta contraction was measured as described previously [10]. Briefly, the aortas were cleaned of all adipose and connective tissue, the abdominal region was cut into rings (3 mm long), and their weights were measured. To induce the contraction changes, we freshly added a 2 μg/mL dose of AVP into the

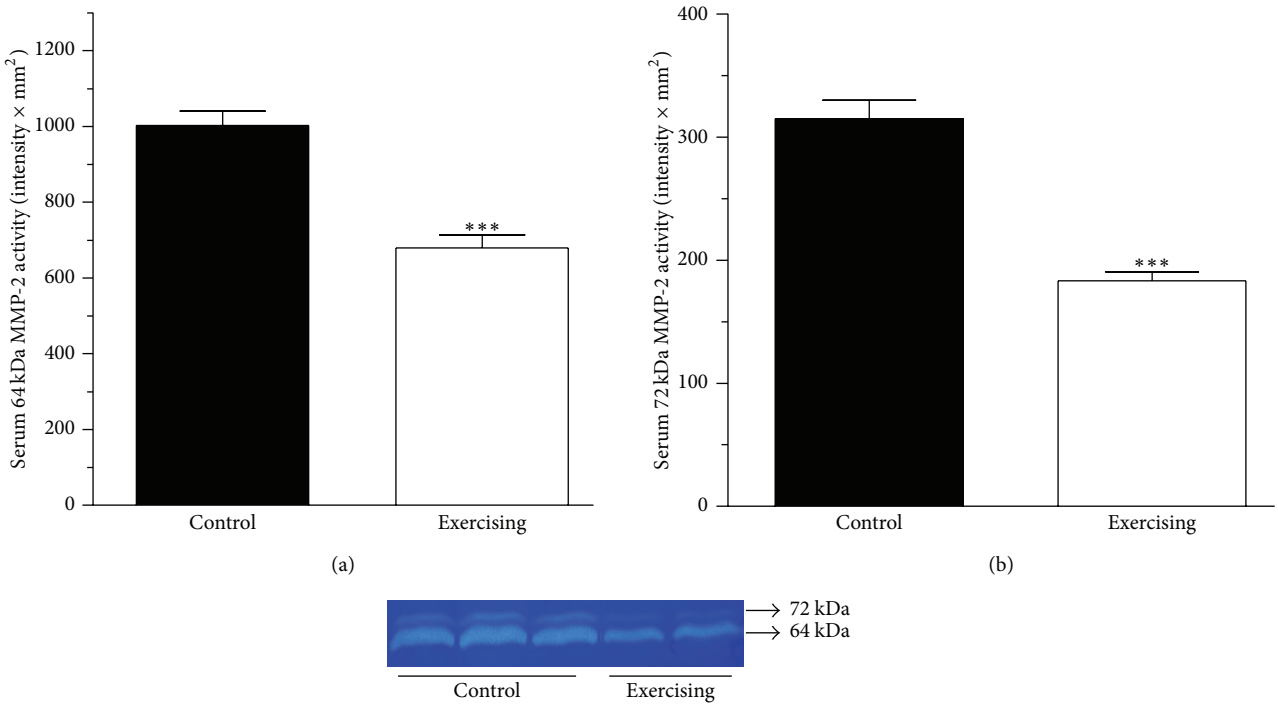

Figure 1: Serum matrix metalloproteinase-2 activity (64 kDa and 72 kDa MMP-2, expressed as intensity × mm^2) in control (■) and exercising (□) rats. Data are means ± SEM, n = 12-13. Statistical significance: $^{***}P < 0.001$ as compared with the control group. (a) Serum 64 kDa matrix metalloproteinase-2 activity (64 kDa MMP-2, expressed as intensity × mm^2) in control (■) and exercising (□) rats. (b) Serum 72 kDa matrix metalloproteinase-2 activity (72 kDa MMP-2, expressed as intensity × mm^2) in control (■) and exercising (□) rats. The representative image presents a zymographic picture.

incubation buffer [10]. The isometric tension was measured through the transducer, which was connected to an ISOSYS computerized program system (Experimetria, UK, London) for continuous recording of the blood vessel tension. The contractile response to vasopressin was expressed in terms of the tension of the aorta ring (g/mg ring weight).

2.7. Ischemia/Reperfusion Protocol. After cervical dislocation the hearts were rapidly excised and then perfused in Langendorff mode at a constant pressure of 75 mmHg with Krebs-Henseleit buffer, gassed with a mixture of 5% CO_2 + 95% O_2 at 37°C. After normoxic perfusion for 10 min, local ischemia was induced by occlusion of the left anterior descending coronary artery (LAD) for 30 min. This was followed by reperfusion for 120 min. The coronary effluent was collected during the first 5 min of reperfusion and was concentrated in Amicon Ultra Centrifugal concentrating vessels (5000 g, 4°C, Millipore, MA, USA) for MMP-2 activity determination via gelatin zymography. At the end of the protocol the hearts were stained with 1% Evans blue and then were frozen at −20°C overnight.

2.8. Infarct Size Determination. Infarct size was measured after regional ischemia induced by LAD occlusion *ex vivo* [11]. Frozen hearts were cut into 2 mm thick cross-sectional slices. These slices were stained with 1% 2,3,5-triphenyltetrazolium chloride (TTC) for 10 min at 37°C. After TTC staining, the slices were transferred to a formalin (10%) solution for 10 min and then placed in phosphate buffer (pH = 6). Following this incubation, both sides of each slice were photographed with a digital camera. Infarct size was calculated as the percentage of the area at risk.

2.9. Statistics. The results shown in the figures are expressed as means ± S.E.M. Differences between groups were determined with two-tailed Student's t-test and P values less than 0.05 were considered significant.

3. Results

3.1. Sera 64 kDa and 72 kDa MMP-2 Activity. Serum samples were collected from the lateral tail vein. We found that the 6-week voluntary physical exercise significantly ($P < 0.001$) decreased the serum MMP-2 activity (that of 64 kDa MMP-2 from 1002.71 ± 37.50 to 679.73 ± 34.35 intensity × mm^2 and that of 72 kDa MMP-2 from 314.93 ± 14.80 to 183.33 ± 7.12 intensity × mm^2, n = 12-13). Data are shown in Figure 1.

3.2. Measurement of Basal Blood Pressure. The basal blood pressure measured in the right carotid artery proved to be similar in the control and exercising rats (from 94.71 ± 4.13 to 91.48 ± 1.69, n = 14–18). Data are presented in Figure 2(a).

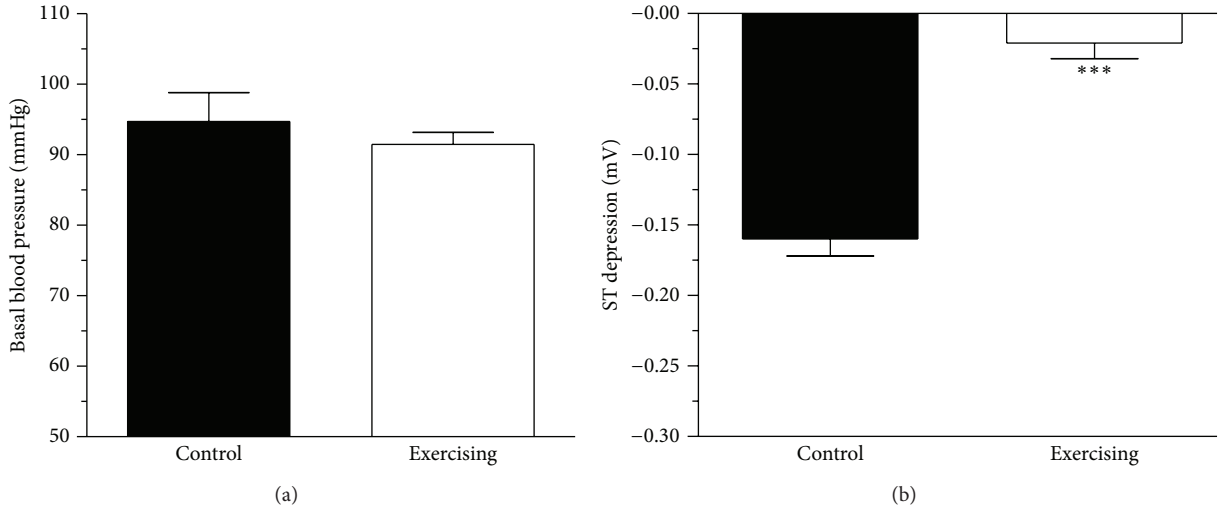

Figure 2: (a) The basal blood pressure (mmHg) in control (■) and exercising (□) animals. Data are expressed as means ± S.E.M., $n = 14$–18. (b) The effect of recreational physical exercise on ST segment changes (measured in a lead II standard surface ECG; expressed in mV) following intravenous injection of epinephrine (10.0 μg/kg) and 30 s later phentolamine (15.0 mg/kg). Data are shown as means ± S.E.M, $n = 14$–18. Statistical significance: $^{***}P < 0.001$ as compared with the control group.

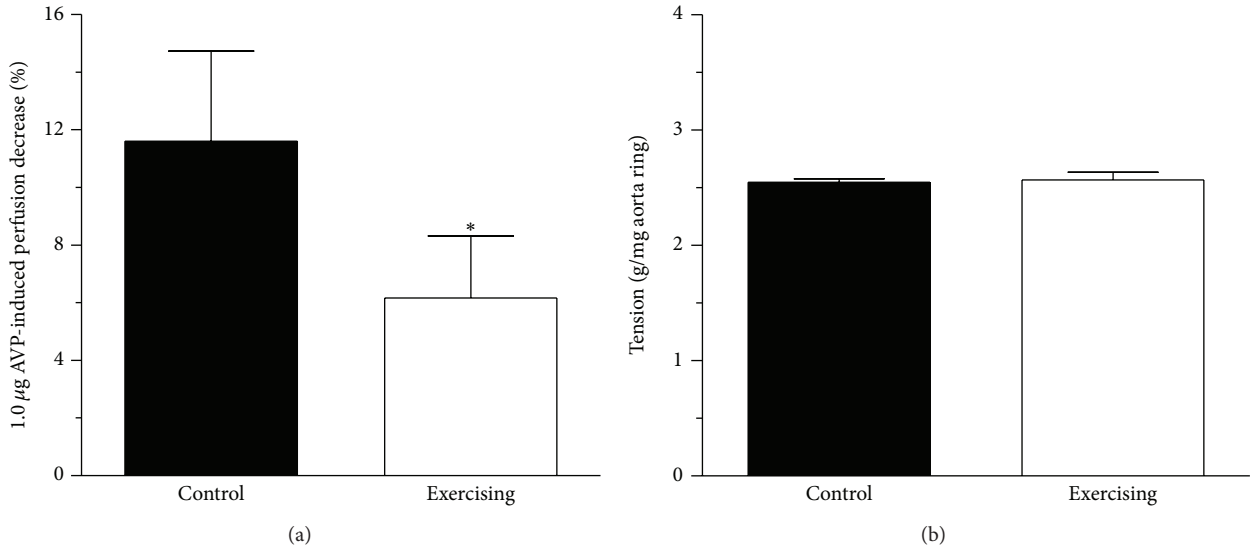

Figure 3: (a) The Langendorff heart perfusion decrease expressed as a percentage in response to 1.0 μg arginine-vasopressin (AVP) in control (■) and exercising (□) rats. Means ± S.E.M., $n = 8$–10. Statistical significance: $^{*}P < 0.05$ as compared with the control group. (b) The effect of 2.0 μg AVP-induced aorta ring contraction (expressed as g/mg aorta ring weight) on control (■) and exercising (□) animals. Results are shown as means ± S.E.M., $n = 9$-10.

3.3. Experimental Angina Provoked by Epinephrine Plus Phentolamine. The administration of phentolamine 30 s after epinephrine caused a robust ($P < 0.001$) ST segment depression in the control rats (−0.16 ± 0.012 mV, $n = 14$–18). As a result of 6-week physical exercise we have found an improvement in ST segment changes (−0.021 ± 0.011 mV, $n = 14$–18, $P < 0.001$). Data are shown in Figure 2(b).

3.4. Measurement of Isolated Heart Perfusion Provoked by AVP. The perfusion measured according to Langendorff is illustrated in Figure 3(a). No differences in basal perfusion or in response to 0.01 or 0.1 μg AVP were observed between the control and the exercising animals. However, 1.0 μg AVP revealed a significantly ($P < 0.05$) improved perfusion in the exercising group (from 11.6 ± 3.14 to 6.17 ± 2.16%, $n = 8$–10).

3.5. Measurement of Aorta Contraction Provoked by AVP. Results observed in the experiment involving the surviving aorta ring contraction are demonstrated in Figure 3(b). In response to the 2.0 μg/mL dose of AVP, we found no significant difference between the control and the exercising

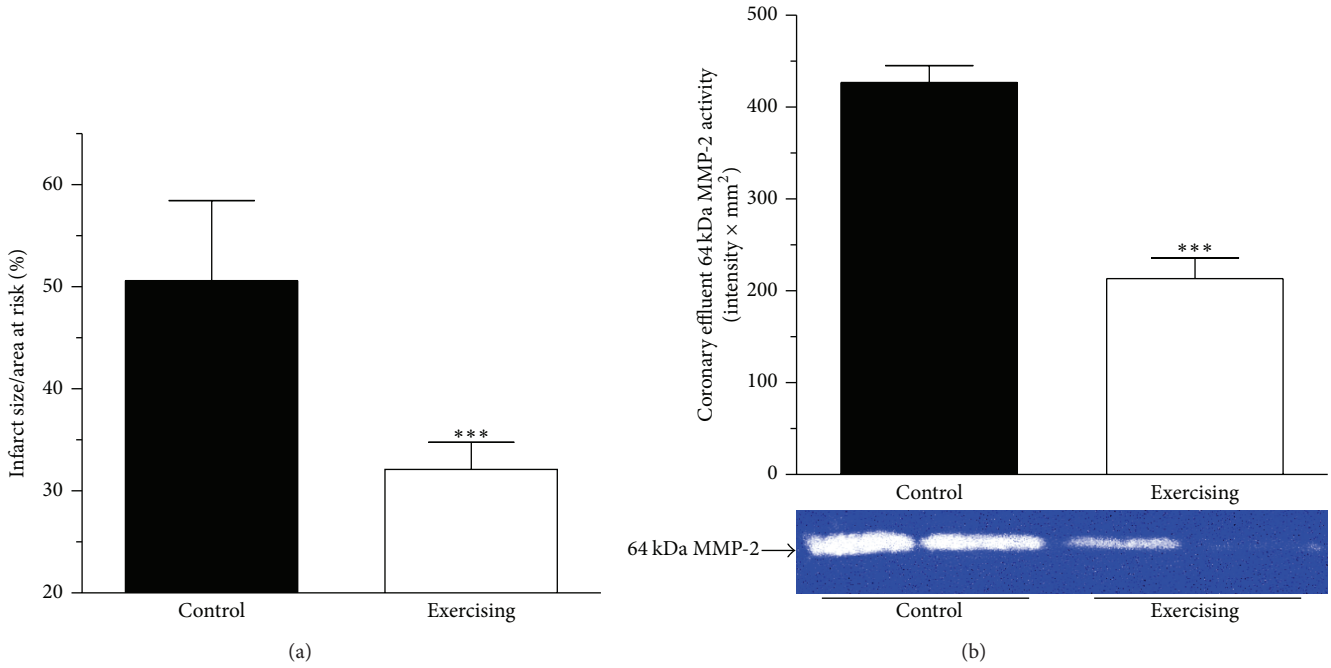

FIGURE 4: (a) Infarct size is demonstrated as a percentage of the area at risk in control (■) and exercising (□) groups. Means ± S.E.M., n = 11-12. Statistical significance: $^{***}P < 0.001$ as compared with the control animals. (b) Densitometrically assessed MMP-2 activity in coronary effluent collected from isolated perfused hearts during the first 5 min of reperfusion (64 kDa MMP-2, expressed as intensity × mm^2) in control (■) and exercising (□) rats. Means ± S.E.M., n = 10. Statistical significance: $^{***}P < 0.001$ as compared with the control group.

animals (from 2.55 ± 0.03 to 2.57 ± 0.07 g/mg aorta ring, n = 9-10).

3.6. Determination of Infarct Size. Figure 4(a) shows the infarct size after 30 min of LAD occlusion and 120 min of reperfusion expressed as a percentage of the area at risk. 6 weeks of voluntary exercise training significantly ($P < 0.001$) reduced the infarct size in the exercising animals as compared with the control group (from 50.6 ± 7.86% to 32.12 ± 2.66%, n = 11-12).

3.7. Coronary Effluent MMP-2 Activity. The coronary effluent MMP-2 activity was determined after the 30-minute LAD occlusion, during the first 5 min of reperfusion. In the exercising group the activity of the 64 kDa MMP-2 isoform was significantly ($P < 0.001$) decreased from 426.94 ± 18.37 to 213.32 ± 22.45 intensity × mm^2, n = 10). Data are presented in Figure 4(b).

4. Discussion

We demonstrated that 6 weeks of voluntary exercise was able to decrease the levels of serum and coronary effluent MMP-2 activity, reduce the myocardial infarct size, and improve the angina susceptibility of the heart. Voluntary wheel-running additionally resulted in an improvement in myocardial perfusion.

We used a voluntary wheel-running model where the animals were able to self-select the time, duration, and intensity of exercise in a nonstressful environment. Previously, 3-4 weeks of voluntary wheel-running had been demonstrated to induce robust physiological hypertrophy, which provokes a beneficial adaptive response of the cardiovascular system [12, 13]. We therefore employed a 6-week exercise protocol to provide sufficient stimuli for adaptation.

Regular physical exercise results in physiological left ventricular hypertrophy which contributes to several cardiovascular benefits. Resting bradycardia has been considered to be the hallmark cardiovascular effect of exercise-training adaptation [14, 15]. Changes after regular exercise might result in an improvement in bioenergetics and metabolic status and modification in endogenous defense system. There is a broad consensus that antioxidative mechanisms play a key role in cardioprotection through training-induced upregulation [16, 17]. However, besides the antioxidant defenses, another mechanism, MMP-2 secretion, can be modulated by exercise. Most previous studies have reported data concerning MMP activity after various training models in either the skeletal muscle [18] or the cardiac tissues [19]. Less information is available concerning the influence of exercise-induced MMP-2 in the circulation. To investigate this question, serum and released MMP-2 activity into the coronary effluent were analyzed. Urso et al. measured the serum MMP-2 activity after a single bout of exercise but did not find a change in their study [20]. In our present investigation involving wheel-running animals, we hypothesized that exercise would influence the serum MMP-2 activation. Our zymography analyses revealed that 6 weeks of voluntary exercise training decreased

the serum levels of the 64- and 72 kDa MMP-2 forms in the exercising animals as compared with the control group. Previous human studies have demonstrated that physical exercise can affect the levels of MMPs following acute and chronic interventions. Under coronary risk events, the elevations of MMP-2 and MMP-9 are correlated with increased inflammation [21]. The decrease in MMPs could be related to expression of the tissue inhibitors of metalloproteinases (TIMPs), protease degradation, and reduction of proinflammatory cytokine tumor necrosis factor-alpha (TNF-α) [22]. The lowering levels of inflammatory biomarkers mediate the inhibitory effect of exercise on MMP-2 and MMP-9 levels [23]. Lucotti et al. have investigated the effects of aerobic exercise and found that the exercise program caused about 20% reduction in TNF-α and MMP-2 levels [24]. Thus, the alterations on MMP's circulating concentrations might be a good reflection of the exercise effect on inflammation markers. The decrease in the level of serum MMP-2 is a systemic effect which might be contributing to the multiple adaptation mechanisms of physical exercise.

It is well known that the increased activity of MMP-2 during cardiac I/R contributes to the disruption of the endothelial layer and increase of its secretion into the coronary circulation. Cheung et al. first reported that MMP-2 can be released into the coronary effluent of perfused rat hearts. The release of MMP-2 peaked during the first and fifth minutes of reperfusion and was enhanced with increasing duration of ischemia [25]. In a previous study, Lalu et al. examined the effect of I/R on the gelatinolytic activity of MMP-2 in the heart ventricles and coronary effluent. Gelatinolytic activities of MMP-2 were detected before and after I/R and it was found that the release of MMP-2 into the coronary effluent was higher after I/R. It is known that in the first minutes of reperfusion there is a burst of reactive oxygen species (ROS) which determine the severity of the reperfusion injury [26]. The release of MMP-2 correlates negatively with the functional recovery [27]. Our data demonstrated that 6-week voluntary exercise was protective against reperfusion damage, since we found that the release of 64 kDa MMP-2 into the perfusate decreased significantly in the exercising rats. However, we did not identify the 72 kDa isoform activity in the coronary effluent.

Increased activity of the MMP-2 into the perfusate contributes to the disruption of the endothelial layer and has a negative impact on the vascular permeability and leads to coronary artery disease and heart failure [28]. However, the decrease in infarct size leads to an improved cardiac function [29–33]. Human epidemiological data clearly suggest that regular exercise reduces the risk of death during clinical I/R injury [34]. The results of our study show that 6-week voluntary wheel-running provides protection against I/R injury by reducing the myocardial infarct size. The extent of the necrotic area in exercised hearts was lessened by more than 50%. Doustar et al. found that 4-week resistance training did not protect the heart against I/R-induced injury [35]. The difference in these results could have resulted from methodological differences, such as the type and duration of exercise. The relationship between MMP-2 and infarct size was supported in a large body of different experimental evidences [4, 36, 37]. Our current data support this possible link between released MMP-2 activity and reduced infarct size.

That exercise training resulting in cardiovascular benefits that causes functional and structural adaptations is widely investigated. During exercise, increased blood flow and shear stress augment endothelium dependent vasodilatation through the upregulation of endothelial nitric oxide synthase (eNOS) [28]. The nitric oxide (NO), particularly derived from eNOS, has been implicated in the cardioprotection offered by exercise [38]. Increased arterial dilation induced by NO improves myocardial oxygen supply [39] and may indicate additional endothelium-dependent functions that prevent ischemic events. Exercise-induced NO may be a potential inducer of heme oxygenase (HO) enzyme system. In earlier investigations, many researches demonstrate the beneficial roles of the HO enzyme system in cardiovascular function [10, 40, 41]. In a previous study, Sun et al. found that exercise-induced elevation of vascular HO and enhanced HO-related dilation demonstrate the direct participation of the HO system in cardiovascular adaptation [42]. In a diet-induced obese mouse model, Hafstad et al. showed that the impairment of left ventricular (LV) function and mechanoenergetics were normalized by moderate-intensity training. They demonstrated that the changes were associated with altered myocardial substrate utilization and improved mitochondrial capacity as well as reduced oxidative stress, fibrosis, and intracellular matrix metalloproteinase-2 content [43].

To prove the adaptive and protective effects of voluntary wheel-running exercise, cardiac parameters were measured in this study. We detected the ST segment depression, which is considered a reliable ECG finding and has been associated with a worse prognosis for patients with coronary artery disease [44]. 6 weeks of voluntary exercise diminished the ST segment depression and therefore improved the ischemia susceptibility of the heart.

We have observed that basal normoxic heart perfusion was similar in the exercising group and the control group. We therefore utilized AVP, which can regulate the hemodynamic parameters by inducing moderate vasoconstriction. In response to 1.0 μg AVP, a significant improvement in heart perfusion was observed after exercise training. The AVP-induced perfusion changes imply that a 6-week period of voluntary exercise may be effective in producing functional and structural adaptations in the cardiovascular system.

We also measured the AVP-induced aorta ring contraction and the basal blood pressure. In our earlier study, the surviving aorta ring contraction was measured by incubation with AVP (2.0 μg/mL) in male and female rats [10]. The 2.0 μg/mL AVP dose was earlier the most effective one for the detection of gender differences, so we used this treatment in the present investigation [10]. Despite our results revealing favorable adaptation in the cardiovascular system after voluntary exercise training, no differences in aorta contraction were observed between the control and the exercising animals.

Similarly, no alteration in basal blood pressure was detected between the control and the exercising rats. In

another investigation, Roque et al. measured the hemodynamic parameters after swimming training in normotensive rats and also found no blood pressure changes after exercise [45]. The effect of aerobic exercise training on the blood pressure of normotensive animals and humans seems to be minimal and our results are in agreement with those of other studies. Other investigations clearly demonstrated that hypertensive rats and rats with metabolic syndrome had higher blood pressure levels than did normotensive rats in which aerobic exercise did not induce any change in the blood pressure [46, 47].

In this current study, wheel-running exercising decreased the activities of both 64 kDa and 72 kDa MMP-2 in the serum and also the release of MMP-2 from the heart into the coronary effluent as a consequence of 30-minute LAD occlusion. Similar to the decreases in the MMP-2 values, the infarct size was also reduced. Furthermore, such a training period seems to be a potent stimulus for functional recovery in respect of the myocardial perfusion and ischemic susceptibility of the heart.

In conclusion, our results show adaptive and cardioprotective effects of voluntary wheel-running exercise. The reduced activity of serum MMP-2 might be contributing to the multiple adaptation mechanisms of 6-week physical exercise. The fact that the infarct size is decreased suggests that liberation of MMP-2 into the perfusate could be part of cardioprotective effects. Moreover, our training program was able to improve the angina susceptibility of the heart and causes functional recovery detected by AVP-induced perfusion changes.

Conflict of Interests

The authors declare that there is no conflict of interests in relation to this work.

Authors' Contribution

All authors participated in the design and interpretation of the studies in the analysis of the data and in the drafting and review of the paper. Renáta Szabó and Anikó Pósa contributed equally to this paper as first authors.

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Research Article

Dietary Supplementation with the Microalga *Galdieria sulphuraria* (Rhodophyta) Reduces Prolonged Exercise-Induced Oxidative Stress in Rat Tissues

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We studied the effects of ten-day 1% *Galdieria sulphuraria* dietary supplementation on oxidative damage and metabolic changes elicited by acute exercise (6-hour swimming) determining oxygen consumption, lipid hydroperoxides, protein bound carbonyls in rat tissue (liver, heart, and muscle) homogenates and mitochondria, tissue glutathione peroxidase and glutathione reductase activities, glutathione content, and rates of H_2O_2 mitochondrial release. Exercise increased oxidative damage in tissues and mitochondria and decreased tissue content of reduced glutathione. Moreover, it increased State 4 and decreased State 3 respiration in tissues and mitochondria. *G. sulphuraria* supplementation reduced the above exercise-induced variations. Conversely, alga supplementation was not able to modify the exercise-induced increase in mitochondrial release rate of hydrogen peroxide and in liver and heart antioxidant enzyme activities. The alga capacity to reduce lipid oxidative damage without reducing mitochondrial H_2O_2 release can be due to its high content of C-phycocyanin and glutathione, which are able to scavenge peroxyl radicals and contribute to phospholipid hydroperoxide metabolism, respectively. In conclusion, *G. sulphuraria* ability to reduce exercise-linked oxidative damage and mitochondrial dysfunction makes it potentially useful even in other conditions leading to oxidative stress, including hyperthyroidism, chronic inflammation, and ischemia/reperfusion.

1. Introduction

To date the most important products of the health food market contain antioxidant substances, since their intake seems to be negatively correlated with the risk of cancer, strokes, and neurodegenerative affections. Moreover, natural antioxidants have been proposed as substituent for the synthetic antioxidants, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), which are suspected to be responsible for serious side effects such as liver damage and carcinogenesis [1, 2]. Algae are a source of fat soluble as well as water soluble antioxidants [3], so health and pharmaceutical industry are focusing their attention on the potential use of algal mass farming for the production of natural antioxidant mixture.

Galdieria (Cyanidiophyceae, Rhodophyta) is a genus of unicellular algae inhabiting hot springs and other low pH environments [4]. *G. sulphuraria*, widely diffused in thermal acidic environments of the world, has shown optimal growth conditions at pH 1.5 and temperatures in the range 35–45°C [5]. Moreover, *G. sulphuraria* is able to grow in the dark, utilizing a wide range of carbon sources [6]. It contains high concentration of phycobiliproteins, among which C-phycocyanin (C-PC) is a strong antioxidant which also has positive effects on inflammation and heart and kidney injuries [7]. The antioxidant and therapeutic potential of C-PC are related to their molecular structure similar to bilirubin, a natural antioxidant that protects lipids from oxidation [8]. The high C-PC content found in *G. sulphuraria* strain grown in heterotrophic conditions has suggested that *G. sulphuraria* could be a promising candidate for the production of this pigment [9]. Moreover, the recent finding that *G. sulphuraria* from heterotrophic cultures shows favorable macro- and micronutrient profiles has suggested its use in

food preparations rich in bioavailable proteins and dietary fibers [10].

Interestingly, *G. sulphuraria* thrives in acidic hot springs, where sulphur compounds are largely present. The volcanic emissions contain H_2S, which is progressively oxidized to sulphur and H_2SO_4, causing a sharp decrease of pH values. The large occurrence of sulphur compounds in the environment could enhance the intracellular production of glutathione in *Galdieria* as suggested by preliminary data obtained for *G. phlegraea*, another *Galdieria* species inhabiting the same sites (Carfagna et al., unpublished). The high levels of substances with high antioxidant capacity suggest a possible utilization of *G. sulphuraria* coming from heterotrophic cultures as ingredient in preparation of healthy foods to improve their antioxidant capacity [10] and, therefore, their ability to counteract oxidative stress conditions which an aerobic organism can undergo.

To test this hypothesis, in the present paper we evaluated the ability of *G. sulphuraria* supplementation to reduce the oxidative stress induced by acute long-lasting exercise in rat tissues. Moreover, because the oxidative damage has been found to be associated with a reduction in tissue respiratory function [11], we evaluated the capacity of *G. sulphuraria* supplementation to preserve the mitochondrial functionality of exercised rats.

2. Materials and Methods

2.1. Algal Strain and Medium. The *G. sulphuraria* strain 064 was from the ACUF collection of the Department of Biology of the University of Studies of Napoli "Federico II" (http://www.biologiavegetale.unina.it/acuf.html). Allen medium [12] supplemented with $(NH_4)_2SO_4$ as nitrogen source was adopted. As carbon substrate, glycerol was selected, at 2% (W/V) concentration, a cheap organic source of carbon, also available as a byproduct from biodiesel transesterification processes [13]. For mass culture, 6 cylindrical photobioreactors were used, 1 L bubble glass columns (0.04 m ID, 0.8 m high), covered with aluminum foils [10]. The working volume was set at 0.9 L. Air was sparged at the photobioreactor bottom by means of a porous ceramic diffuser at volumetric flow rate range of 20 nL/h; 0.2 μm filters were adopted to sterilize air flow inlet and outlet. The photobioreactors were housed in a thermostated chamber at temperature of 36 ± 1°C. The growth of *G. sulphuraria* in the cylindrical photobioreactors was divided into three phases: batch culture, which lasted about two weeks (Allen medium with 2% glycerol was supplemented to the culture one time during this phase to restore the initial nitrogen and glycerol concentration); fed-batch culture, which lasted about one month (to prevent nitrogen and glycerol starvation, a fixed volume (90 mL) of ten-fold concentrated Allen medium with glycerol was periodically added to the culture; the integration did not dilute the broth since the added liquid volume balanced the periodic culture sampling); semicontinuous culture, which lasted about three months (a prefixed volume of microalgae suspension—broth and cells—was weekly replaced with fresh medium; the test simulates closely continuous cultures and the average dilution rate was assessed as the ratio between the replaced suspension volume and the photobioreactor working volume (0.9 L)). During the course of the experiment, the variation of the oxygen ppm in the medium was followed with a Hanna HI 9142 oximeter.

Cell dry weight determination was made with duplicate samples of cultures. During the semicontinuous phase of growth the samples collected every week were centrifuged in an ALC pK121 centrifuge at 4000 g for 10 minutes and washed one time with a 0.5 NaCl solution and two times with distilled water, to remove culture medium constituents. Then, supernatants were discarded, and the algae were frozen at −80°C, lyophilized, ground in a mill, and weighed.

2.2. Determination of Glutathione (GSH) Content in Algal Extracts. For glutathione analyses samples of the cells (about 100 mL) from semicontinuous cultures were collected by centrifugation (4000 g for 15 min); the packed cells were resuspended in 2 mL of cold extraction buffer containing 0.1 N HCl and 1 mM EDTA and broken by passing through a French pressure cell (11,000 psi). The homogenate was centrifuged at 15,000 g at 4°C for 15 min, and the clear supernatant was used as crude extract. Thiols were reduced at room temperature for 1 h by mixing 400 μL of the supernatants with 600 μL of 200 mM 2(N-cyclohexylamino)-ethanesulfonic acid (CHES) (pH 9.3) and 100 μL of 3 mM DTT. Aliquots (330 μL) were derivatized in the dark for 15 min by adding 20 μL of 15 mM monobromobimane in acetonitrile. The reaction was stopped by adding 250 μL of 0.25% (v/v) methanesulfonic acid and samples were centrifuged for 15 min (14,000 rpm) [14]. Derivatized thiols were separated and quantified by reverse-phase HPLC using the method described by Newton et al. [15]. Quantification was made against a calibration curve for GSH. Thiol levels were expressed as μmol g^{-1} cell. The concentration of protein in algal cells was determined using the Bio-Rad protein assay based on the Bradford method [16] with bovine serum albumin as the standard.

2.3. Determination of C-Phycocyanin (C-PC) Content in Algal Extracts. Cells (50 mL of culture) were harvested by low speed centrifugation (4000 g for 5 min) and washed two times in 50 mmol L^{-1} Na-phosphate buffer (pH 7.0) to remove culture medium constituents. The packed cells were resuspended in 5 mL of cold extraction buffer (50 mmol L^{-1} Na-phosphate buffer, pH 7.2) and broken by passing through a French pressure cell (11,000 psi). The homogenate was centrifuged at 15,000 g at 4°C for 15 min, and the clear supernatant was used as crude extract. Cell debris and proteins in crude cell extracts were precipitated by ammonium sulphate (0–50%) at 4°C under stirring overnight. The precipitate was pelleted by centrifugation for 30 min at 15,000 g at 4°C. The pellet was redissolved in 2.5 mmol L^{-1} Na-phosphate buffer (pH 7.0) and desalted on a Sephadex G25 column (Pharmacia PD10). The C-PC content was measured at 618 and 680 nm and the concentration was determined as described by Kursar and Alberte [17]. For the experiments of food intake samples for semicontinuous cultures of *G. sulphuraria* were periodically collected, centrifuged as previously described, and stored at −80°C. Then cells were lyophilized,

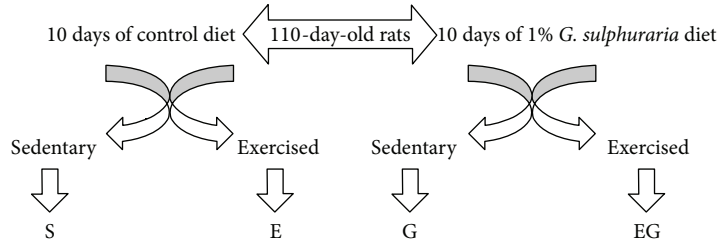

Figure 1: Schematic protocol of rat treatments; 110-day-old animals were randomly assigned to one of two dietary regimens of ten days, receiving either the control diet or a *G. sulphuraria* supplemented diet, consisting of commercial rat chow to which the algae were added to a final concentration of 10 g/Kg. One half of the animals on both the control and supplemented diets were subjected to a 6 h swimming exercise before the sacrifice. The animals swam in a plastic container that was 100 cm high, filled to a depth of 45 cm with water maintained at a temperature between 35 and 36°C. A weight equivalent to the 2% of their body weight was tied to the tail of each rat. Sedentary animals were sacrificed at rest having been kept for 6 h in a small chamber holding about 3 cm of water at 35°C. S: sedentary untreated rats; SG: sedentary *G. sulphuraria* treated rats; E: exercised untreated rats; EG: exercised *G. sulphuraria* treated rats.

and the dried material was pulverized with Ika grinder mill.

2.4. Animals. The experiments were carried out on 120-day-old male Wistar rats, supplied by Nossan (Correzzana, Italy) at day 100 of age. All rats were subjected to the same conditions (one per cage, constant artificial circadian cycle of 12 h of light and 12 h of darkness, and 50 ± 10% relative humidity) and fed the same diet, a commercial rat chow purchased from Nossan, and water on an *ad libitum* basis. From day 110, animals were randomly assigned to one of two dietary regimens, receiving either the control diet or a *G. sulphuraria* supplemented diet, consisting of commercial rat chow to which the algae were added to a final concentration of 10 g/Kg. The animals were maintained on their respective diets for 10 days. One half of the animals on both the control and supplemented diets were subjected to swimming exercise, so we obtained four rat groups: control sedentary (S), algae fed sedentary (SG), exercised (E), and algae fed exercised (EG) rats (Figure 1). Exercised rats were sacrificed immediately after a 6 h swimming exercise. The animals swam in a plastic container that was 100 cm high, filled to a depth of 45 cm with water maintained at a temperature between 35 and 36°C. A weight equivalent to the 2% of their body weight was tied to the tail of each rat. Sedentary animals were sacrificed at rest having been kept for 6 h in a small chamber holding about 3 cm of water at 35°C.

The treatment of animals in these experiments was in accordance with the guidelines set forth by the University's Animal Care Review Committee.

2.5. Tissues Preparation. The animals were sacrificed by decapitation while under anesthesia induced by intraperitoneal injection of chloral hydrate (40 mg/100 g body weight). Exercised rats were sacrificed immediately after the end of the swim session. Liver, heart, and gastrocnemius muscle were excised and placed into ice-cold homogenisation medium (HM) (220 mM mannitol, 70 mM sucrose, 1 mM EDTA, 0.1% fatty acid-free albumin, and 10 mM Tris, pH 7.4). The heart great vessels and valves were trimmed away and the ventricles and atria were cut open and rinsed free of blood. Heart, gastrocnemius muscle (red portion), and liver were freed from connective tissue and the tissues were weighed, finely minced, and washed with HM. Heart and gastrocnemius muscle fragments were incubated for 5 min with HM containing 0.1 mg mL^{-1} nagarse and washed. Finally, all tissues were gently homogenised (20% w:v) in HM using a glass Potter-Elvehjem homogeniser set at a standard velocity (500 rpm) for 1 min. Tissue homogenates were used for analytical procedures.

2.6. Preparation of Mitochondria. The homogenates, diluted 1 : 1 with HM, were freed of debris and nuclei by centrifugation at 500 g for 10 min at 4°C. The resulting supernatants were centrifuged at 3000 g for 10 min. The mitochondrial pellets were resuspended in washing buffer (WB) (220 mM mannitol, 70 mM sucrose, 1 mM EGTA, and 20 mM Tris, pH 7.4) and centrifuged at the same sedimentation velocity. Mitochondrial preparations were washed in this manner twice before final suspension in WB. Mitochondrial protein was measured by the biuret method [18].

2.7. Oxygen Consumption. Oxygen consumption of homogenates and mitochondria was monitored at 30°C by Hansatech respirometer in 1.0 mL of incubation medium (145 mM KCl, 30 mM Hepes, 5 mM KH_2PO_4, 3 mM $MgCl_2$, and 0.1 mM EGTA, pH 7.4) with 50 μL of 20% (w/v) homogenate or 0.25 mg of mitochondrial protein per mL and succinate (10 mM), plus 5 μM rotenone, or pyruvate/malate (10/2.5 mM) as substrates, in the absence (State 4) and in the presence (State 3) of 500 μM ADP.

2.8. Oxidative Damage. The extent of the lipid peroxidative processes in tissue homogenates and mitochondrial preparations was determined by measuring the level of lipid hydroperoxides (HPS) according to Heath and Tappel [19]. Determination of protein oxidative damage was performed measuring protein-bound (CO) carbonyl levels by the procedure of Reznick and Packer [20] for homogenates and by the modified procedure of Schild et al. [21] for mitochondria.

2.9. Antioxidants. Glutathione peroxidase (GPX) activity was assayed at 37°C according to Flohé and Günzler [22] with

H_2O_2 as substrate. Glutathione reductase (GR) activity was measured at 30°C according to Carlberg and Mannervik [23]. Reduced (GSH) and oxidized (GSSG) glutathione concentrations were measured as described by Griffith [24].

2.10. Mitochondrial H_2O_2 Release. The rate of mitochondrial H_2O_2 release during respiration was measured at 30°C following the increase in fluorescence (excitation at 320 nm, emission at 400 nm) due to oxidation of *p*-hydroxyphenylacetate (PHPA) by H_2O_2 in the presence of horseradish peroxidase (HRP) [25] in a computer-controlled Jasko fluorometer equipped with a thermostatically controlled cell-holder. The reaction mixture consisted of 0.1 mg mL^{-1} mitochondrial proteins, 6 U mL^{-1} HRP, and 200 μg mL^{-1} PHPA, in a medium containing 145 mM KCl, 30 mM Hepes, 5 mM KH_2PO_4, 3 mM $MgCl_2$, and 0.1 mM EGTA, pH 7.4. As respiratory substrates the following were used: 10 mM succinate, plus 5 μM rotenone, or 10 mM pyruvate plus 2.5 mM malate added after 30 seconds of stabilization to start the reaction. Measurements with succinate or pyruvate plus malate in the presence of 500 μM ADP were also performed.

2.11. Data Analysis. The data, expressed as mean ± standard error, were analyzed with a two-way analysis of variance method. When a significant *F* ratio was found, the Bonferroni test was used to determine the statistical significance between means. Probability values (P) < 0.05 were considered significant.

3. Results

The strain 064 of *G. sulphuraria* grown in heterotrophic conditions during the semicontinuous phase of cultivation reached a biomass of 32 g L^{-1} dry weight. In the cells collected during this phase glutathione and C-phycocyanin concentrations of 1.76 ± 0.66 μmol g $cell^{-1}$ and 0.28 ± 0.08 mg g $cell^{-1}$, respectively, were found.

Body weights of rats were 398 ± 5, 396 ± 15, 382 ± 14, and 400 ± 15 g for S, SG, E, and EG groups, respectively, and were not significantly modified by treatments, suggesting that the food intake was the same for all groups and that algal supplementation has no toxic effects.

3.1. Oxidative Damage. The levels of lipid hydroperoxides and protein-bound carbonyls are reported in Figure 2. Prolonged aerobic exercise increased the level of lipid hydroperoxide in all tissues of alga unfed rats and in liver and muscle of alga fed rats. Moreover, it increases the levels of lipid hydroperoxide in the mitochondria independently of alga supplementation. Alga supplementation lowered lipid hydroperoxide content in the tissues of both sedentary and exercised rats and in heart and muscle mitochondria of sedentary and in mitochondria of all tissues of exercised rats.

Protein carbonyl content was increased by exercise in tissues and mitochondria of alga treated and untreated rats. *G. sulphuraria* intake reduced protein carbonyl content in heart mitochondria of sedentary rats and in all tissues and in mitochondria from heart and muscle of exercised rats.

3.2. Oxygen Consumption. The rates of O_2 consumption in tissues homogenates and mitochondria in the presence of succinate are reported in Figure 3.

Prolonged exercise increased State 4 oxygen consumption in liver and heart tissues and in mitochondria of alga untreated rats and in heart and muscle tissues and in heart mitochondria from alga treated rats. Exercise lowered the rates of State 3 oxygen consumption in all tissues and in mitochondria from liver and muscle only in alga untreated rats. *G. sulphuraria* supplementation decreased State 4 oxygen consumption in heart and muscle homogenates and in heart mitochondria of sedentary rats and in liver and heart preparations from exercised rats. The State 3 oxygen consumption, which was not changed by *G. sulphuraria* supplementation in sedentary group, was increased by the alga in tissue homogenates and in liver and muscle mitochondria of exercised rats.

In Figure 4 the rates of O_2 consumption in tissue homogenates and mitochondria in the presence of pyruvate plus malate as substrates are reported.

In the presence of pyruvate/malate prolonged exercise increased State 4 oxygen consumption in all tissues and mitochondria of alga untreated rats and in heart and muscle homogenates and in muscle mitochondria of alga treated rats. Prolonged exercise lowered State 3 oxygen consumption in all tissue homogenates and in liver and muscle mitochondria of alga untreated rats. In alga treated rats exercise lowered State 3 respiration in liver and muscle tissues and in muscle mitochondria. *G. sulphuraria* intake decreased State 4 oxygen consumption in heart homogenate and in muscle mitochondria of sedentary rats and in all preparations of exercised rats. Moreover, *G. sulphuraria* increased State 3 oxygen consumption in all tissues of sedentary rats and in all tissues and mitochondria of exercised rats.

3.3. Mitochondrial H_2O_2 Release. The rates of mitochondrial H_2O_2 release are reported in Figure 5. Prolonged exercise increased the rates of both succinate and pyruvate/malate supported H_2O_2 release in mitochondria of liver heart and muscle, during both State 4 and State 3 respiration in alga untreated and treated rats. *G. sulphuraria* lowered H_2O_2 release in the presence of pyruvate/malate during State 3 respiration in muscle mitochondria of sedentary rats. In exercised group in the presence of succinate as respiratory substrate, the alga consumption lowered H_2O_2 release during State 4 in muscle mitochondria and during State 3 in liver mitochondria. In the presence of pyruvate/malate as respiratory substrates, alga lowered H_2O_2 release during State 4 in muscle mitochondria and during State 4 and State 3 in liver mitochondria.

3.4. Tissue GSH Levels and Antioxidant Enzymes Activities. The tissue GSH content is reported in Figure 6, upper panel. Prolonged exercise lowered the GSH content in liver, heart, and muscle independently of alga treatment. Alga supplementation increased GSH content in sedentary and in exercised rats. The tissue GSH/GSSG ratio is reported in Figure 6, lower panel. Prolonged exercise reduced the GSH/GSSG

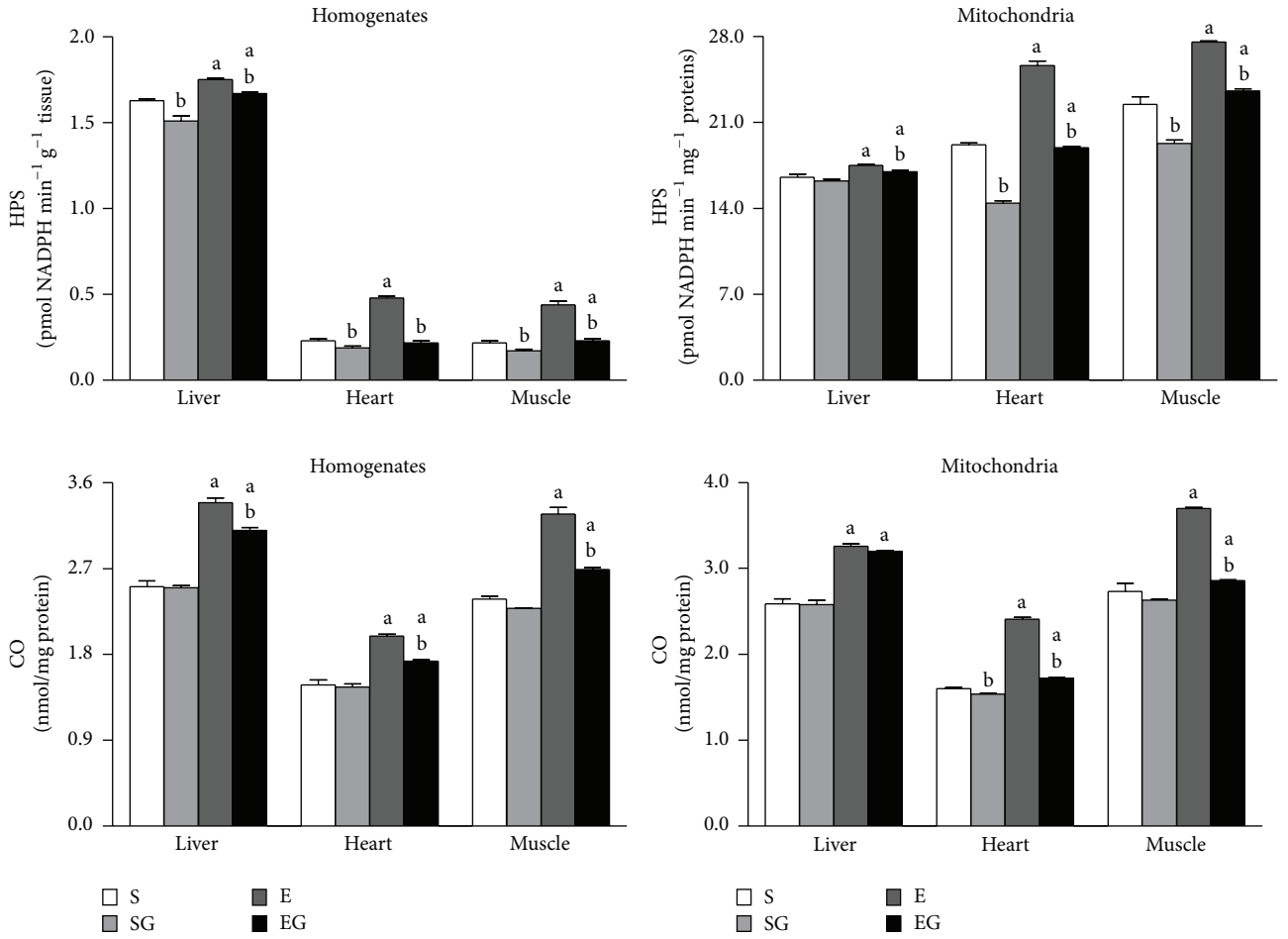

Figure 2: Effect of prolonged exercise and *G. sulphuraria* treatment on the oxidative damage of liver, heart, and skeletal muscle, homogenates, and mitochondria. Values are mean ± S.E.M. For each value eight rats were used. Lipid hydroperoxides (HPS) are expressed as pmol NADPH min^{-1} per g of tissue or mg of mitochondrial protein. Protein-bound carbonyls (CO) are expressed as nmol/mg protein. S: sedentary untreated rats; SG: sedentary *G. sulphuraria* treated rats; E: exercised untreated rats; EG: exercised *G. sulphuraria* treated rats. [a]Significant difference for exercised rats versus respective sedentary controls; [b]significant difference for *G. sulphuraria* treated animals versus respective untreated controls. The level of significance was chosen as $P < 0.05$.

ratio in liver, heart, and muscle of alga untreated rats and in muscle of alga treated rats. Alga supplementation increases GSH/GSSG ratio in liver and heart of both sedentary and exercised rats. Moreover, prolonged exercise increased GPX and GR (Figure 7) activities in liver and GR activity in heart, irrespective of algal supplementation.

4. Discussion

In physiological conditions antioxidant systems preserve redox homeostasis necessary for normal cell functions, but when free radical and reactive oxygen species (ROS) generation exceeds the cell antioxidant capacity, oxidative stress develops [26] leading to tissue damage and dysfunction.

Since high free radical production during acute exercise was firstly demonstrated by Davies et al. [27], research in the area has greatly grown and it is now clear that the consequent proteins and lipids oxidative damage is responsible for tissues damage, decrease in muscle force production, and fatigue appearance. Conversely, regular exercise induces adaptations which protect against oxidative stress conditions [26] and reduce inflammation [28]. Because the alterations induced by acute exercise are prevented by antioxidant supplementation [29], exercise provides an excellent model to study the dynamic balance between oxidative challenge and antioxidant defense in a biological system. On the other hand, epidemiological and human studies suggest that a diet rich in multiple vitamins is more strongly correlated with a low risk of cancer and other diseases than one rich in an individual vitamin. For such a reason, in the present work, to prevent the exercise-induced oxidative stress, we used dried biomass of *G. sulphuraria* heterotrophic cultures, which has been found to exhibit high antioxidant properties because of its content of antioxidants, including vitamin E and phycobiliproteins [10].

The results reported in the present paper agree with previous reports indicating that acute swimming administration

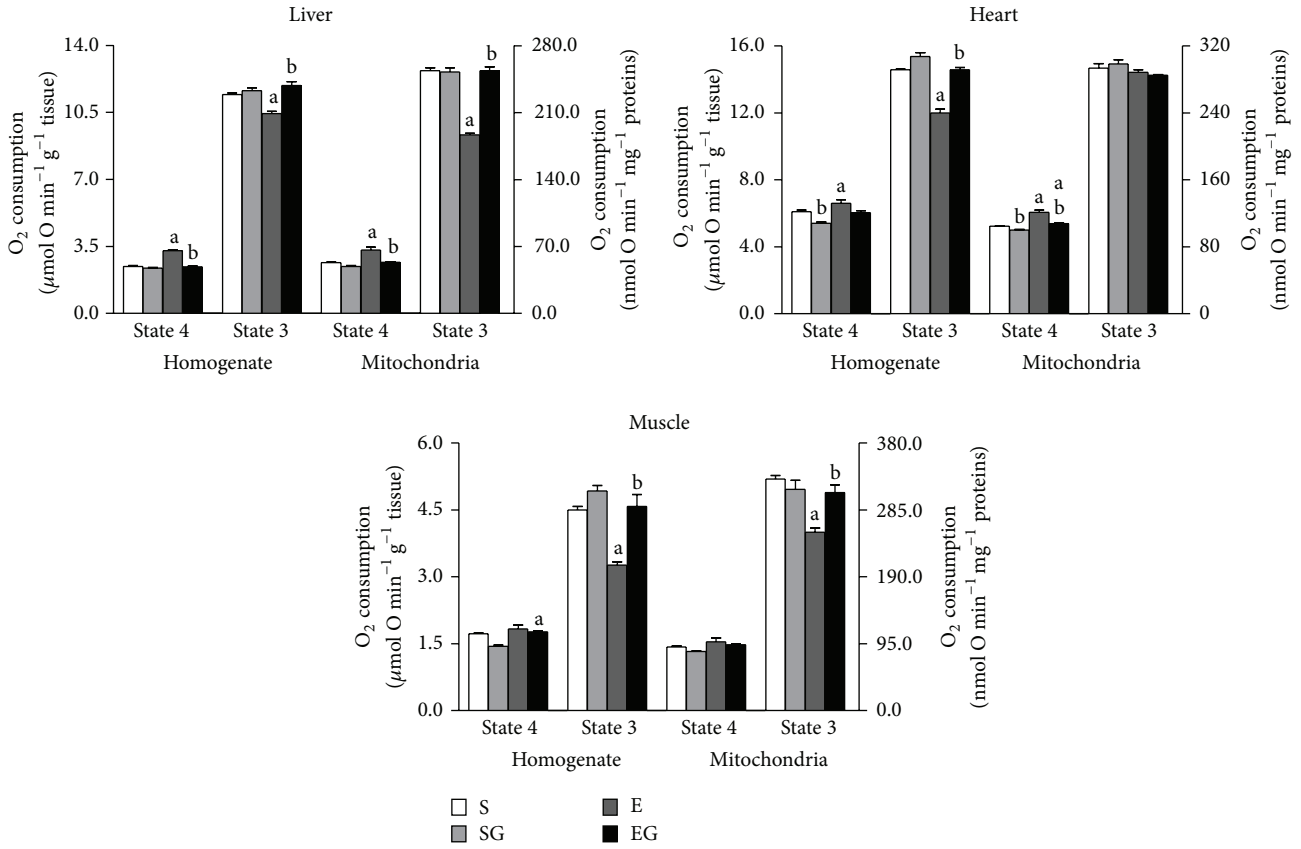

Figure 3: Effect of prolonged exercise and *G. sulphuraria* treatment on the rates of O_2 consumption of liver, heart, and skeletal muscle, homogenates, and mitochondria, in the presence of Complex II substrate (succinate). Values are mean ± S.E.M. For each value eight rats were used. Oxygen consumption is expressed in μmol O min^{-1} per g of tissue and nmol O min^{-1} per milligram mitochondrial protein. Rates of O_2 consumption were measured in the absence (State 4) and in the presence (State 3) of ADP. S: sedentary untreated rats; SG: sedentary *G. sulphuraria* treated rats; E: exercised untreated rats; EG: exercised *G. sulphuraria* treated rats. [a]Significant difference for exercised rats versus respective sedentary controls; [b]significant difference for *G. sulphuraria* treated animals versus respective untreated controls. The level of significance was chosen as $P < 0.05$.

produces increased lipid peroxidation in homogenates from rat muscle, heart, and liver [11, 27, 30, 31] and mitochondria from skeletal muscle [32] and, for the first time, show that exercise also increases lipid peroxidation in mitochondria from liver and cardiac muscle.

Protein oxidation, which was reported to both increase [11, 33] and remain unchanged in homogenates from cardiac and skeletal muscles [34] and liver [33, 34] from running rats, was increased by swimming in all examined tissues and mitochondria. Interestingly, we found that the exercise-induced increase in the lipid oxidative marker in heart and muscle homogenates was about 100%, whereas that found in liver homogenate was lower. This result is likely dependent on the higher antioxidant capacity of the liver in comparison with heart and skeletal muscle [30].

It was previously found that algae supplementation can protect from oxidative stress induced by various pathological conditions. Thus, *Dunaliella salina* reduced the increase in serum levels of a marker of lipid peroxidation, the malondialdehyde (MDA), induced by a diet supplemented with 2% cholesterol [35]. Moreover, the increase in plasma MDA concentration, induced by cadmium administration, was reduced by concomitant *Chlorella vulgaris* supplementation [36].

In this paper we report, for the first time, that *G. sulphuraria* supplementation lowers tissue oxidative damage. However, *G. sulphuraria* supplementation differently affected the levels of lipid and protein oxidative damage in both sedentary and exercised rats. In sedentary rats, the supplementation reduced lipid peroxidation in homogenates and mitochondria but did not affect protein oxidation. In exercised rats, the increase in oxidative damage to lipids was greatly reduced by supplementation, whereas protein oxidative damage was reduced to a lesser extent. These results suggest a specific ability of the alga to preserve lipids from oxidative damage. Such an ability can be attributed to the type of antioxidants contained in *G. sulphuraria* and to the repair processes to which the oxidized lipids are subjected. The C-phycocyanin is able to scavenge various radicals, including peroxyl radicals [37], which are generated during the peroxidative reactions. Vitamin E is the major peroxyl radical scavenger in biological lipid phases. Glutathione,

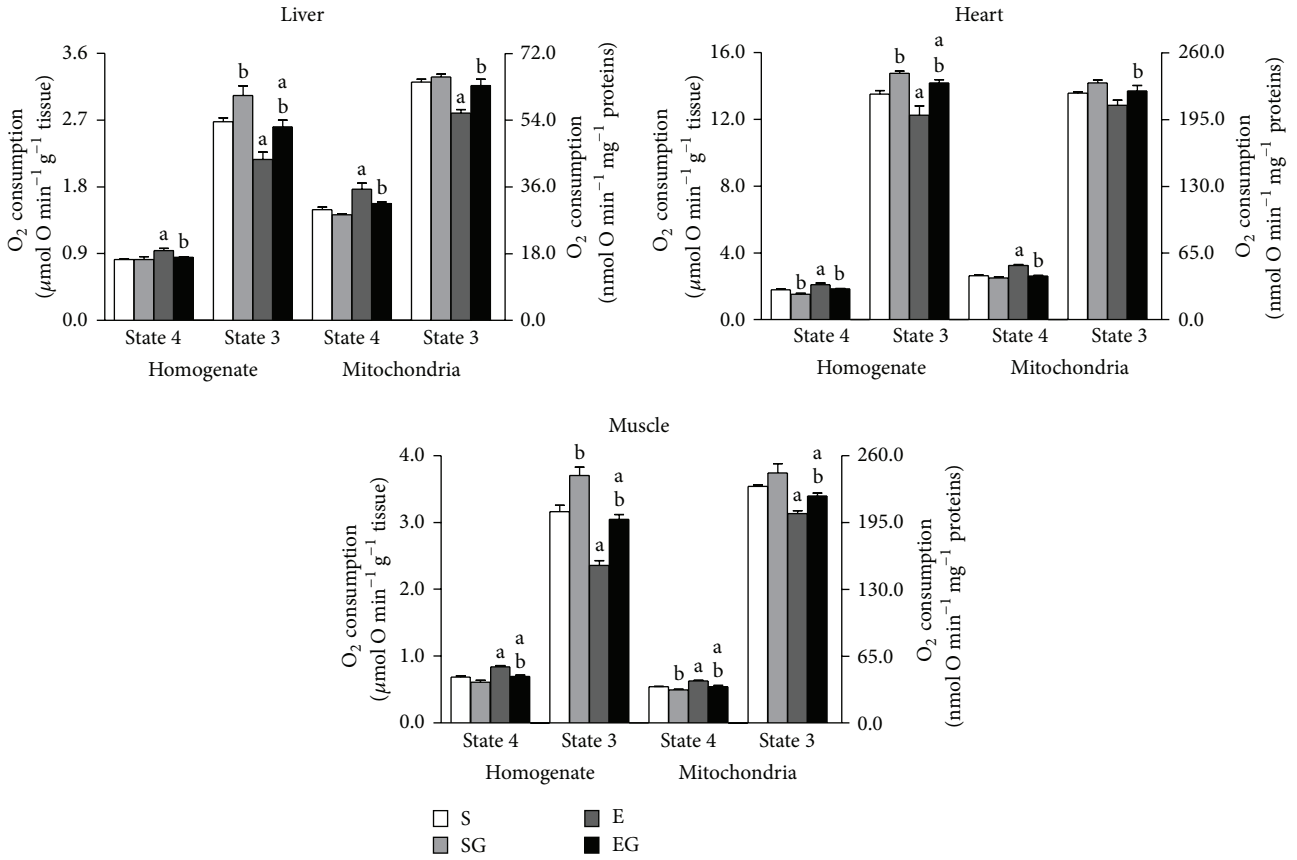

Figure 4: Effect of prolonged exercise and *G. sulphuraria* treatment on the rates of O_2 consumption of liver, heart, and skeletal muscle, homogenates, and mitochondria, in the presence of Complex I substrates (pyruvate/malate). Values are mean ± S.E.M. For each value eight rats were used. Oxygen consumption is expressed in μmol O min^{-1} per g of tissue and nmol O min^{-1} per milligram mitochondrial protein. Rates of O_2 consumption were measured in the absence (State 4) and in the presence (State 3) of ADP. S: sedentary untreated rats; SG: sedentary *G. sulphuraria* treated rats; E: exercised untreated rats; EG: exercised *G. sulphuraria* treated rats. [a]Significant difference for exercised rats versus respective sedentary controls; [b]significant difference for *G. sulphuraria* treated animals versus respective untreated controls. The level of significance was chosen as $P < 0.05$.

whose oral administration positively influences GSH plasma levels [38], is the cofactor used by the glutathione peroxidase (GPX4) to metabolize the phospholipid hydroperoxides, a process which should lead to their conversion to alcohols and subsequent removal by phospholipase A_2 [39].

The exercise-induced increase in the oxidative processes was associated with respiration impairment, revealed by an almost general increase in State 4 and decrease in State 3 respiration in all preparations. The increase in State 4 respiration rate represents a compensatory response to the increased leak of protons back into the mitochondrial matrix. The two major pathways of proton leak, the basal proton conductance of the mitochondrial membrane and the inducible proton conductance mediated by specific leak proteins [40], appear to be activated by exercise. Indeed, adenosine monophosphate, whose levels increase during exercise, acts on adenine nucleotide carrier (ANT) to induce H^+ leak [41]. Moreover, levels of specific uncoupling proteins (UCPs), catalyzing inducible proton conductance, have been found to be increased in cardiac [42] and skeletal muscle [43]. A stronger relationship among exercise, oxidative damage, and proton conductance is supplied by the observation that peroxynitrite, the product of reaction between species generated during exercise, such as superoxide and nitric oxide, increases proton leak enhancing the lipid peroxidation [44].

The observation that reactive oxygen species are able to damage respiratory chain components [45] and that reactive nitrogen species (RNS) inhibit mitochondrial function [46] suggests that the exercise-induced decrease in State 3 respiration is due to a direct action of such species. The ability of the *G. sulphuraria* supplementation to reduce tissue and mitochondrial oxidative damage can explain the attenuation or, in some cases, the prevention of the exercise-induced changes of State 4 and State 3 oxygen consumption in tissue preparations.

It has been proposed that mitochondrial respiratory chain is a major cellular source involved in ROS production during exercise [47]. Respiratory chain produces superoxide anion radical which is converted to hydrogen peroxide (H_2O_2) by superoxide dismutase [48]. The hydrogen peroxide escaping antioxidant removal systems is released in the cytosol and

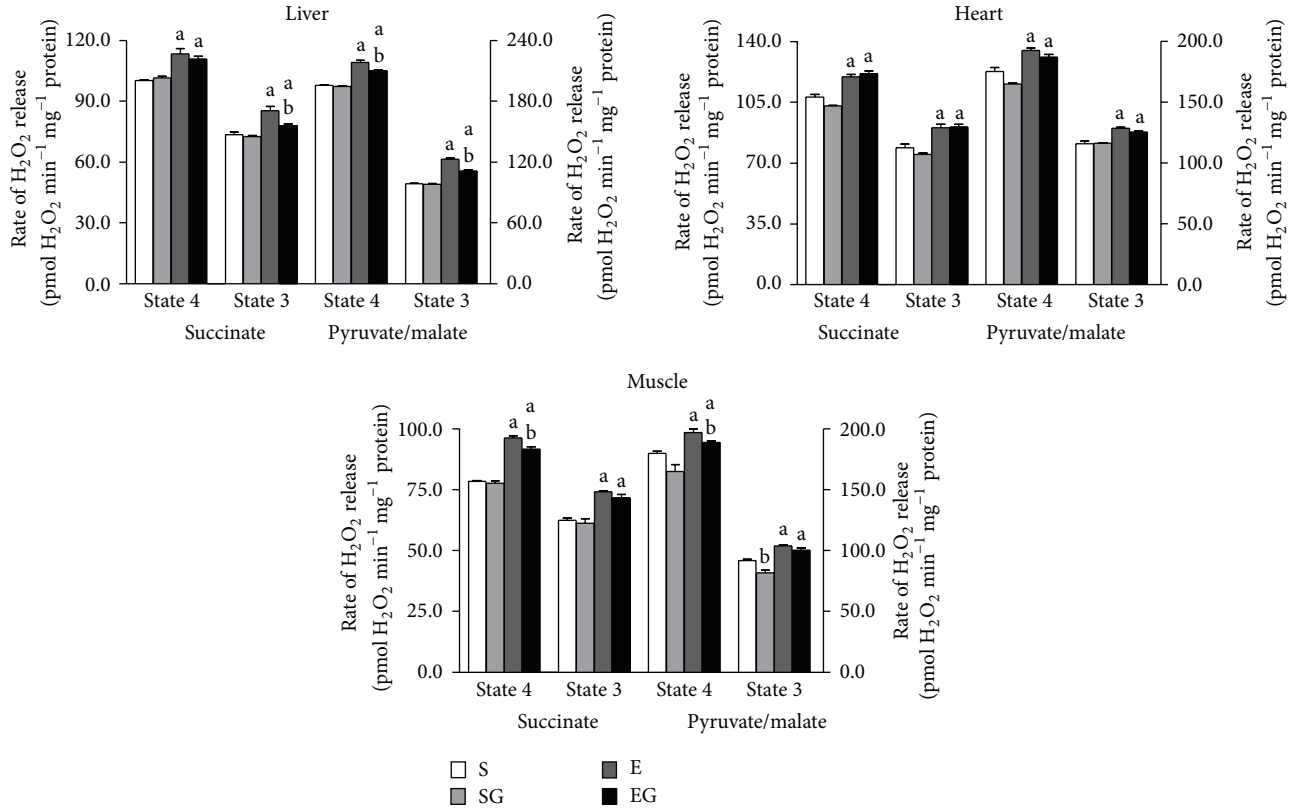

Figure 5: Effect of prolonged exercise and *G. sulphuraria* treatment on the rates of H_2O_2 release by rat liver, heart, and skeletal muscle mitochondria in State 4 and State 3 of respiration, in the presence of succinate or pyruvate plus malate, as respiratory substrates. Values are mean ± S.E.M. For each value eight rats were used. Mitochondrial H_2O_2 release rate is expressed as pmol min^{-1} per mg of mitochondrial protein. S: sedentary untreated rats; SG: sedentary *G. sulphuraria* treated rats; E: exercised untreated rats; EG: exercised *G. sulphuraria* treated rats. [a]Significant difference for exercised rats versus respective sedentary controls; [b]significant difference for *G. sulphuraria* treated animals versus respective untreated controls. The level of significance was chosen as $P < 0.05$.

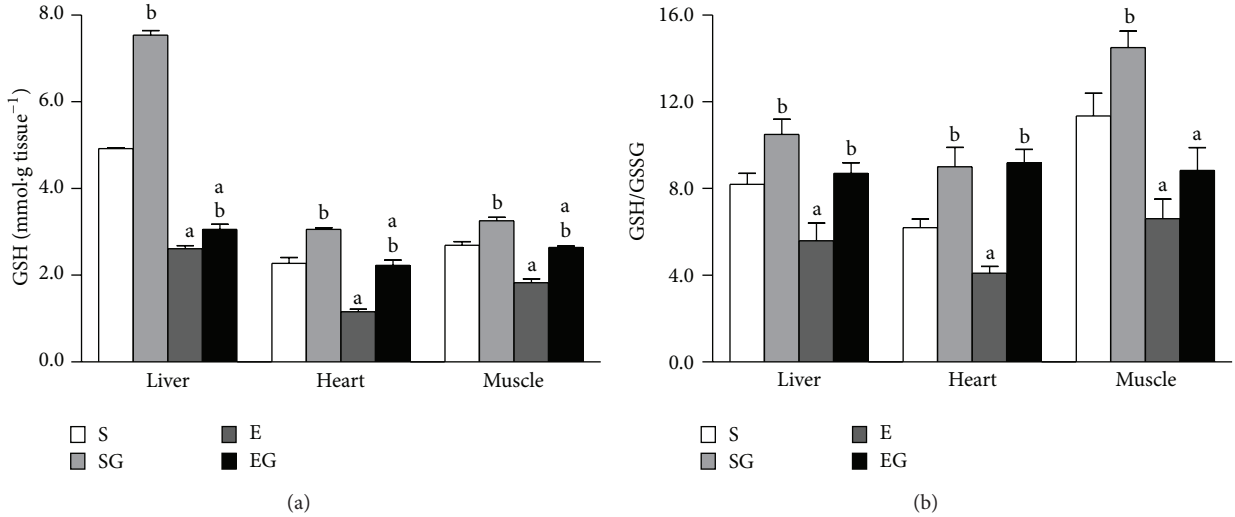

Figure 6: Effect of prolonged exercise and *G. sulphuraria* treatment on rat liver, heart, and skeletal muscle content of reduced glutathione (GSH) (a) and GSH/GGSG ratio (b). Values are mean ± S.E.M. For each value eight rats were used. GSH is expressed as nmol GSH per g of tissue. S: sedentary untreated rats; SG: sedentary *G. sulphuraria* treated rats; E: exercised untreated rats; EG: exercised *G. sulphuraria* treated rats. [a]Significant difference for exercised rats versus respective sedentary controls; [b]significant difference for *G. sulphuraria* treated animals versus respective untreated controls. The level of significance was chosen as $P < 0.05$.

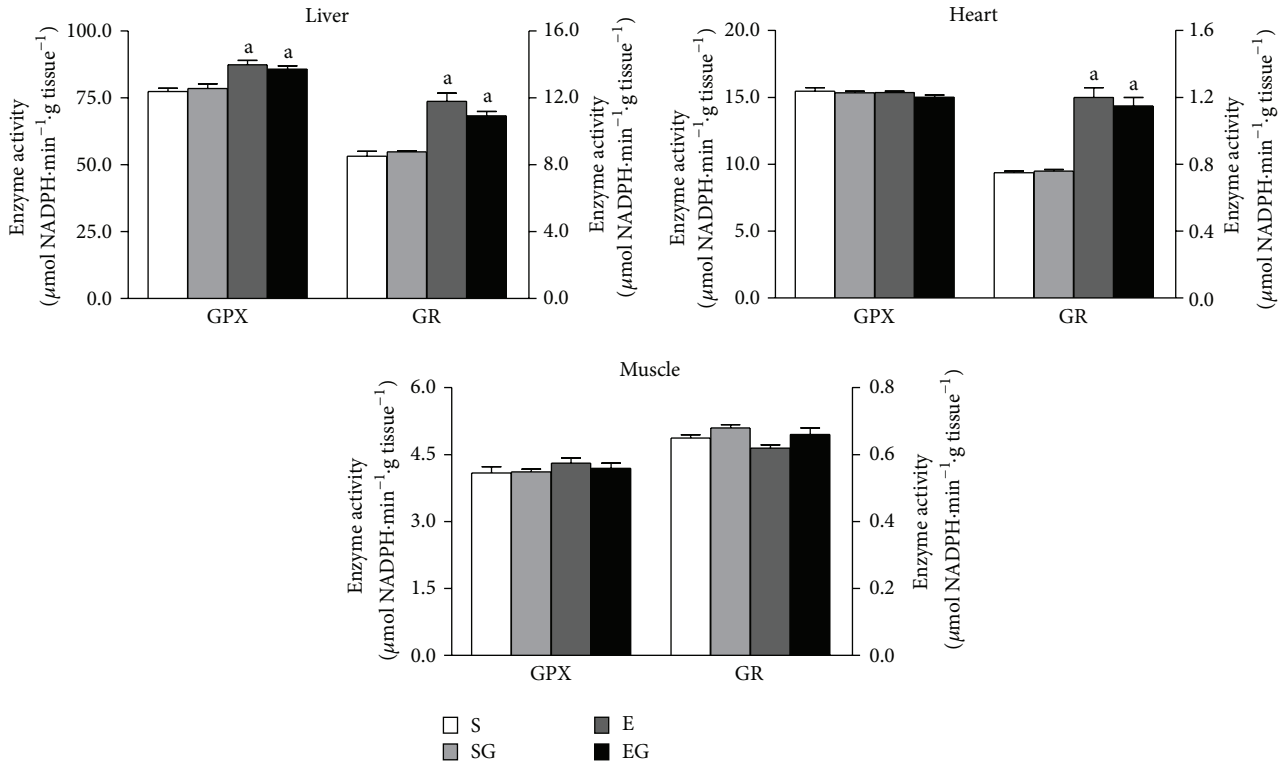

FIGURE 7: Effect of prolonged exercise and *G. sulphuraria* treatment on rat liver, heart, and skeletal muscle enzyme activities of gluthatione peroxidase (GPX) and reductase (GR) activities. Values are mean ± S.E.M. For each value eight rats were used. GPX and GR activities are expressed as μmol NADPH min^{-1} per g of tissue. S: sedentary untreated rats; SG: sedentary *G. sulphuraria* treated rats; E: exercised untreated rats; EG: exercised *G. sulphuraria* treated rats. [a]Significant difference for exercised rats versus respective sedentary controls; [b]significant difference for *G. sulphuraria* treated animals versus respective untreated controls. The level of significance was chosen as $P < 0.05$.

converted into hydroxyl radical, which plays a major role in determining the extent of tissue oxidative damage.

We found, according to previous reports [32, 42], that prolonged exercise increases the mitochondrial H_2O_2 release rate in cardiac and skeletal muscle. However, we also showed that this also happens in liver. The exercise-induced liver oxidative damage was frequently attributed to ROS production from source different from the mitochondria. Indeed, the exercise and subsequent recovery period appeared to mimic the ischemia/reperfusion phenomenon, in which xanthine dehydrogenase-xanthine oxidase conversion and coupling of xanthine and uric acid formation from hypoxanthine with the univalent oxygen reduction to superoxide are involved. This idea is supported by the observation that liver lipid peroxidation and uric acid content are reduced in exercised rats treated with allopurinol, a xanthine oxidase inhibitor [49]. However, in liver another source of ROS production during reperfusion could be the respiratory chain, which, with resumption of respiration, having high amounts of reducing equivalent and low availability of ADP, should produce ROS at high rate damaging its own components. This, in turn, should block electron flux in some units of respiratory chain, thus increasing ROS production rate. This hypothesis should supply an explanation for the increase in mitochondrial oxidative damage and the higher rate of ROS production found after exercise in isolated liver mitochondria. The fact that *G. sulphuraria* scarcely reduces the exercise-induced increase in mitochondrial ROS production in liver as well as in the other tissues, notwithstanding the reduction in oxidative damage, can be due to the presence in the alga of great amounts of substance able to scavenge peroxyl radicals or metabolize lipid hydroperoxides. Moreover, the reduction of oxidative damage caused by the alga might be the result of its ability to reduce ROS production by a source different from mitochondria or by other antioxidant effects. Indeed, C-phycocyanin inhibits NADPH oxidase expression [50], an enzyme producing $O_2^{\bullet -}$ which is found at many sites in skeletal muscle and is activated by muscle contraction [51]. Conversely, it is apparent that the alga does not affect the oxidative damage stimulating the activity of antioxidant enzymes such as glutathione peroxidase and glutathione reductase even though it is not possible to exclude the fact that it can exert an influence on activity of other antioxidant systems.

In conclusion the data here reported indicate that the food supplementation with the alga *G. sulphuraria* protects tissues from the oxidative damage induced by acute exercise. Such a capacity seems to depend on the particular antioxidant content of the alga which contains important sources of glutathione and C-phycocyanin. The latter antioxidant seems to be particularly able to protect lipids from oxidative damage [52]. This suggests the possibility that *G. sulphuraria*

supplementation can protect from oxidation plasma lipoproteins reducing cardiovascular risk factors.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Training Effects on ROS Production Determined by Electron Paramagnetic Resonance in Master Swimmers

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Acute exercise induces an increase in Reactive Oxygen Species (ROS) production dependent on exercise intensity with highest ROS amount generated by strenuous exercise. However, chronic repetition of exercise, that is, exercise training, may reduce exercise-induced oxidative stress. Aim of this study was to evaluate the effects of 6-weeks high-intensity discontinuous training (HIDT), characterized by repeated variations of intensity and changes of redox potential, on ROS production and antioxidant capacity in sixteen master swimmers. Time course changes of ROS generation were assessed by Electron Paramagnetic Resonance in capillary blood by a microinvasive approach. An incremental arm-ergometer exercise (IE) until exhaustion was carried out at both before (PRE) and after (POST) training (Trg) period. A significant ($P < 0.01$) increase of ROS production from REST to the END of IE in PRE Trg (2.82 ± 0.66 versus 3.28 ± 0.66 μmol·min^{-1}) was observed. HIDT increased peak oxygen consumption (36.1 ± 4.3 versus 40.6 ± 5.7 mL·kg^{-1}·min^{-1} PRE and POST Trg, resp.) and the antioxidant capacity (+13%) while it significantly decreased the ROS production both at REST (−20%) and after IE (−25%). The observed link between ROS production, adaptive antioxidant defense mechanisms, and peak oxygen consumption provides new insight into the correlation between ROS response pathways and muscle metabolic function.

1. Introduction

Cells are exposed to a large variety of Reactive Oxygen Species (ROS) from both exogenous and endogenous sources. At appropriate concentration, ROS are known to act as important signaling molecules essential to cell function, playing various regulatory roles in cells [1]. Nevertheless the effects of ROS are dose dependent and when ROS generation exceeds antioxidant defenses oxidative damage is observed [2].

Exercise is associated with an increase in oxygen uptake by whole body and particularly by skeletal muscle [3], utilized, among others, into mitochondria for substrate metabolism and ATP production. As reported [4], an increase of 10-fold in the rate of whole body oxygen consumption and an increase of more than 100-fold in the oxygen flux in active muscles, during whole-body exercise, result in increased ROS formation, shifting the cellular environment from a reduced to an oxidized state, independently of physical activity types (aerobic, anaerobic, or resistance) [5]. Many factors might contribute to the oxidative stress induced by exercise also influencing the oxidative rate, such as recruited muscle groups, types of contraction, exercise frequency and intensity, and exercising population. Physical exercise is one

of the most characteristic examples demonstrating that ROS are not necessarily harmful, considering that the well-known benefits of regular exercise on muscle function and health are accompanied by repeated episodes of oxidative stress [6]. The promoting effects of regular exercise on different cellular functions include the upregulation of antioxidant and oxidative damage repairing systems and induction of trophic factors [7]. Finally, training can play positive or negative effects on oxidative stress, depending on training load and specificity [8].

Previously it was demonstrated that high-intensity discontinuous and continuous moderate-intensity training induced similar beneficial effects in master runners, reducing the resting levels of oxidative stress biomarkers and inducing changes in total antioxidant capacity level [9].

Many investigators have assumed that skeletal muscle provides the major source of ROS generation during exercise [5]. Nevertheless, other tissues such as heart, lungs, or blood may also contribute to total body ROS generation during exercise [6]. Recent reports have indicated the potential role that blood may play at rest or during exercise on ROS production [10]. The whole blood or parts of it: plasma [11], erythrocytes [12], neutrophils [11, 13], lymphocytes [14], and platelets [15], have reported an increased production of various reactive species after exercise. However, the majority of the relevant human studies measured the redox status by using plasma. This probably can be ascribed to two reasons: (1) the assumption that plasma better reflects tissue redox status [16] and (2) the easiness of plasma collection. During exercise, ROS are generated by both blood and muscle and it is reasonable to assume that a corresponding systemic steady state level is reached in blood. The same may hold true for exchanges among blood constituents [6] once certain basic assumptions are met: reactive species with adequate half-life have the ability to cross membranes and generate new reactive species at the vicinity of the considered compartments.

Usually, direct measurements of free radical and reactive species production are very difficult due to their high reactivity and low steady-state concentration [17]. Consequently, for the assessment of oxidative stress, indirect methods are mainly used. Indeed, Electronic Paramagnetic Resonance (EPR) spectroscopy is the only technique available to directly detect the "instantaneous" presence and to quantitate ROS concentration in biological samples. Nevertheless ROS half-life ($t_{1/2}$ (s): superoxide [$O_2^{\bullet -}$] 10^{-4}; nitric oxide [$NO^{\bullet}$] $4 \cdot 10^{-1}$, at room temperature) is too short when compared to the EPR time scale so they are EPR invisible. This is only when "trapped" and transformed in a more stable radical species that they become EPR detectable. Moreover, in EPR spectra, signal areas are proportional to the number of the excited electron spins, leading to absolute concentration levels, when a stable radical compound is adopted as reference.

The present study aimed at examining the effects of high-intensity discontinuous training exercise on ROS production and on antioxidant capacity in master swimmers by applying reliable, rapid, and microinvasive EPR measurement of the instantaneous concentration of ROS [18, 19] and antioxidant power using a novel redox sensor to measure the levels of reducing species [20] directly in human peripheral blood. Possible correlation between metabolic and ROS production levels was also investigated.

2. Materials and Methods

2.1. Subjects. Sixteen master swimmers (males, mean age 30 ± 5 years; nonsmokers) of the Saronno Swimming Club were recruited. Athletes had a training experience of 11 ± 4 years and they were specialized in front crawl on distances between 50 and 400 m. All athletes belonged to the master swimmer category as established by both Féderation Internationale de Natation Amateur (FINA: http://www.fina.org/) (25-year-old subjects and over) and Italian Swimming Federation (FIN: http://www.federnuoto.it/). No special diet, minerals, vitamins, or other kinds of supplementation were administered to swimmers. During the experimental phase of the study antioxidant supply was excluded and participants sustained only the programmed training protocol. Furthermore, participants abstained from food (6 h) and physical activity, alcohol, and caffeine consumption (24 h) prior to testing and were not currently taking any medications or supplements. Subjects were tested after a week of tapering (PRE), characterized by low-intensity training of short duration.

The anthropometric characteristics and the calculated body mass index (BMI), body fat, and free fat masses, determined by bipolar bioimpedentiometry (Tanita), were assessed. A written informed consent was signed by all participants, after being informed of all risks, discomforts, and benefits associated with the study. Procedures were in accordance with the Declaration of Helsinki, and institutional review board approval was received for this study.

2.2. Experimental Protocol. All subjects visited the laboratory two times: before (PRE Trg (Trg = training)) and after (POST Trg) 6 weeks of high-intensity discontinuous training (HIDT). On the experimental day, the subjects arrived at the laboratory 2.5 h after consuming a standardized breakfast [77 percent energy (*E*%) carbohydrate; 11 *E*% protein; 12 *E*% fat].

All tests were performed under close medical supervision and subjects were continuously monitored by 12-lead electrocardiography (ECG). Participants sat on the arm crank ergometer (Monark 891E, Stockholm, Sweden) with the crankshaft in line with the shoulder joint [21]. The ergometer presented adjustable seat and handlebars, which were set to suit each subject. All subjects were instructed to remain seated during the test. Subjects performed an incremental exercise (IE) up to voluntary exhaustion. In brief, this protocol involved a starting power output of 15 W with increases of 15 W every 1 min up to voluntary exhaustion. Arm-ergometer workload was adjusted by manually placing weights on the attached basket. Cadence was set at 60 rpm. Pulmonary ventilation ($V'E$, expressed in BTPS), O_2 uptake ($V'O_2$), and CO_2 output ($V'CO_2$), both expressed in STPD, were determined breath-by-breath by a computerized metabolic cart (SensorMedics Vmax29c, Bilthoven, Netherlands). Expiratory flow measurements were performed by a mass flow sensor (hot wire anemometer),

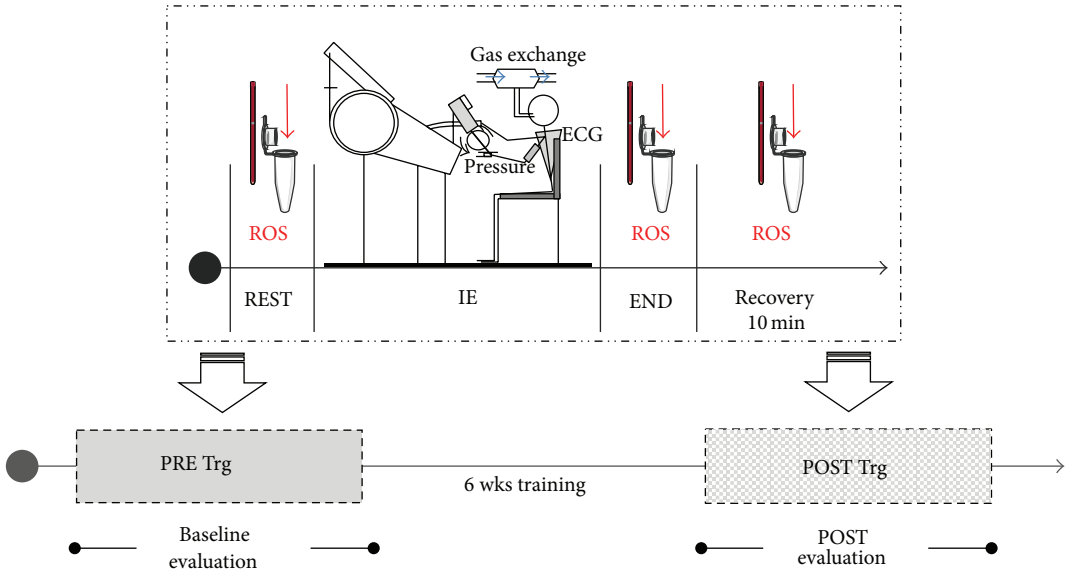

Figure 1: Sketch of the experimental protocol adopted to measure ROS production rate in swimmers. The data were collected at REST, at the END of the incremental arm-ergometer exercise (IE), carried out up to voluntary exhaustion, and at 10 min of the recovery period (see upper part of the figure) both before (PRE Trg) and after (POST Trg) training (lower part of the figure).

calibrated before each experiment by a 3-litre syringe, at three different flow rates. Tidal volume and $V'E$ were calculated by integration of the flow tracings recorded at the mouth. $V'O_2$ and $V'CO_2$ were determined by continuously monitoring PO_2 and PCO_2 at the mouth throughout the respiratory cycle and from established mass balance equations, after alignment of the expiratory volume, expiratory gases tracings, and A/D conversion. Calibration of O_2 and CO_2 analysers was performed before each experiment by utilizing gas mixtures of known composition. Digital data were transmitted to a personal computer and stored on disk. Gas exchange ratio was calculated as $V'CO_2/V'O_2$. $V'O_2$ peak was determined as the average of the last 20 s values. Heart rate (HR) was determined by ECG. Blood pressure (BP) was measured using a standard cuff sphygmomanometer. Severe hypertension (systolic BP value > 250 mmHg) or falling BP during exercise was adopted as criteria for terminating the test. At rest, at the end of exercise, and at 1, 3, and 5 min during the recovery period, blood lactate concentration ($[La]_b$) was determined using an enzymatic method (Biosen 5030; EKF Diagnostic, Eppendorf, Milan, Italy) on 20 μL of capillary blood obtained at the ear lobe.

Voluntary exhaustion was defined as the inability to maintain the armful frequency, despite vigorous encouragement by the operators, as well as by maximal levels of self-perceived exertion using the validated Borg scale [22].

2.3. EPR Measurements. At rest, at the end of IE and after 10 minutes of recovery, ROS production rate was determined in 50 μL capillary blood by means of a recently developed EPR microinvasive method [18, 19]. The capillary blood samples were collected at both PRE and POST Trg periods. The experimental protocol adopted for ROS detection in master swimmers is shown in Figure 1.

In summary, EPR experiments were carried out by using e-scan spectrometer (Bruker, Germany), operating at the common X-Band microwave frequency (~9.8 GHz). Acquisition EPR parameters were microwave frequency: 9.652 GHz; modulation frequency: 86 kHz; modulation amplitude: 2.28 G; sweep width: 60 G; microwave power: 21.90 mW; number of scans: 10; receiver gain: $3.17 \cdot 10^1$. The instrument was interfaced to a temperature and gas controller unit (Bio III, Noxigen Science Transfer & Diagnostics GmbH, Germany) allowing temperature to be kept at the constant chosen level (37°C). Radical signals generated by the reaction of the 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine probe (CMH, Noxygen Science Transfer & Diagnostics, Germany) with the blood radicals were acquired and the spectra sequentially transformed for about 6 min in order to calculate the ROS production rate. The calculated spectral data were transformed in absolute concentration levels (μmol·min^{-1}) by recording the CP$^\bullet$ (3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy) stable radical signal adopted as reference (10 μM). All EPR data were handled using the software standardly supplied by Bruker (Win-EPR version 2.11).

2.4. Antioxidant Capacity. Reducing capacity in blood was measured by a redox sensor in 10 μL of capillary blood. The electrochemical measurements were performed using a commercial EDEL potentiostat electrochemical analyser (Edel Therapeutics, Switzerland) in a three-electrode arrangement. The working electrode (WE) was a screen-printed carbon electrode operating in conjunction with a screen-printed counter and a silver/silver-chloride (Ag/AgCl) reference one. This technique is an electrochemical-based method responding to all water-soluble compounds in biological fluids, which can be oxidized within a defined potential range [23, 24].

Table 1: Weekly training contents were classified in three intensity zones based on the individual anaerobic threshold (IAT).

Training contents	HIDT	
	Distance (m)	% of total
Zone 1 (100–105% IAT)	600	10
Zone 2 (110–120% IAT)	2700	45
Zone 3 (>130% IAT)	2700	45
Total amount	6000	100

Blood sample was loaded onto a chip and an increasing potential between 0 and 1.2 V at a scan rate of 100 $mV \cdot s^{-1}$ (versus Ag/AgCl reference electrode) was applied while the resulting current was measured at the working electrode. The result was then pseudotitrated to account for the most biologically relevant antioxidants [20]. Data are expressed in nW.

2.5. Training Intervention. The training protocol adopted in our study, that is, HIDT, is characterized by brief intermittent bouts of vigorous activity, interspersed by periods of rest or low intensity exercise. HIDT causes repeated O_2 consumption fluctuations related to changes of exercise intensity as opposed to continuous endurance training where O_2 consumption is nearly constant during the exercise.

Subjects trained 3 times per week during 6 weeks in an indoor 25 m swimming pool. Training contents were classified in three intensity zones based on the individual anaerobic threshold (zone 1, 100–105% IAT; zone 2, 110–120% IAT; zone 3, >130% IAT). Total training volume and training amount at different intensity zones are presented in Table 1. The athletes' coach participated in the schedule of training programs and conducted all training sessions. Dry-land training (resistance, athletics, and cross training) was not performed.

At the start and end of each training session swimmers performed controlled warm-up (500 m per session) and cool-down (300 m per session), respectively. Excluding these phases in which subjects swam freely, all training sessions were conducted in front crawl.

2.6. Statistical Analysis. Descriptive statistics such as mean ± SD were used to summarize continuous variables. Data were analyzed using parametric statistics following mathematical confirmation of a normal distribution using Shapiro-Wilks W test. Experimental data were compared by ANOVA variance analysis followed by Bonferroni's multiple comparison test to further check among groups' significance (GraphPad Prism 6, Software Inc. San Diego, CA). The relationship between selected dependent variables was assessed using Pearson correlation coefficients. $P < 0.05$ statistical significance level was accepted.

Prospective calculation of power to determine subjects' number was made by using Freeware G*Power software (http://www.psycho.uni-duesseldorf.de/abteilungen/aap/gpower3/). At a power of 80% the number of subjects of 10 was calculated, which is well below the number of subjects recruited for this study.

Table 2: Mean (±SD) values of the investigated variables obtained in the two sessions: PRE Trg (before training) and POST Trg (after training). BMI: body mass index; $V'O_2$ peak: peak oxygen consumption; HR: heart rate; $[La]_b$ peak: blood lactate peak concentration; BORG: scale measure of perceived exertion.

	PRE	POST
Weight (kg)	78.6 ± 5.0	78.8 ± 5.1
Height (cm)	182.2 ± 4.7	182.2 ± 4.7
BMI ($kg \cdot m^{-2}$)	23.7 ± 2.0	23.8 ± 2.0
Fat mass (kg)	13.3 ± 3.5	13.0 ± 2.9
Free fat mass (kg)	65.3 ± 2.3	65.7 ± 2.9
Peak power output (W)	175 ± 23	200 ± 26[#]
$V'O_2$ peak ($L \cdot min^{-1}$)	2.87 ± 0.41	3.21 ± 0.56[#]
$V'O_2$ peak ($mL \cdot Kg^{-1} \cdot min^{-1}$)	36.15 ± 4.26	40.64 ± 5.73[#]
HR peak ($beats \cdot min^{-1}$)	175.5 ± 5.38	174.90 ± 7.93
$[La]_b$ peak (mM)	10.05 ± 2.00	11.85 ± 2.53
Borg scale	16.8 ± 1.68	17.2 ± 1.39

[#]Statistically significant difference at $P < 0.01$.

3. Results

3.1. Exercise and Training Effects. Anthropometric characteristics and the main physiological variables recorded during arm cranking are reported in Table 2. After 6 wks of HIDT, $V'O_2$ and power output peaks significantly ($P < 0.001$) increased in POST Trg versus PRE Trg.

The kinetics of ROS production data estimated by the EPR spectra recorded at rest, immediately after the IE, and at 10 min of recovery are shown in Figure 2.

After IE, ROS production increased significantly with respect to REST ($P < 0.01$) in PRE Trg (2.82 ± 0.66 versus 3.28 ± 0.66 $\mu mol \cdot min^{-1}$) while the increase was not significant in POST Trg (2.24 ± 0.14 versus 2.46 ± 0.12 $\mu mol \cdot min^{-1}$). Thereafter ROS production attained the resting levels in the time course of recovery, although in PRE Trg ROS level was found still more significantly ($P < 0.05$) higher (3.13 ± 0.30 $\mu mol \cdot min^{-1}$) at 10 minutes of recovery in relation to REST.

HIDT induced a significant ($P < 0.001$) decrease in the ROS production rate at REST in POST Trg compared to PRE Trg (2.24 ± 0.14 versus 2.82 ± 0.66, resp.). Moreover, the attained peak value (END) resulted significantly ($P < 0.001$) lower in POST Trg than in PRE Trg despite a similar trend. Finally, a significant difference ($P < 0.001$) in the time course of recovery (10 minutes after exercise: 3.13 ± 0.30 versus 2.29 ± 0.11, resp.) between ROS production in PRE Trg and POST Trg was observed. Stacked plots of the EPR spectra recorded in PRE Trg at REST and at the END of exercise (a) and in POST Trg at REST and at the END of exercise (b) are shown in Figure 3. An increase of the signal amplitude (a. u). at the end of exercise with respect to rest and a decrease of the signal amplitude in POST Trg with respect to PRE show up from the spectra.

Antioxidant capacity changes after IE are displayed in Figure 4 as well. This parameter was found significantly increased with respect to the REST at the END and at 10

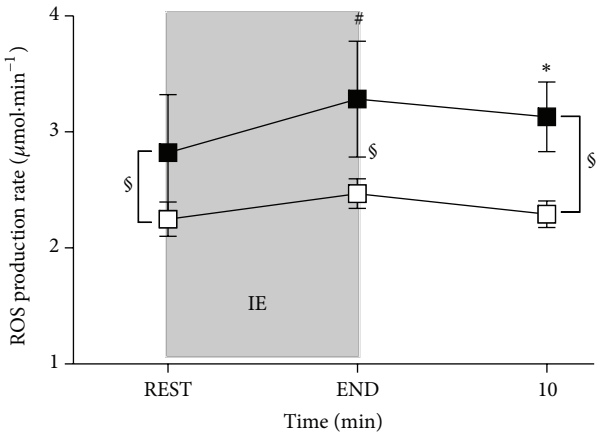

Figure 2: Time course of ROS production rate (μmol·min^{-1}) detected by EPR technique before (REST) and immediately after the IE (END) and at 10 minutes of recovery. The data obtained during two sessions of IE are shown: PRE Trg (full squares) and POST Trg (empty squares). Changes over time were significant at $P < 0.05$ during recovery (10 minutes after exercise) in PRE Trg (∗ symbol); $P < 0.01$ comparing peak levels in PRE Trg versus REST (# symbol); $P < 0.001$ between PRE Trg and POST Trg at REST, END, and 10 minutes of recovery (§ symbol).

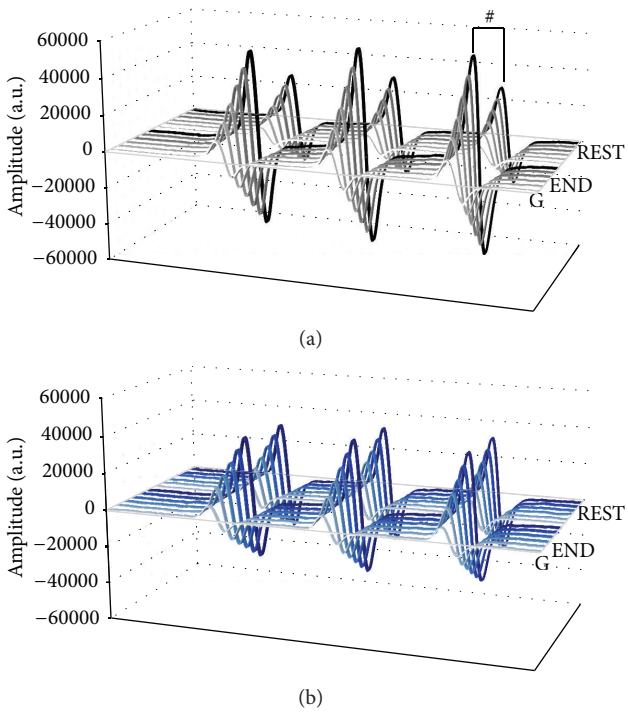

Figure 3: Stacked plots of the EPR spectra recorded at REST and at the END of exercise in PRE Trg (a) and in POST Trg (b). In each panel an increase of the signal amplitude (a. u.) at the end of exercise with respect to rest. A decrease of the signal amplitude in POST Trg with respect to PRE Trg shows up. # symbol ($P < 0.01$) shows the difference between REST versus END in PRE Trg.

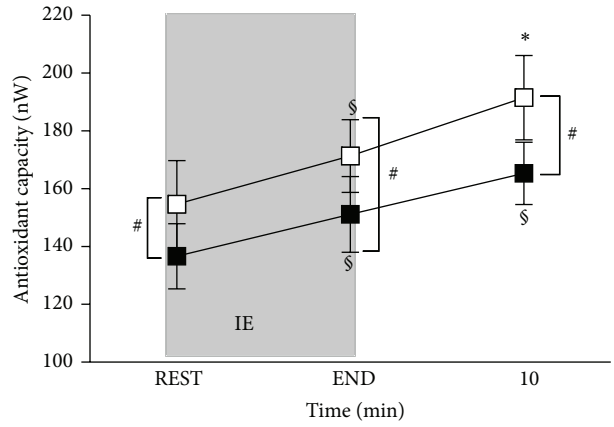

Figure 4: Time course of antioxidant capacity (nW) before (REST) and immediately after the IE (END) and at 10 minutes of recovery: PRE Trg (full squares) and POST Trg (empty squares). Changes over time were significant in PRE Trg at $P < 0.001$ at the END of exercise and during recovery (10 minutes after exercise) (§); in POST Trg at $P < 0.001$ at the END (§) and $P < 0.05$ during recovery (10 minutes after exercise) (∗); $P < 0.01$ between PRE Trg and POST Trg at REST, END, and 10 minutes of recovery (# symbol).

minutes of recovery, in both PRE (136.6 ± 11.34; 151.1 ± 13.1; 165.3 ± 10.9 nW, resp.) and POST Trg (154.7 ± 15.1; 171.4 ± 12.6; 191.5 ± 14.7 nW, resp.). HIDT induced a significant ($P < 0.01$) increase of antioxidant capacity in POST Trg compared to PRE Trg at REST and END and after 10 minutes of recovery (+13%; +13%; +16%, resp.).

Lastly, a possible correlation between ROS production rate levels, antioxidant capacity, and metabolic data was investigated. An inverse significant relationship between (i) ROS production rate (μmol·min^{-1}) and antioxidant capacity (nW) ($r^2 = 0.48$, $P < 0.0001$) at baseline (see Figure 5(a)) and (ii) ROS peak production rate (μmol·kg^{-1}·min^{-1}) and $V'O_2$ peak (mL·kg^{-1}·min^{-1}) ($r^2 = 0.61$, $P < 0.0001$) (see Figure 5(b)) was found by Pearson's product-moment correlation.

4. Discussion

Many experimental works have analyzed the redox biology of exercise with high relevance to the area of Sport Science [17]: the general benefits of physical exercise are widely known and understood [25] but it is important to emphasize that exercise may generate an excessive production of free radicals [26]. As well-known and widely reported in the literature, compared to enzymatic methods able to measure end point biomarkers of oxidative stress damage (oxidized proteins and membrane lipids), EPR is the only technique allowing the direct detection and quantification of ROS. However despite the great interest in measuring ROS in biology and medicine, EPR technique has not till now been widely used because of several technical and methodological problems [27]. The observation that muscular exercise increases ROS production in skeletal muscles was for the first time reported by Davies et al. [28]. In the following years, a lot of studies on animals and

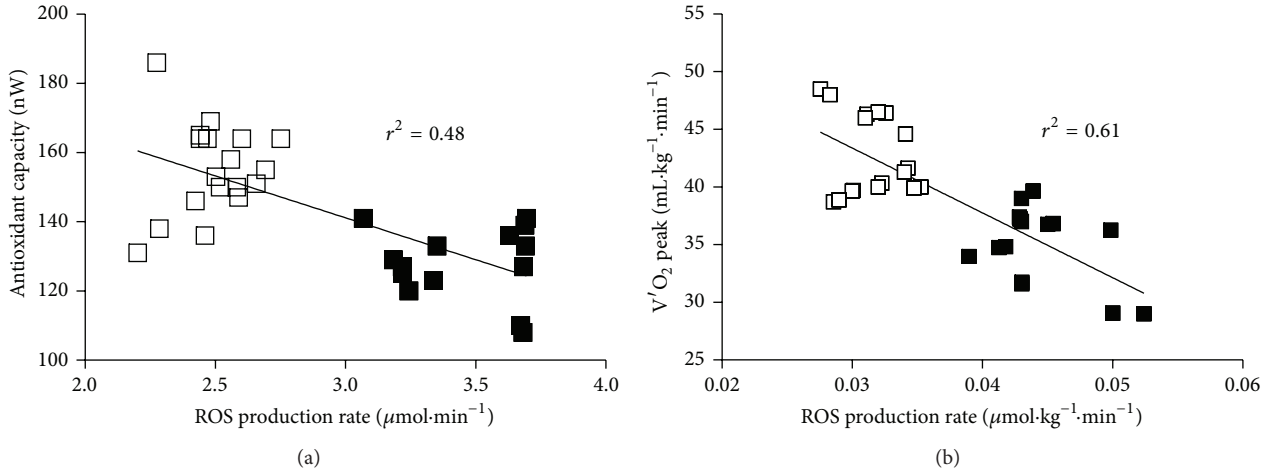

Figure 5: Panel plots of relationship between ROS production rate (μmol·min^{-1}) and (a) antioxidant capacity (nW) and (b) peak ROS production rate (μmol·min^{-1}·kg^{-1}) and $V'O_2$ peak (mL·kg^{-1}·min^{-1}) recorded at the end of IE in the two sessions: before (PRE Trg, full squares) and after (POST Trg, empty squares) training. The linear regression fit (solid line) is also shown and so is the correlation coefficient (r^2) reported in each panel. A significant linear relationship in the ROS production between antioxidant capacity ($P < 0.0001$) and $V'O_2$ peak ($P < 0.0001$) values was estimated.

humans have showed an increase of free radicals production after aerobic or anaerobic exercise in both sedentary or athletes subjects, according to exercise intensity [8, 29].

This increase was also observed in this study using an innovative method [18, 19] that employed EPR technique to attain a rapid and microinvasive measurement of ROS concentration in human peripheral blood. Compared with other spin trap and/or probe molecules, CMH was considered the spin probe of choice to quantify ROS in a most physiological way. Indeed, it shows greatest efficacy for trapping $O_2^{\bullet-}$ radicals, the reaction being much faster ($1.2 \times 10^4\ M^{-1}\ s^{-1}$) and producing stable CM-nitroxide, thereby enabling the reaction with both extra- and intracellular $O_2^{\bullet-}$. Moreover CMH detects ROS from all cellular compartments, including mitochondria [30].

During PRE and POST Trg sessions a significant increase of ROS production was found at the end of IE (+16% and +10%, resp.); this was followed by a gradual decrease in the magnitude of the ROS production in both sessions, returning toward resting values after 10 min (+11% and +2%, resp.). This finding is in agreement with the idea that increased ROS generation caused by physical exercise overwhelms the body capacity to detoxify ROS and that, upon chronic training, adaptive responses, including the one of the antioxidant defense system, better controls ROS production both at rest and after IE. Indeed antioxidant capacity significantly improved at REST (+13%) and maintained high levels 10 min after the end of the exercise (+16%).

One of the aims of this study was to investigate, by means of the same mini-invasive measurement method adopted for ROS production levels determination, whether alterations in redox homeostasis can be monitored to assess the fitness of intensively training athletes.

Aiming at minimizing the invasiveness of the method and hence to improve its potential for routine applications, oxidative stress markers (e.g., thiobarbituric acid substances, protein carbonyls) determination, requiring more invasive venous blood samples, was herein avoided. This choice was also supported by the linear correlation between ROS production rate and the above-mentioned biomarkers concentration previously observed at rest [18, 19]. In addition, the time-course changes of the same oxidative stress biomarkers were found delayed and of longer duration with respect to ROS production kinetics so that no correlation was possible in dynamic conditions [18].

The relationship between metabolic measurements and ROS production rate, before and after an exercise training program, was for the first time attempted in order to check the relation between antioxidant adaptive pathways and muscle metabolic function and at the same time investigate whether alterations in redox homeostasis can be monitored to assess the fitness of intensively training athletes. Indeed, in the examined athletes, exhibiting clear physiological training effects, a significant statistical inverse correlation was observed between ROS production rate and $V'O_2$ peak (Figure 5(b)) determined during an IE. In support to our data, Bloomer et al. [31] demonstrated that peak protein carbonyl concentration value is a function of total $V'O_2$.

It is known that $V'O_2$ peak is one of the important parameters of physical fitness: thus an improvement of $V'O_2$ after training accompanied with an increase in the antioxidant capacity and a subsequent decrease in ROS production can enhance the redox homeostasis thus producing beneficial effects on the response of human body to physical exercise. Moreover Venditti et al. [32] reported that chronic endurance training reduces H_2O_2 production from skeletal muscle mitochondria isolated from gastrocnemius muscles of Wistar rats, by reducing the production at complex I of the electron transport chain. Similarly in the present study ROS production lowered according to the training degree as seen

in Figure 5(b) where subjects' fitness can be evaluated by the $V'O_2$ peak values.

Finally the obtained results support that such HIDT protocol, characterized by repeated variations of intensity [33] associated with changes of redox potential, ATP/ADP ratio, and, consequently, disturbances of cellular homeostasis, can play a positive effect on oxidative stress leading to decrease in lipid peroxidation and DNA damage [9] and on antioxidant capacity reducing ROS production.

5. Conclusions

The study showed that 6 weeks of HIDT training improves exercise (+12% $V'O_2$ peak) and antioxidant (+13%) capacity and significantly ($P < 0.001$) decreases baseline ROS production (−20%). Results also show that after identical exercise trained individuals produced lower levels of ROS related to higher level of antioxidant capacity compared to an untrained state. A novel insight into the correlation between ROS production response pathways and muscle metabolic function has been attained. The adopted microinvasive procedure for ROS rate production measurement by EPR appeared to be a reliable method to evaluate oxidative stress adaptation to acute exercise and training.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Acute Exercise Induced Mitochondrial H_2O_2 Production in Mouse Skeletal Muscle: Association with p^{66Shc} and FOXO3a Signaling and Antioxidant Enzymes

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Exercise induced skeletal muscle phenotype change involves a complex interplay between signaling pathways and downstream regulators. This study aims to investigate the effect of acute exercise on mitochondrial H_2O_2 production and its association with p^{66Shc}, FOXO3a, and antioxidant enzymes. Male ICR/CD-1 mice were subjected to an acute exercise. Muscle tissues (gastrocnemius and quadriceps femoris) were taken after exercise to measure mitochondrial H_2O_2 content, expression of p^{66Shc} and FOXO3a, and the activity of antioxidant enzymes. The results showed that acute exercise significantly increased mitochondrial H_2O_2 content and expressions of p^{66Shc} and FOXO3a in a time-dependent manner, with a linear correlation between the increase in H_2O_2 content and p^{66Shc} or FOXO3a expression. The activity of mitochondrial catalase was slightly reduced in the 90 min exercise group, but it was significantly higher in groups with 120 and 150 min exercise compared to that of 90 min exercise group. The activity of SOD was not significantly affected. The results indicate that acute exercise increases mitochondrial H_2O_2 production in the skeletal muscle, which is associated with the upregulation of p^{66Shc} and FOXO3a. The association of p^{66Shc} and FOXO3a signaling with exercise induced H_2O_2 generation may play a role in regulating cellular oxidative stress during acute exercise.

1. Introduction

Skeletal muscle has the remarkable ability to adapt to changes in cellular environmental influences during exercise [1]. Studies have shown that muscle stimulation can induce diverse metabolic and morphological adaptations, which are important mechanisms for controlling skeletal muscle phenotype changes [2]. For example, studies have shown that resistance exercises caused muscle hypertrophy and increased muscle strength [3], while endurance exercises increased muscle oxidative capacity [4]. Even a single bout of exercise can induce various effects including metabolic improvement [5]. Although the exact mechanism of exercise induced skeletal muscle adaptation remains to be elucidated, it has been shown that such adaptation involves a complex interplay between signaling pathways and downstream regulators, leading to specific molecular and cellular responses [6].

One mechanism affecting exercise related skeletal muscle phenotype changes involves reactive oxygen species (ROS), in particular hydrogen peroxide (H_2O_2) related redox activity [7]. H_2O_2 is a major component of ROS, generated during mitochondrial respiration. Electron leakage at specific redox centres during mitochondrial electron transfer chain reactions has been shown to be responsible for generating a significant fraction cellular ROS [8]. As a by-product of oxidative metabolism, H_2O_2 has certain damaging effects on cellular components such as DNA, proteins, and lipids

in pathological conditions [9]. For example, Haycock et al. [10] reported that mitochondrial proteins such as succinate dehydrogenase and cytochrome oxidase showed particular susceptibility to H_2O_2, which induced mitochondrial dysfunction and oxidative damage in skeletal muscle. On the other hand, exercise induced ROS/H_2O_2 production in skeletal muscles has been shown to cause modification of mitochondrial signaling pathways [11, 12]. There is now genetic and biomolecular evidence indicating that ROS generation in mitochondria can also be finely controlled to play an important role in a wide variety of physiological processes by regulating signal transduction, gene expression, and redox reaction [13]. Among these, p^{66Shc} has been shown to orchestrate mitochondrial redox signaling by acting as a ROS sensor to regulate its redox function within mitochondria [14, 15]. p^{66Shc} is a peculiar protein, acting specifically in the mitochondrion as a redox enzyme that generates H_2O_2 by sequestering electrons from the respiratory chain [16]. It regulates cellular H_2O_2 content through changes in H_2O_2 purification ability, membrane oxidase activity, and mitochondrial respiratory chain proton leak, so that levels of intracellular H_2O_2 maintain homeostasis in mammalian cells [17]. Studies in p^{66Shc}-deficient fibroblast and endothelial cells have found a remarkable resistance of these cells to exogenous oxidative stress and ROS-induced apoptosis [18]. $p^{66Shc-/-}$ mice appeared to be protected from oxidative stress-induced apoptosis, diabetic renal damage, and age-dependent increase in emotionality and pain sensitivity [19, 20]. p^{66Shc} has been proposed to control oxidative stress response in mammals [21]. Further studies found that $p^{66Shc-/-}$ mice showed resistance to a number of oxidative stress-related pathological damages, such as ischemia/reperfusion injury, vascular injury and atherosclerosis, hind limb ischemia, and alcohol-related liver damage [22–24]. However, it is still not very clear about the relationship between p^{66Shc} and exercise induced H_2O_2 generations.

Mammalian cells have a sophisticated system for scavenging ROS to nontoxic forms to defence cells against oxidative stress induced by high levels of ROS. This antioxidant defence system is composed of antioxidant enzymes such as superoxide dismutase (SOD) and catalase [25] and certain transcription factors, such as Forkhead box O3a (FOXO3a) (also named forkhead rhabdomyosarcoma-like 1, FOXO3a). FOXO3a is a member of the FOXO family proteins, which has been implicated in the regulation of oxidative stress in diverse physiological processes including stress resistance and metabolism [26]. It has been demonstrated that FOXO3a reduced ROS generation by the transcriptional activation of SOD [27] and catalase [28]. The general antioxidant effect of FoxOs has been shown in its capacity to extend lifespan in nutrient-restricted organisms (such as *Caenorhabditis elegans*) and organism survival under various environmental stress [29]. However, the relationship between FOXO3a and H_2O_2 generation and antioxidant enzymes in exercise has not been reported.

Previous studies indicate that the physiological actions of H_2O_2 depend on its cellular concentrations. At low concentrations it affects cell proliferation and differentiation [6]; at higher concentrations it has been shown to induce certain antioxidant genes [30], but at pathological concentrations, it can cause cell damage or cell death [27]. Thus, it is important to understand the regulation of intracellular H_2O_2 production and related signaling pathways, including antioxidant enzyme activities during exercise. However, currently the molecular mechanism of endogenous H_2O_2 generation and its relationship to antioxidant cellular signaling pathways in skeletal muscles during exercise still remains to be elucidated, in particular regarding the relationship between acute exercise induced H_2O_2 generation and relevant signaling pathway. In this study we aim to investigate the association of H_2O_2 generation with p^{66Shc} and FOXO3a signalling and antioxidant enzymes in acute exercise. We hypothesise that p^{66Shc} is involved in the regulation of H_2O_2 production during acute exercise. We have investigated how acute exercise affected H_2O_2 production and the expression of p^{66Shc} and FOXO3a genes and proteins and their relationship to activity of antioxidant enzymes (SOD and catalase) in skeletal muscles of ICR/CD-1 mice.

2. Materials and Methods

2.1. Animal Groups. Eight-week-old male ICR/CD-1 mice were purchased from the Shanghai SLAC Experimental Animal Centre (Shanghai, China). Mice were fed on a standard chow diet and housed in a standard pathogen-free environment under controlled temperature (21 ± 3°C) and light (12 : 12 h light-dark cycle) at East China Normal University Animal Facility. Animals were acclimatised for a week before being randomly assigned to one of the following experimental groups: sedentary control (control, $n = 6$) and acute exercise for different period (45, 90, 120, and 150 min, respectively, $n = 6$ in each group). The use of animals and all experimental procedures were approved by the Experimental Animal Care and Use Committee at East China Normal University (SCXK 2007-0005). Experiments were performed in accordance with the Guidelines for the Use of Laboratory Animals published by the People's Republic of China Ministry of Health.

2.2. Exercise Protocol. The acute incremental exercise model used in this study was performed according to an established animal model described in previous studies [31]. Briefly, ICR/CD-1 mice from the exercise group were accustomed for 3 days of training on a treadmill. In the training (familiarisation) period, mice ran for 15 min at 8.2 m/min at a 0° incline on day 1; 15 min at 8.2 m/min at a 0° incline followed by another 15 m/min at a 5° incline on day 2; 15 min at 15 m/min at a 5° incline followed by another 15 min at 19.3 m/min at a 10° incline on day 3. After the familiarisation period, mice were subjected to an exercise program according to the following, with the first load (0°, 8.2 m/min, 53% $VO_{2\,max}$), then the second load (5°, 15 m/min, 64% $VO_{2\,max}$) and the third load (10°, 19.3 m/min, 76% $VO_{2\,max}$) exercise, respectively, each for 15 min, until reaching the preset 45 min, 90 min, 120 min, and 150 min. During the exercise, tails of mice are stimulated by brush to ensure mice run at preset speed and incline.

2.3. Tissues and Mitochondria Isolation. After completing the exercise program, mice were sacrificed rapidly by cervical dislocation. Gastrocnemius muscles and musculus quadriceps femoris were dissected. Left gastrocnemius muscles were used for mitochondrial isolation. Other muscles were immediately frozen in liquid nitrogen and then stored at −80°C. The frozen tissues were used for protein content determination, real-time PCR, and Western blotting.

Mitochondria were isolated as described previously [32, 33]. Briefly, muscles were homogenized in precooled isolation buffer (0.075 M sucrose, 0.225 M sorbitol, 1 mM EGTA, 0.1% fatty acid-free bovine serum albumin, and 10 mM TrisHCl, pH 7.4, 4°C) (1 mL buffer per 100 mg tissue). Homogenate was centrifuged at 1200 ×g for 10 min at 4°C using a Beckman centrifuge (Avanti J-26XP). The supernatant fraction was decanted and saved. The pellet was washed once with 2 volumes of isolation buffer. The supernatant fractions were combined and centrifuged at 9,000 ×g for 10 min at 4°C. The mitochondrial pellet was washed and centrifuged twice at 15,000 g for 2 min at 4°C with isolation buffer. Mitochondrial protein content was assayed using BSA (bovine serum albumin) as a standard according to Bradford. Freshly isolated mitochondria were used immediately for measuring H_2O_2, SOD and catalase activity assays.

2.4. Determination of Mitochondrial H_2O_2 Content. The hydrogen peroxide content in the skeletal muscle mitochondria was measured by *colorimetric* method as previously described [12], using a commercial kit, based on the reaction with molybdic acid (Jiancheng Biotech Inc., Nanjing, China). Adduct was measured spectrophotometrically at 405 nm in a plate reader (TECAN infinite M200, USA) in strict accordance with manufacturer's instructions.

2.5. Expression of p^{66shc} and FOXO3a mRNAs. The expression of p^{66Shc} and FOXO3a mRNAs was determined by quantitative real-time PCR (qPCR), as previously described [34]. Briefly, total RNA was extracted and purified by a commercial kit (Trizol, Invitrogen, Chromos, Singapore). Double-stranded cDNA was synthesised from 1 μg of total RNA using ReverTra Ace qPCR RT Kit (Toyobo Co., Ltd, Osaka, Japan). Real-time PCR was conducted using Toyob SYBR-green PCR kit (Toyobo) and the StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). PCR was performed in a fluorescence temperature cycler containing 0.8 μL upstream and downstream primers, respectively; 4 μL ddH_2O, 3.0 μL template; and 10 μL 2.0x Master SYBR green I (containing Taq DNA polymerase, reaction buffer, dNTP mix, SYBR green I dye, and 10 mmol/L $MgCl_2$) in a total volume of 20 μL. Amplification occurred over a three-step cycle (denaturation at 95°C for 15 s, annealing at 61°C for 30 s, and extension and data collection at 72°C for 45 s) for 35 cycles. p^{66Shc} FOXO3a mRNAs were standard against that of β-actin. Relative expression level of each sample was calculated according to formula $2^{-\Delta Ct}$. Primers pairs were designed based on GenBank reference sequences and synthetized by Shanghai Sangon Biological Technology Co. Ltd, with the following sequences (5′ to 3′), forward primer: caactctaagttccctttca, reverse primer: gctgctgtacccaatcccac (p^{66Shc} [35]), forward primer: taggctgcactgggggtaa, reverse primer: actgatcagagctacaagac (FOXO3a [36]), forward primer: tgttaccaactgggacgaca, reverse primer: ctatgggagaacggcagaag (β-actin).

2.6. Expression of p^{66shc} and FOXO3a Proteins. The expression of p^{66Shc} and FOXO3a proteins was determined by Western blotting as described previously [34]. Briefly, frozen muscles (muscles from two individual animals in each group, combined into one sample) were homogenised (1 : 9 w/v) in ice-cold buffer (20 mM HEPES, pH 7.4, 100 mM KCl, 50 mM β-glycerophosphate, 50 mM NaF, 1 mM dithiothreitol, 0.5 mM Na_3VO_4, 0.2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine). Homogenates were centrifuged for 5 min at 1000 ×g at 4°C, and then supernatant was centrifuged at 10,000 ×g at 4°C for 10 min, resolved in SDS buffer, and boiled for 5 min at 100°C [34]. The protein content was quantified using bicinchoninic acid assay with bovine serum albumin as the standard (Shanghai Sangon, Shanghai, China). Equal amounts of protein (30 μg/lane) were electrophoresed in 12% SDS-polyacrylamide (120 V; Bio-Rad, Hercules, CA, USA), and proteins were transferred (1 h, 1.2 mA/cm^2, Criterion blotter; Bio-Rad) onto a polyvinylidenedifluoride membrane. After Ponceau S staining and destaining, the membranes were blocked in 5% nonfat dry milk powder (Shanghai Sangon) in Tris-buffered saline, containing 0.1% Tween 20 (TBST) for 1 h at room temperature. Thereafter, a 1 : 200 dilution of the primary specific antibody (p^{66Shc} : sc-1695, p-FOXO3a-ser253 : sc-34894, Santa Cruz Biotechnology) in 5% TBST was added and incubated overnight at 4°C on a shaker. After the membranes were washed three times for 10 min each in 5% TBST, membranes were incubated with a 1 : 2000 dilution of the horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) in 5% TBST for 1 h at room temperature. Membranes were washed three times in TBST for 10 min each [37]. Visualisation of the reaction bands was performed with 3, 3′-diaminobenzidine staining (Shanghai Sangon) and scanned densitometrically. Quantification was performed with a gel image processing system (GIS-2008; Tanon, Shanghai, China). GAPDH was used to standardize amounts of protein loaded.

2.7. Activity of SOD and Catalase. The total SOD activity (U/mg protein) in mitochondria of the left gastrocnemius muscles of ICR/CD-1 mice was measured using a commercial kit (Jiancheng Biotech Inc., Nanjing, China) in strict accordance with instructions. The adduct was measured spectrophotometrically at 550 nm with a plate reader (TECAN infinite M200, USA).

Similarly, the activity of catalase (U/mg protein) in mitochondria of the left gastrocnemius muscles of ICR/CD-1 mice was measured using a commercial kit (Jiancheng Biotech Inc., Nanjing, China), which is based on the reaction of ammonium molybdate with H_2O_2 to form a light yellow complex compound. Adduct was measured spectrophotometrically at

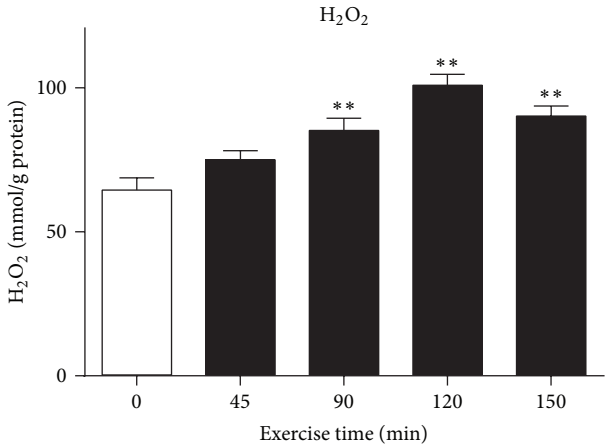

FIGURE 1: Mitochondrial H_2O_2 content in skeletal muscles of ICR/CD-1 mice subjected to acute exercise for different periods. Data are presented as mean ± SEM ($n = 6$). $^{**}P < 0.01$ compared to the sedentary control group (C).

405 nm with a plate reader (TECAN infinite M200, USA) in strict accordance with instructions.

2.8. Statistical Analysis. Data were expressed as the means ± standard error of the means (SEM). Statistical analysis was performed using SPSS21. Data were analysed by one-way analysis of variance (ANOVA), followed by Tukey post hoc tests for multiple comparisons. Correlation was analysed by linear regression, and correlation coefficient (r) and P value were calculated. For all tests the significance level was set at $P < 0.05$ or $P < 0.01$.

3. Results

3.1. Mitochondrial H_2O_2 Content in Skeletal Muscles of ICR/CD-1 Mice with Acute Exercise. The acute exercise did not cause a significant change in the mitochondrial H_2O_2 content in gastrocnemius muscles of ICR/CD-1 mice with 45 min exercise, compared with that of the sedentary control. However, acute exercise caused a significant increase in mitochondrial H_2O_2 content in mice with 90, 120, and 150 min exercise, respectively, compared to the sedentary control ($P < 0.01$, Figure 1). The effect was time-dependent with the maximal peak effect at 120 min exercise (Figure 1).

3.2. Expression of p^{66shc} and FOXO3a Transcript Genes in Skeletal Muscles of ICR/CD-1 Mice with Acute Exercise. The expressions of p^{66Shc} and FOXO3a transcript genes were not significantly changed in the skeletal muscles of ICR/CD-1 mice with 45 min and 90 min acute exercise. However the expression of p^{66Shc} mRNA was significantly increased in mice with 120 min and 150 min exercise, respectively, compared to the sedentary control ($P < 0.05$, Figure 2). Similarly, the expression of FOXO3a mRNA was significantly increased in groups with 120 and 150 min exercise, compared to the sedentary control ($P < 0.05$, $P < 0.01$, Figure 2).

3.3. Expression of p^{66shc} and FOXO3a Proteins in Skeletal Muscles of ICR/CD-1 Mice with Acute Exercise. The expressions of p^{66Shc} and FOXO3a proteins in the skeletal muscles of ICR/CD-1 mice were not significantly changed in groups with 45 min and 90 min acute exercise (Figure 2). However the expressions of p^{66Shc} proteins significantly increased in mice with 120 min and 150 min exercise, compared to the sedentary control ($P < 0.01$, Figure 2). Similarly, the expression of FOXO3a protein was significantly increased in mice with 120 min and 150 min exercise, respectively ($P < 0.01$, Figure 2), compared to the sedentary control. The maximal effect was observed with 120 min exercise for p^{66Shc} proteins and 150 min exercise for FOXO3a proteins (Figure 2).

3.4. Correlation between H_2O_2 Content and Expressions of p^{66shc} and FOXO3a mRNA in Skeletal Muscles of ICR/CD-1 Mice with Acute Exercise. There was a positive correlation between mitochondrial H_2O_2 content and expression of p^{66Shc} mRNA ($r = 0.4723$, $P < 0.01$, Figure 3) in skeletal muscles of exercised ICR/CD. Similarly, there was a positive correlation between mitochondrial H_2O_2 content and expression of FOXO3a mRNA ($r = 0.5623$, $P < 0.01$, Figure 3) in skeletal muscles of exercised ICR/CD-1 mice.

3.5. Mitochondrial SOD and Catalase Activities in Skeletal Muscles of ICR/CD-1 Mice with Acute Exercise. The SOD activity was not significantly changed in groups with 45, 90, 120, and 150 min acute exercises, compared to the sedentary control ($P > 0.05$, Figure 4). The catalase activity was slightly reduced in groups with 90 min acute exercise, compared to the sedentary control ($P < 0.05$, Figure 4). However, the catalase activity was significantly higher in mice with 120 and 150 min exercise, compared to that of 90 min exercise group ($P < 0.05$, $P < 0.01$, Figure 4).

4. Discussion

It has been known that ROS generated during mitochondrial respiration in muscle after exercise can regulate endogenous antioxidant defence genes through the activation of redox-sensitive transcription factors [13]. For example, PGC1a, a transcriptional coactivator of genes involved in mitochondrial respiration and biogenesis and regulating antioxidant defence genes [38], can be transiently induced by endurance exercise [39]. ROS has also been linked to an increase in muscle-produced cytokines and release Ca^{++} from the sarcoplasmic reticulum during moderate exercise [40]. Among different species of ROS, H_2O_2 has recently been recognised as an important physiological regulator, key to regulating biological processes such as mitochondrial antioxidant defence [27]. The involvement of H_2O_2 in endurance exercise induced changes in signaling pathways in skeletal muscles has been recently reported for PLA2 [9]. However, the exact mechanism which links H_2O_2 generation and antioxidant signaling in skeletal muscle during acute exercise still needs to be elucidated. The main finding of the present study is that the acute exercise induced a time-dependent increase

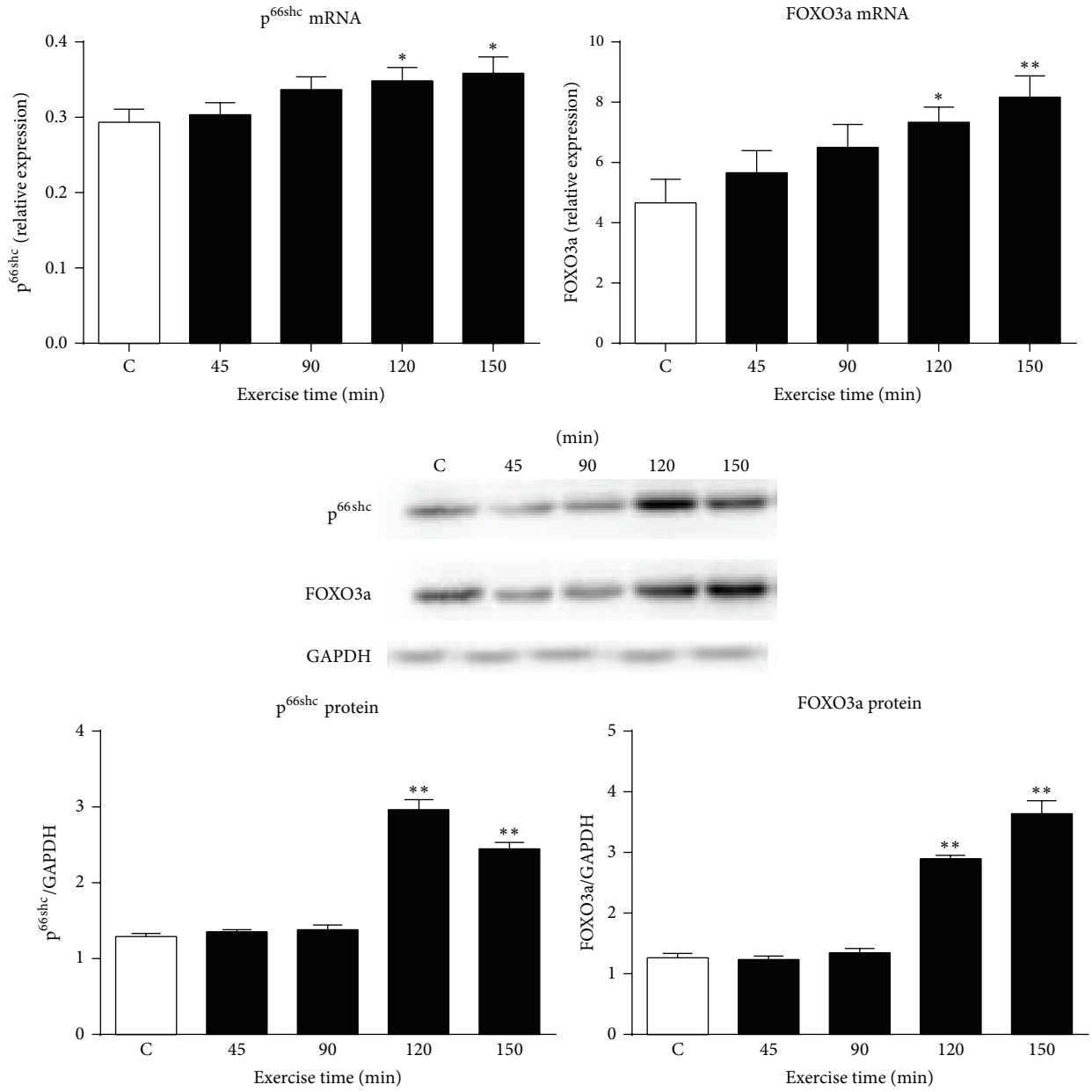

Figure 2: Expression of p^{66Shc} and FOXO3a mRNA and proteins in skeletal muscles of ICR/CD-1 mice, subjected to varying periods of acute exercise. Data are presented as mean ± SEM ($n = 3$–6). $^{*}P < 0.05$, $^{**}P < 0.01$ compared to the sedentary control group (C).

in mitochondrial H_2O_2 content, which was associated with an increased expression of p^{66Shc} and FOXO3a genes and proteins, and a time-dependent change in mitochondrial catalase activities, which highlights an interplay of mitochondria H_2O_2 production and modulation of p^{66Shc} signaling, and antioxidant enzyme activity induced by acute exercise in mouse skeletal muscle.

Previous studies have demonstrated that H_2O_2 generated during exercise can cause cell signalling change, inducing alterations in gene expression by directly modifying target proteins, or by changing their intracellular redox state [13], such as via modulation of signaling pathways for growth factors and myokine production [41]. In the present study, we observed that acute exercise caused a time-dependent increase in mitochondrial H_2O_2 content in skeletal muscles of ICR/CD-1 mice over the 45–150 min exercise period, with the maximal effect seen after 120 min exercise. In addition, the expression of p^{66Shc} and FOXO3a genes and proteins in skeletal muscles of ICR/CD-1 mice showed a similar time-dependent manner to that of H_2O_2 content change, indicating a role of p^{66Shc} and FOXO3a in H_2O_2 production in the acute exercise. This is further supported by a close correlation between p^{66Shc} and FOXO3a gene expressions and H_2O_2 content. Previous studies have shown that cells and tissues derived from p^{66Shc}-null mice accumulate significantly less oxidative stress, and the deletion of p^{66Shc} gene in mice

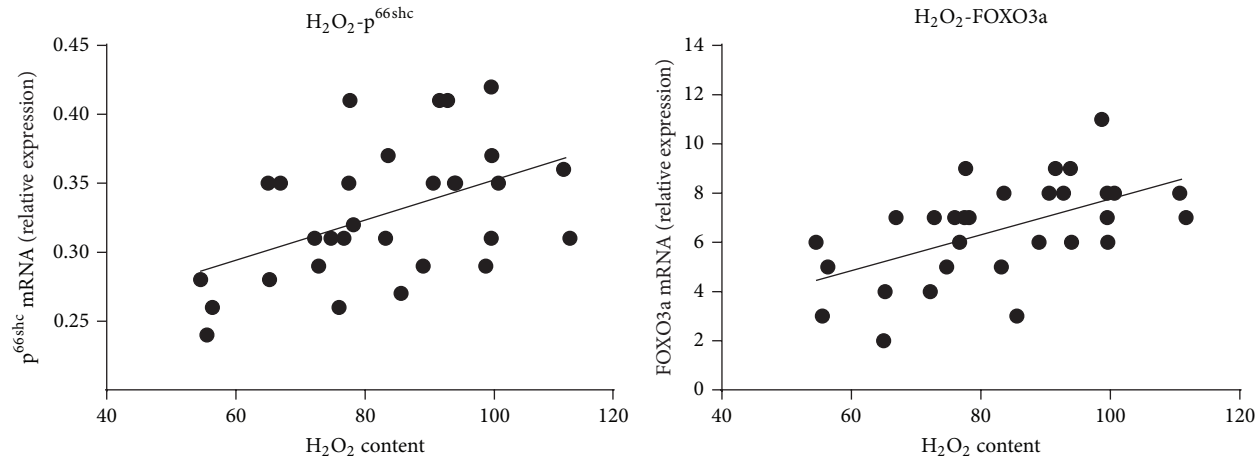

Figure 3: Correlation between expression of p^{66Shc} and FOXO3a mRNA and mitochondrial H_2O_2 content (mmol/g protein) in skeletal muscles of exercised ICR/CD.

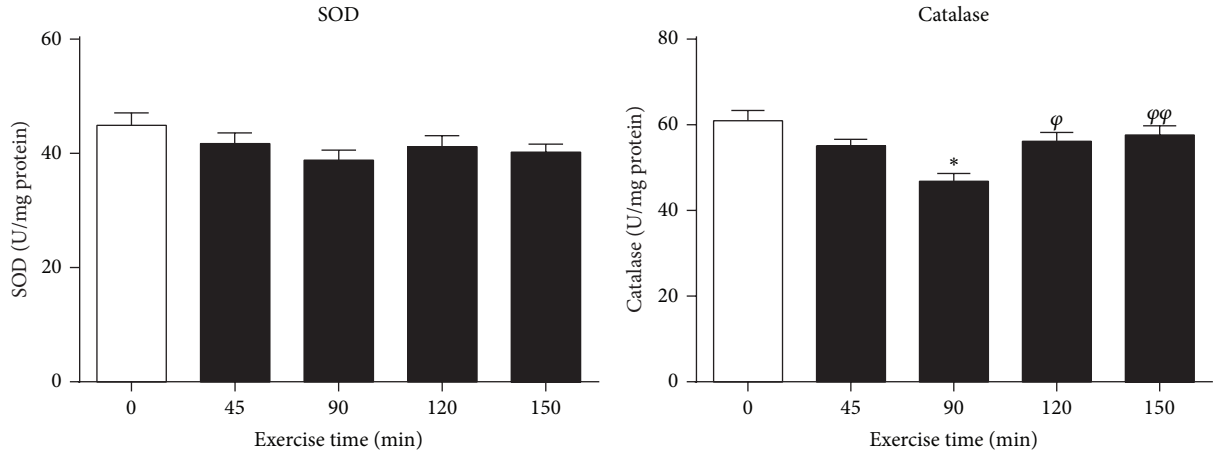

Figure 4: Mitochondrial SOD activity and catalase activity in skeletal muscle of ICR/CD-1 mice subjected to acute exercise over different periods. Data are presented as mean ± SEM ($n = 6$). $^{*}P < 0.05$ compared to the sedentary control group (C). $^{\varphi}P < 0.05$, $^{\varphi\varphi}P < 0.01$ compared to the 90 min exercise group.

resulted in the decreased formation of mitochondrial H_2O_2 [16]. Our finding is also consistent with a latest study demonstrating that a prolonged swimming exercise promoted cellular oxidative stress and p^{66Shc} phosphorylation in rat heart [42] and supporting the hypothesis that acute exercise increases H_2O_2 levels involving upregulating p^{66Shc} signaling. p^{66Shc} is a peculiar protein, acting specifically in the mitochondrion as a redox enzyme that generates H_2O_2 by sequestering electrons from the respiratory chain [16]. It regulates H_2O_2 content through changes in H_2O_2 purification ability, membrane oxidase activity, and mitochondrial respiratory chain proton leak, so that levels of intracellular H_2O_2 maintain homeostasis in mammalian cells [17]. It has been known that a small fraction of p^{66Shc} translocates from the cytosol into the mitochondria, where it directly transfers electrons from cytochrome c to molecular oxygen, thus producing H_2O_2 [16]. Studies have demonstrated that PKC b, a protein kinase activated by oxidative stress, phosphorylated p^{66Shc} at serine 36, allowing its interaction with prolyl isomerase pin-1, which then physically translocated p^{66Shc} across the outer mitochondrial membrane [43]. Thus, oxidative stress-triggered p^{66Shc} phosphorylation and localization to mitochondria may play an important role in H_2O_2 generation during acute exercise. Our finding is similar to that of Ding et al. [12], who reported that acute exercise increased H_2O_2 content in triceps surae skeletal muscle in rats. In addition, there is a possibility that other metabolic and/or mitochondrial pathways may also be involved in regulation of H_2O_2 in the skeletal muscle during the acute exercise, as it has been shown that H_2O_2 generated by p^{66Shc} accounts for about 30% of the total pool of intracellular H_2O_2 [16]. Interestingly, previous studies have reported similar changes for other ROS species. For example, McArdle et al. found that aerobic contractile activity induced a release of

superoxide anions from mouse gastrocnemius muscle *in vivo* [44]. One important observation in the present study is an association between H_2O_2 production and the downstream regulator FOXO3a, which supports the possibility that redox-dependent FOXO3a activation is regulated by intracellular H_2O_2 in a p^{66Shc}-dependent manner during acute exercise. The possible link between H_2O_2 induced cell responses and FOXO3a was suggested previously [45]. Our finding is consistent with that by van der Horst et al. who showed that ROS induced a FOXO3a dependent antioxidant response [46, 47], indicating the p^{66Shc}-H_2O_2-FOXO3a signalling interplay may play an important role in modulating cellular functions during exercise [48].

FOXO3a is a key transcription factor that translocates to nucleus and activates transcription by specifically binding to the consensus sequence TTGTTTAC in the promoters of target genes [49], causing an activation of transcription of the two essential antioxidant enzymes mitochondrial superoxide dismutase (MnSOD) and catalase, which scavenge superoxide and hydrogen peroxide, respectively [50]. It has been demonstrated that MnSOD and catalase are transcriptional targets of FOXO3a [51], and these enzymes are involved in the regulation of the cell cycle and the defence against oxidative stress [52]. Previous studies showed that increase in FOXO3a expression protected mitochondria dysfunction from hyperglycemia-induced oxidative stress in human lens epithelial cells [26]. FOXO3a reduced H_2O_2-induced cellular oxidation and increased mitochondrial MnSOD protein expression (but no change in cytoplasmic copper/zinc SOD (CuZnSOD) expression) in DL23 cells [53] and controlled the expression of proteins involved in the DNA repair mechanisms [54]. In the present study, we found no significant changes of total SOD activity in all exercise groups; this finding is similar to a previous study which found no significant change of soleus muscle SOD activity by endurance exercise in rats [55]. On the contrary, Itoh et al. [56] reported a decrease in diaphragm SOD activity in rat with an acute exercise. Feoli et al. [57] also reported a decrease in serum SOD activity in volunteers with acute exercise. In addition, an increase of SOD activities in skeletal muscles and cardiac mitochondria after exercise has also been reported [58, 59]. The reason for these discrepancies is not clear, but it may be related to differences in exercise patterns, muscle types, and SOD assays used in these studies. For example, a 10-fold difference in the relative sensitivity among different SOD assays has been reported [60]. On the other hand, it is possible that FOXO3a may cause a change of a particular type of SOD activity (e.g, MnSOD), rather than total SOD activity [53]; thus further study is necessary to investigate the pattern of SOD changes and possible mechanisms in acute exercise. Similarly, the change of activity of catalase, another essential antioxidant enzyme targeted by FOXO3a target genes [17, 49] during the exercise, is also controversial. It was reported earlier that acute exercise significantly increased mitochondrial catalase activity, compared to the sedentary control [59]. Karanth and Jeevaratnam [55] also reported that swimming training increased muscle catalase content in rats. In contrast, Choi and Cho reported that catalase activity was significantly lower after 6 weeks of treadmill exercise [61]. It is not clear if this discrepancy related to the variations in catalase activities under different experimental conditions [62]. In the present study, we observed a slight decrease in catalase activity in the 90 min acute exercise group, then a significant increase in catalase activity in the 120 min and 150 min exercise groups, compared with that of the 90 min exercise group. It is possible that the initial decrease in the catalase activity is caused by the increased ROS production in mice with relative short period of exercise, and such decrease is corrected with the increase in FOXO3a-associated catalase enzymes [17] after longer period exercise. This seems to be in line with the observation that the slight decrease in catalase activity occurred earlier than the change of FOXO3a protein expression, while the increase in catalase activity occurred in the period with increased FOXO3a expressions. Further study with a longer period of exercise (e.g., >150 min) may help to elucidate FOXO3a-mediated increase in mitochondria catalase activity and related mechanisms. Thus, our findings indicate that mitochondrial catalase and FOXO3a related regulation of antioxidant enzymes may serve as important protective mechanisms in reducing acute exercise induced cellular oxidative stress.

In conclusion, our study demonstrates that acute exercise causes an increase in mitochondrial H_2O_2 production, which is associated with the upregulation of p^{66Shc} and FOXO3a in the skeletal muscles of ICR/CD-1 mice. Activation of the p^{66Shc}-H_2O_2-FOXO3a signaling pathway by acute exercise may underline the molecular mechanism of regulating cellular oxidative stress resistance during exercise. Given that oxidative stress has been implicated in various diseases and aging, the mechanism behind this link may have therapeutic implications. A further understanding of the mechanism of acute exercise induced modulation of H_2O_2 production and associations to p^{66Shc} signaling and ROS-FOXO3a-antioxidant enzymes may help to develop interventions to improve exercise outcomes and control oxidative stress-related diseases or conditions such as diabetes and aging.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Assessment of Eccentric Exercise-Induced Oxidative Stress Using Oxidation-Reduction Potential Markers

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The aim of the present study was to investigate the use of static (sORP) and capacity ORP (cORP) oxidation-reduction potential markers as measured by the RedoxSYS Diagnostic System in plasma, for assessing eccentric exercise-induced oxidative stress. Nineteen volunteers performed eccentric exercise with the knee extensors. Blood was collected before, immediately after exercise, and 24, 48, and 72 h after exercise. Moreover, common redox biomarkers were measured, which were protein carbonyls, thiobarbituric acid-reactive substances, total antioxidant capacity in plasma, and catalase activity and glutathione levels in erythrocytes. When the participants were examined as one group, there were not significant differences in any marker after exercise. However, in 11 participants there was a high increase in cORP after exercise, while in 8 participants there was a high decrease. Thus, the participants were divided in low cORP group exhibiting significant decrease in cORP after exercise and in high cORP group exhibiting significant increase. Moreover, only in the low cORP group there was a significant increase in lipid peroxidation after exercise suggesting induction of oxidative stress. The results suggested that high decreases in cORP values after exercise may indicate induction of oxidative stress by eccentric exercise, while high increases in cORP values after exercise may indicate no existence of oxidative stress.

1. Introduction

Eccentric exercise is an active contraction of a muscle occurring simultaneously with lengthening of the muscle and induces severe muscle damage characterised by decreased muscle force production [1, 2], increased serum creatine kinase (CK) activity [1, 2], and inflammation response [3–5]. Specifically, eccentric exercise induces damage to skeletal muscle in a fiber specific manner [6]. Reactive species (RS) have been shown to play an important role in both the initiation and the progression of muscle fiber injury after eccentric exercise [7, 8]. The generation of RS during an extended bout of eccentric exercise has been attributed to different mechanisms such as xanthine and NADPH oxidase production, ischemia reperfusion, prostanoid and catecholamine metabolism, disruption of iron-containing proteins, and excessive calcium accumulation [9]. Moreover, the infiltration of neutrophils and macrophages to the site of injury [10] after eccentric exercise generate RS [11], which damage the muscle tissue [8].

However, although it is taken as granted that eccentric exercise induces oxidative stress, great differences have been shown in the extent of oxidative stress between different individuals after eccentric exercise [12]. Moreover, Margaritelis et al. [12] have reported that eccentric exercise can induce reductive stress or negligible stress in a considerable number of people. These differences impose the need for finding markers that could predict the severity of oxidative stress

induced by eccentric exercise. In our previous studies [13, 14] as well as in other studies, increases in oxidative stress markers have been reported in blood after eccentric exercise [15–17]. For example, eccentric exercise has been shown to increase malondialdehyde (MDA), protein carbonyl (CARB), and F2-isoprostane levels [13, 15, 17] and decrease glutathione levels (GSH) [12, 13]. However, most of these markers assess a specific damage induced by RS, and so there is also the need for markers that could measure the total redox status of an individual after eccentric exercise.

Thus, the aim of the present study was to investigate if markers based on the measurement of oxidation-reduction potential (ORP) can be used for predicting the total oxidative stress induced by eccentric exercise. ORP assessment was made by a new methodology used by the Luoxis' proprietary RedoxSYS Diagnostic System. RedoxSYS Diagnostic System measures ORP which is an integrated measure of the balance between total oxidants (e.g., oxidized thiols, superoxide radical, hydroxyl radical, hydrogen peroxide, nitric oxide, peroxynitrite, and transition metal ions) and total reductants (e.g., free thiols, ascorbate, a-tocopherol, b-carotene, and uric acid). Thus, ORP is an overall measure of the oxidative stress to which a biological system is subjected [18]. The RedoxSYS Diagnostic System enables robust and rapid assessment of oxidative stress in a single drop of plasma via measurement within four minutes of two distinct elements to determine ORP, the static ORP (sORP), and the capacity ORP (cORP). sORP is the standard potential between a working electrode and a reference electrode with no driving current (or extremely small current) which is proportional to the balance of reductants and oxidants and is what is classically termed ORP (i.e., a homeostatic parameter capturing the current balance of oxidants and reductants in a biological specimen). Low sORP values mean that the biological sample is in the normal range of oxidative stress, while higher than normal sORP values mean that the biological sample is in a higher state of oxidative stress. cORP is the measure of antioxidant reserve available in the body's system; high capacity values mean that the biological sample has antioxidant reserves in the normal range; lower than normal cORP values mean that the biological sample has below normal antioxidant reserves. Specifically, the RedoxSYS Diagnostic System measures the ORP with a three-electrode system, a working electrode, a counter electrode, and a reference electrode. First, a negligible amount of current is applied between the working and counter electrodes, and the ORP is measured between the working and reference electrodes. Once the ORP reading reaches equilibrium, the sORP is established and measured in millivolts (mV). Then, a linearly increasing current is applied to the sample, between the counter and working electrodes. The time from the beginning of the current sweep to the maximum rate of change in ORP is referred to as transition time and the integrated current to this time is the cORP, measured in microcoulombs (μC). In one of our previous studies, the RedoxSYS Diagnostic System has been shown to be effective for assessing oxidative stress induced after a marathon race [19].

The redox status of the participants in the present study was also assessed by "conventional" oxidative stress markers such as GSH, catalase activity, thiobarbituric acid-reactive substances (TBARS), CARB, and total antioxidant capacity (TAC). Thus, ORP values were compared with those of "conventional" oxidative stress markers to examine if the former could predict eccentric exercise-induced oxidative stress.

Moreover, two different methodologies were used for examining if ORP markers could be used for the monitoring eccentric exercise-induced oxidative stress. In the first methodology, all the participants were examined as one group before and after exercise. However, there is recently growing evidence that there is a marked heterogeneity in responses to eccentric exercise-induced oxidative stress between different individuals [12]. Similarly, in the present study, it was observed that the participants could be divided into two groups, those with high increase and those with large decrease in cORP after exercise compared to before exercise. Thus, for the analysis of the results a second methodology was followed in which pre- and postexercise comparison for all tested oxidative stress markers was made separately for each of these groups.

2. Materials and Methods

2.1. Participants. Nineteen young volunteers (gender: 10 men and 9 female; age: 24.4 ± 4.0 years; height: 168.6 ± 7.5 cm; weight: 69.4 ± 4.0 kg) participated in the present investigation. Subjects were excluded from the study, if they had any history of musculoskeletal injury to the lower limbs that would limit the ability to perform the exercise session. Smoking and consumption of nutritional supplementation the last three months before the study initiation were also exclusion criteria. During their first visit, body mass was measured to the nearest 0.5 kg (Beam Balance 710, Seca, United Kingdom) while the subjects were lightly dressed and barefoot. Standing height was measured to the nearest 0.5 cm (Stadiometer 208, Seca). Volunteers were instructed to abstain from any strenuous exercise during their participation in the study as well as for five days prior and 3 days following the exercise session. Subjects were also advised to refrain from taking anti-inflammatory or analgesic medications for the duration of the study. A written consent was obtained from all participants, after they were informed for the risks, discomforts, and benefits involved in the study. The procedures were in accordance with the Helsinki Declaration of 1975, as revised in 2000.

2.2. Eccentric Exercise Protocol. The eccentric exercise session was performed on an isokinetic dynamometer (Cybex Norm, Ronkonkoma, NY). The exercise protocols were undertaken from the seated position (120° hip angle) with the lateral femoral condyle aligned with the axis of rotation of the dynamometer. Participants were coupled to the dynamometer by an ankle cuff attached proximal to the lateral malleolus, after they were stabilized according to the manufacturer's instructions. Participants completed 5 sets of 15 eccentric maximal voluntary contractions (knee range, 0° full extension to 90° flexion) at an angular velocity of 60°/s. A 2 min rest interval was used between sets and the total workout time was 15 min. Feedback of the intensity and duration of eccentric exercise

was provided automatically by the dynamometer. During the exercise session, the subjects were verbally encouraged to maximally activate their knee extensors, even though, because of fatigue, the performance was declined as the exercise progressed. Before the exercise session, subjects performed an 8 min warm-up consisting of cycling on a Monark cycle ergometer (Vansbro, Sweden) at 70 rpm and 50 W.

2.3. Assessment of Muscle Pain. To confirm the presence of delayed onset muscle soreness (DOMS) immediately after exercise and 24, 48, and 72 h after exercise, participants verbally rated on a scale from 0 ("no soreness") to 10 ("worst soreness imaginable") their perceived pain during walking (DOMSw) and making the squat movement (DOMSsq).

2.4. Muscle Damage. The isokinetic dynamometer was used for the measurement of isometric knee extensor peak torque at 90° knee flexion. The average of the 3 maximal voluntary contractions with the preferred leg was recorded. To ensure that the subjects provided their maximal effort, the measurements were repeated if the difference between the lower and the higher torque values exceeded 10%. There was a 2 min rest between isometric efforts.

2.5. Blood Collection and Handling. All participants performed an acute isokinetic eccentric exercise bout with the knee extensors of their preferred leg. Blood samples (10 mL) were drawn from a forearm vein with subjects in a seated position before, immediately after exercise, and 24 h, 48 h, and 72 h after exercise. Blood was collected in ethylenediamine tetraacetic acid (EDTA) tubes for measuring TAC, TBARS, CARB, and GSH levels and catalase activity. Blood was also collected in heparin tubes for measuring ORP. Blood samples were centrifuged immediately at 1370 g for 10 min at 4°C and the plasma was collected and used for the above measurements. The packed erythrocytes were lysed with distilled water (1 : 1 v/v), inverted vigorously, and centrifuged at 4020 g for 15 min at 4°C and the erythrocyte lysate was collected for measurement of catalase activity. A portion of erythrocyte lysate (500 μL) was treated with 5% trichloroacetic acid (TCA) (1 : 1 v/v), vortexed vigorously, and centrifuged at 28,000 g for 5 min at 4°C. The supernatants were removed, treated again with 5% TCA (1.3 : 1 v/v), and centrifuged again at 28,000 g for 5 min at 4°C. The clear supernatants were transferred to eppendorf tubes and were used for the determination of GSH. Plasma and erythrocyte lysate were stored at 80°C prior to biochemical analyses.

2.6. Assessment of sORP and cORP Using the RedoxSYS Diagnostic System. sORP and cORP values were determined using the RedoxSYS Diagnostic System (Luoxis Diagnostics, Inc., Englewood, CO, USA). In particular, 20 μL of plasma was applied to disposable sensors, which were inserted into the RedoxSYS Diagnostic System that measured and reported within four minutes the sORP and cORP values. sORP captures the integrated balance of oxidants and reductants in a specimen and is reported in millivolts (mV). cORP is the amount of antioxidant reserves and is expressed in microcoulombs (μC).

2.7. Assessment of TAC, TBARS, GSH, Catalase Activity, and CARB. For TBARS determination, a slightly modified assay of Keles et al. [20] was used. According to this method, 100 μL of plasma was mixed with 500 μL of 35% TCA and 500 μL of tris(hydroxymethyl)aminomethane hydrochloride (Tris–HCl) (200 mM, pH 7.4) and incubated for 10 min at room temperature. One milliliter of 2 M Na_2SO_4 and 55 mM thiobarbituric acid solution was added and the samples were incubated at 95°C for 45 min. The samples were cooled on ice for 5 min and were vortexed after adding 1 mL of 70% TCA. The samples were centrifuged at 15,000 g for 3 min and the absorbance of the supernatant was read at 530 nm. A baseline absorbance was taken into account by running a blank along with all samples during the measurement. Calculation of TBARS concentration was based on the molar extinction coefficient of MDA. The intra- and interassay coefficients of variation (CV) for TBARS were 3.9% and 5.9%, respectively.

Protein carbonyls were determined based on the method of Patsoukis et al. [21]. In this assay, 50 μL of 20% TCA was added to 50 μL of plasma and this mixture was incubated in an ice bath for 15 min and centrifuged at 15,000 g for 5 min at 4°C. The supernatant was discarded and 500 μL of 10 mM 2,4-dinitrophenylhydrazine (DNPH) (in 2.5 N hydrochloride (HCl)) for the sample, or 500 μL of 2.5 N HCl for the blank, was added to the pellet. The samples were incubated in the dark at room temperature for 1 h, with intermittent vortexing every 15 min and were centrifuged at 15,000 g for 5 min at 4°C. The supernatant was discarded and 1 mL of 10% TCA was added, vortexed, and centrifuged at 15,000 g for 5 min at 4°C. The supernatant was discarded and 1 mL of ethanol-ethyl acetate (1 : 1 v/v) was added, vortexed, and centrifuged at 15,000 g for 5 min at 4°C. This washing step was repeated twice. The supernatant was discarded and 1 mL of 5 M urea (pH 2.3) was added, vortexed, and incubated at 37°C for 15 min. The samples were centrifuged at 15,000 g for 3 min at 4°C and the absorbance was read at 375 nm. Calculation of protein carbonyl concentration was based on the molar extinction coefficient of DNPH. The intra- and interassay CV for protein carbonyls were 4.3% and 7.0%, respectively. Total plasma protein was assayed using a Bradford reagent from Sigma-Aldrich.

GSH was measured according to Reddy et al. [22]. Twenty microliters of erythrocyte lysate treated with 5% TCA was mixed with 660 μL of 67 mM sodium potassium phosphate (pH 8) and 330 μL of 1 mM 5,5′-dithiobis-2 nitrobenzoate (DTNB). The samples were incubated in the dark at room temperature for 45 min and the absorbance was read at 412 nm. GSH concentration was calculated relative to a calibration curve made using commercial standards. The intra- and interassay CV for GSH were 3.1% and 4.5%, respectively.

Catalase activity was determined using the method of Aebi [23]. Briefly, 4 μL of erythrocyte lysate (diluted 1 : 10) was added to 2991 μL of 67 mM sodium potassium phosphate (pH 7.4) and the samples were incubated at 37°C for 10 min. Five microliters of 30% hydrogen peroxide (H_2O_2) was added to the samples and the change in absorbance was immediately

Table 1: Delayed onset muscle soreness (DOMS) responses following the exercise session.

	Before	After	After 24 h	After 48 h	After 72 h
DOMSw	1.00 ± 0.00	2.63 ± 1.16	3.57 ± 1.16	4.63 ± 1.01	4.63 ± 1.34
DOMSsq	1.00 ± 0.00	3.21 ± 1.27	4.10 ± 1.10	5.00 ± 1.05	4.89 ± 1.10

[a]Values are the mean ± SD; DOMSw: DOMS assessed during walking; DOMSsq: DOMS assessed after performing a squat movement.

read at 240 nm for 130 s. Calculation of catalase activity was based on the molar extinction coefficient of H_2O_2. The intra- and interassay CV for catalase were 6.2% and 10.0%, respectively.

The determination of TAC was based on the method of Janaszewska and Bartosz [24]. In particular, 20 μL of plasma was added to 480 μL of 10 mM sodium potassium phosphate (pH 7.4) and 500 μL of 0.1 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical and the samples were incubated in the dark for 30 min at room temperature. The samples were centrifuged for 3 min at 20,000 g and the absorbance was read at 520 nm. The intra- and interassay CV for TAC were 2.9% and 5.4%, respectively. TAC is presented as mmol of DPPH reduced to 2,2-diphenyl-1-picrylhydrazine (DPPH:H) by the antioxidants of plasma.

2.8. Statistical Analysis. For statistical analysis, data were analyzed by one-way ANOVA followed by Dunnett's test for multiple pairwise comparisons. Moreover, two different statistical comparisons were used for the analysis of the results from the oxidative stress markers before and after eccentric exercise. In the first statistical comparison, all the participants were examined as one group before and after exercise. In the second statistical comparison, the participants were divided into two groups, those with high increase and those with large decrease in cORP after exercise compared to before exercise. Then, pre- and postexercise comparison for all tested markers was made separately for each of these groups. Correlation between DOMS and oxidative stress markers was examined by Spearman's correlation analysis. The level of statistical significance was set at $P < 0.05$. For all statistical analyses SPSS, version 13.0 (SPSS Inc., Chicago, Ill) was used. Data are presented as mean ± SEM.

3. Results and Discussion

3.1. Assessment of Muscle Pain and Muscle Damage. Eccentric exercise increased DOMS from 2.6- to 4.6-fold during walking and from 3.2- to 5-fold when making squat movement (Table 1). The increase in DOMS after eccentric exercise is not completely understood but has been suggested to be caused by various biochemical changes after muscle damage rather than a single event of damage [25]. Thus, the main cause of DOMS is structural muscle damages, fundamentally ruptures within the muscle [25]. This muscle damage induces inflammatory response (e.g., chemokine release, activation of inflammatory cells, increase in prostaglandins, and production of arachidonic acid). These inflammatory compounds interact directly with afferent nerves through pain receptors. When the stimuli from the afferent nerves reach medulla and cerebral cortex, muscle soreness is perceived [25]. Other factors suggested to be involved in the physiological mechanism-induced DOMS are lactic acid and nitric oxides [25]. Moreover, although it is clear that RS are produced after eccentric exercise, it is not clear if there is a direct relationship between them and DOMS [26]. It has been proposed that eccentric exercise-induced inflammation may be the main cause of RS production. Specifically, inflammation causes phagocytic cells to migrate to the damaged tissue and exhibit a respiratory burst resulting in the production of RS such as superoxide and hydrogen peroxide [26]. These RS may cause further damage by killing muscle cells [26]. In the present study, the correlation analysis showed that there was a moderate significant correlation (correlation coefficient $r = 0.648$; $P < 0.01$) between DOMSw and sORP at 48 h after exercise and an inverse correlation between DOMSw and cORP at 48 h ($r = -0.640$; $P < 0.001$) and 72 h ($r = -0.601$; $P < 0.001$) after exercise. Moreover, there was a significant moderate inverse correlation ($r = -0.588$; $P < 0.001$) between DOMSw and catalase activity at 72 h after exercise. The positive correlation between DOMSw and sORP and the inverse correlation between DOMSw and cORP or catalase activity suggest that there was an association of DOMS with oxidative stress. However, this association does not mean necessarily that oxidative stress was an etiologic factor of DOMS. To find out the possible causal relationship between DOMS and RS more experiments are needed in which RS would be inhibited by antioxidant supplementation [26].

Moreover, muscle damage induced by eccentric exercise results in an immediate and prolonged reduction in muscle function, most notably a reduction in force-generating capacity [27]. Thus, isometric torque declined significantly ($P < 0.05$) by 15.8% at the end of the exercise session (before: 243.9 ± 55.4 Nm; after: 205.20 ± 57.54 Nm).

3.2. Oxidative Stress Markers. None of the tested redox markers changed statistically significant postexercise compared to preexercise when all the participants were examined as one group (Table 2). This lack of significance regarding the changes of the oxidative stress markers after exercise can be explained by the great variation that each of them presents between different individuals (Table 2). Moreover, the redox markers in many of the participants in the study changed unexpectedly. For example, in several individuals, TBARS and CARB levels were decreased, while GSH levels were increased after exercise (Figure 1), while according to previous studies the opposite effect was expected [12, 13, 15, 17]. Thus, in all redox markers the participants could be divided into two groups, those with high increase and those with large decrease in the values of the markers after exercise compared to before exercise (Figures 1 and 2). Similar great variation

Table 2: Initial, 24 h, 48 h, and 72 h postexercise values of biomarkers when all participants were examined as one group ($n = 19$) (mean ± SD).

	Before	After 24 h	After 48 h	After 72 h	24 h % change	48 h % change	72 h % change
sORP (mV)	136.1 ± 13.2 (118.9–157.3)	135.5 ± 13.7 (156.4–116)	135.1 ± 16.2 (162.7–112.4)	134.8 ± 16.3 (159.4–104.2)	−0.45 ± 3.76 (−7.54–6.93)	−0.81 ± 5.57 (−12.60–7.45)	−0.97 ± 7.30 (−18.74–9.47)
cORP (μC)	1.05 ± 0.71 (0.64–2.63)	1.08 ± 0.93 (0.34–4.07)	1.17 ± 0.89 (0.31–3.85)	1.19 ± 1.09 (0.32–4.75)	3.37 ± 35.27 (−47.53–87.56)	17.71 ± 52.62 (−38.40–150.00)	15.42 ± 52.16 (−56.27–118.89)
TAC (mmol DPPH/L plasma)	0.94 ± 0.09 (0.70–1.03)	0.99 ± 0.12 (0.70–1.22)	1.00 ± 0.16 (0.70–1.35)	1.04 ± 0.12 (0.89–1.35)	6.05 ± 14.20 (−19.67–45.09)	6.72 ± 15.82 (−11.79–41.83)	12.18 ± 17.30 (−7.15–45.61)
GSH (μmol/g Hb)	3.09 ± 1.75 (0.42–5.60)	3.28 ± 1.70 (0.34–6.20)	3.04 ± 1.60 (0.55–6.00)	2.88 ± 1.51 (0.73–6.10)	15.26 ± 39.99 (−25.78–126.94)	10.54 ± 44.81 (−34.21–150.96)	8.03 ± 48.54 (−32.73–133.83)
Protein carbonyls (nmol/mg protein)	0.70 ± 0.18 (0.29–0.95)	0.74 ± 0.21 (0.38–1.07)	0.75 ± 0.19 (0.43–1.11)	0.76 ± 0.24 (0.43–1.48)	8.21 ± 23.82 (−36.96–57.78)	9.94 ± 26.39 (−35.92–63.16)	12.43 ± 36.8 (−25.17–128.00)
TBARS (μmol/L)	6.7 ± 2.8 (3.4–13.0)	7.5 ± 2.8 (4.0–12.4)	7.4 ± 3.0 (3.1–13.0)	7.9 ± 2.9 (2.9–13.5)	16.95 ± 43.06 (−32.75–178.36)	17.09 ± 37.38 (−72.51–129.85)	27.08 ± 43.46 (−74.40–138.84)
Catalase (U/mg Hb)	151.9 ± 47.6 (92.8–310.4)	154.6 ± 27.0 (114.2–207.2)	142.3 ± 19.3 (105.9–169.9)	137.9 ± 18.6 (108.9–182.4)	9.98 ± 20.06 (−33.24–48.67)	−0.38 ± 26.10 (−45.25–56.04)	−2.18 ± 30.96 (−53.31–84.96)

The numbers in brackets show the minimum and maximum values.

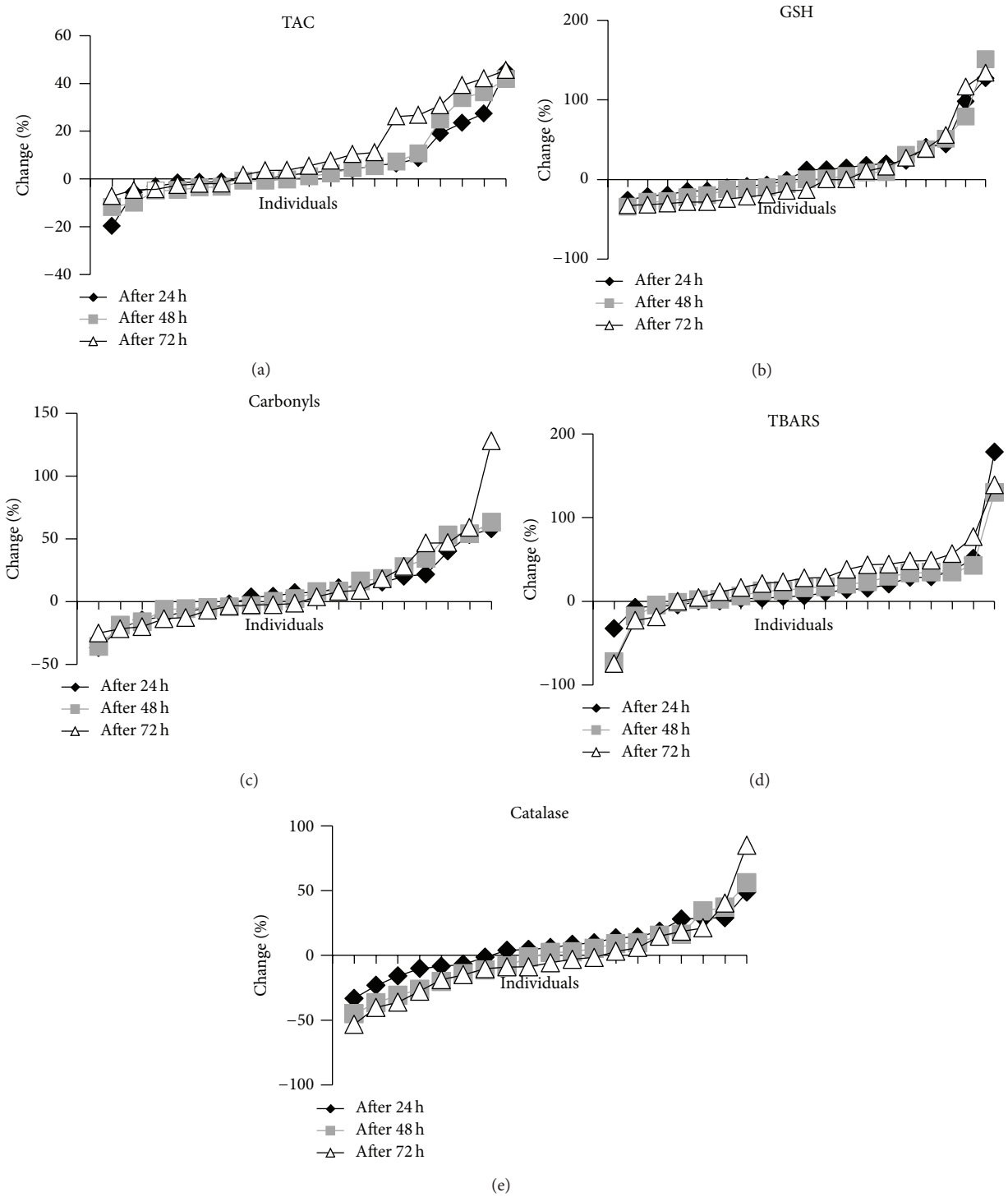

Figure 1: Percent change in redox biomarker levels of each individual at 24 h, 48 h, and 72 h after eccentric exercise. (a) TAC: total antioxidant capacity (in plasma); (b) GSH: reduced glutathione (in erythrocytes); (c) CARB: protein carbonyl levels (in plasma); (d) TBARS: thiobarbituric acid-reactive substances (in plasma); (e) CAT: catalase activity (in erythrocytes).

and unexpected findings have also been exhibited in other studies on eccentric exercise [12]. Specifically, Margaritelis et al. [12] have reported that eccentric exercise can induce even reductive stress or negligible stress in a considerable number of people. This great variation in the response of different individuals to eccentric exercise-induced oxidative stress may be attributed to the high complexity of the regulation of redox homeostasis in humans. That is, many different factors such as genetic, physiological, biochemical, and dietary factors can affect the final outcome of oxidant stimuli [28–31].

Like all markers, cORP responded differently to eccentric exercise between different individuals. Namely, 42.2% of the

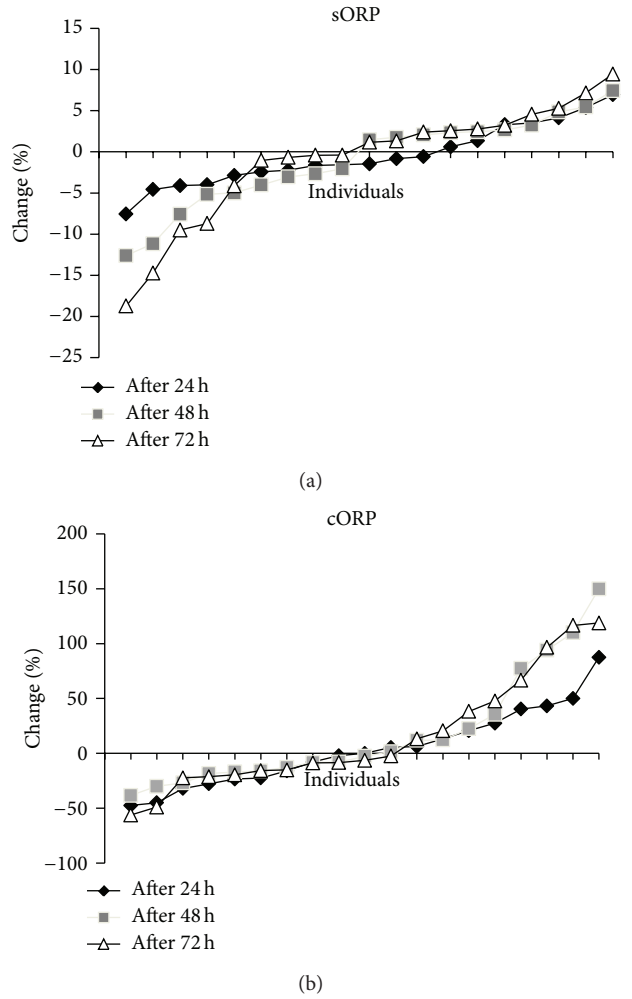

Figure 2: Percent change in ORP marker levels in plasma blood of each individual at 24 h, 48 h, and 72 h after eccentric exercise. (a) sORP: static oxidation-reduction potential; (b) cORP: capacity oxidation-reduction potential.

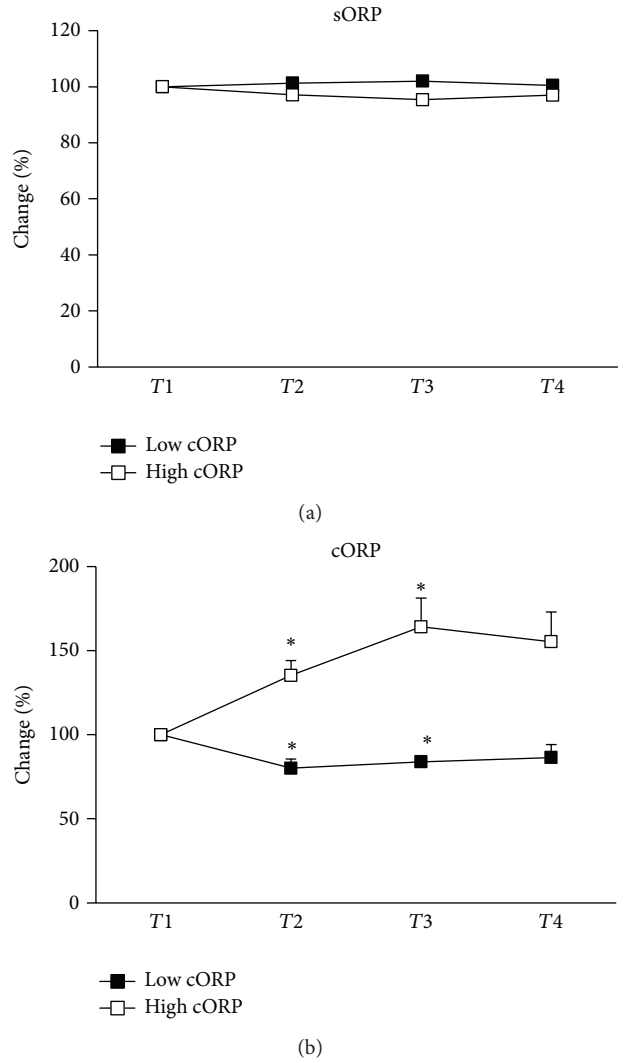

Figure 3: Percentage changes of ORP values of individuals of low cORP and high cORP groups at before exercise ($T1$) and 24 h ($T2$), 48 h ($T3$), and 72 h ($T4$) after eccentric exercise. (a) sORP: static oxidation-reduction potential; (b) cORP: capacity oxidation-reduction potential. *Significantly different compared to preeccentric exercise ($P < 0.05$).

participants exhibited high increase in cORP, while the rest 57.8% of the individuals had large decrease in cORP after exercise (Figure 2). As mentioned cORP is an integrated measure of the antioxidant reserve available in the body's system. Thus, the previous observation about the differential response of cORP to eccentric exercise led us to the hypothesis that some of the participants could confront the eccentric exercise-induced oxidative stress by increasing their antioxidant reserves (i.e., increase in cORP). On the other hand, other participants were not able to cope with oxidative stress or to replace their antioxidant reserves after exercise (i.e., decrease in cORP). For testing this hypothesis, the participants were divided in two groups: (i) the first group had high increase in cORP (high cORP group; $n = 11$); (ii) the second group had high decrease in cORP (low cORP group; $n = 8$). In the high cORP group there was statistical significant increase in cORP values at 24 h and 48 h after exercise, while in the low cORP group there was statistical significant decrease in cORP values at 24 h and 48 h after exercise (Figure 3). Afterwards, in these two groups, the change of the other redox markers was examined separately. Although in all markers the participants could be divided in two groups, cORP was selected for this analysis because it is an integrated measure of the total antioxidant capacity of the organism, while most of the other tested markers assess either a specific antioxidant mechanism (e.g., GSH, catalase) or a specific oxidative damage (e.g., TBARS, CARB). TAC also indicates the total antioxidant capacity, but it is based on the reduction of a free radical (i.e., DPPH) by the antioxidant molecules in plasma. Thus, this method is imperfect, since it evaluates only the reductants found in plasma. However, cORP is based on the amount not only of the reductants but also of the oxidants in the plasma. For this reason, cORP measurement may be a more accurate method than TAC as a holistic approach for

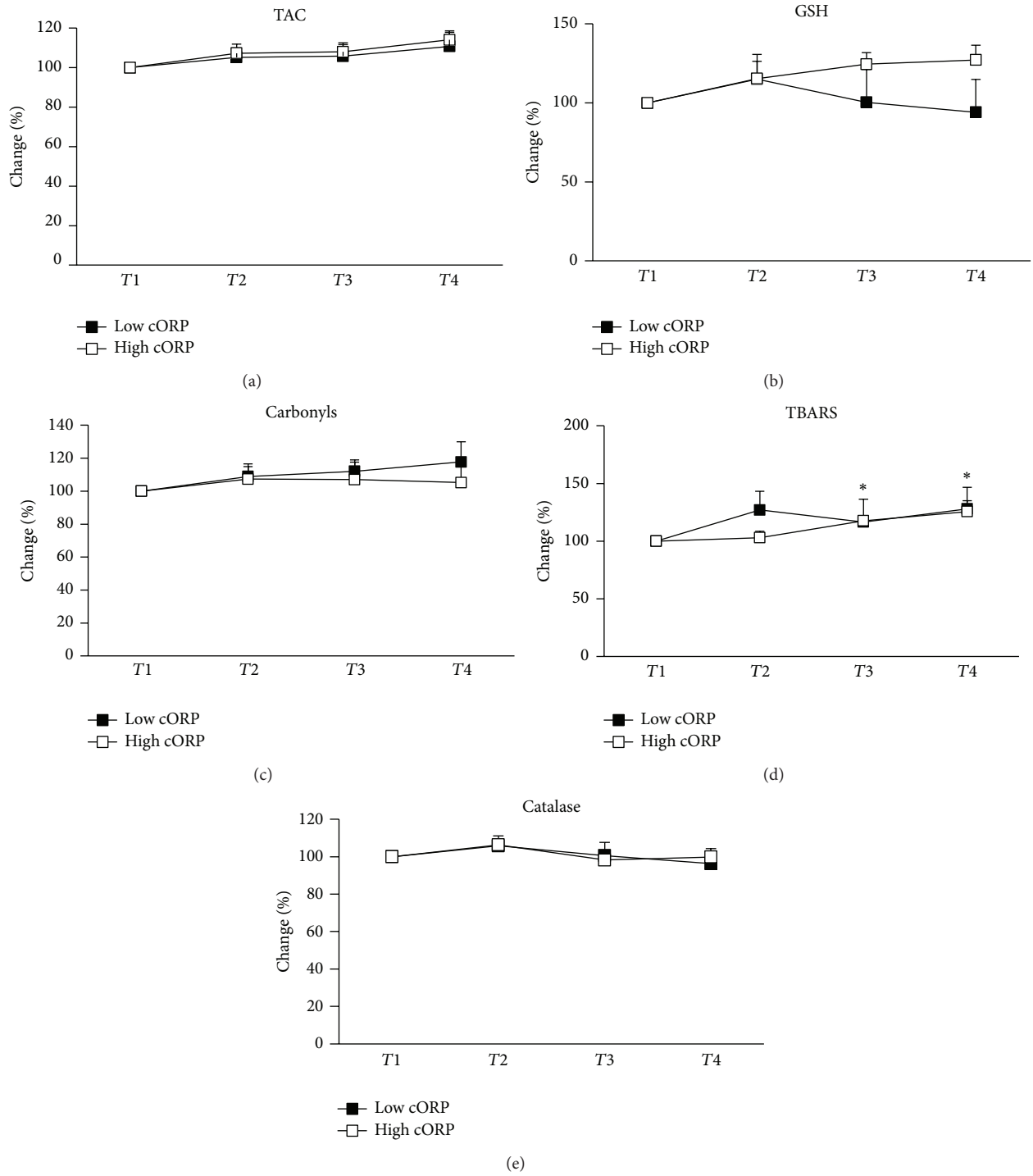

Figure 4: Percentage changes of the redox biomarkers in plasma and erythrocytes of individuals of low cORP and high cORP groups at preexercise ($T1$) and 24 h ($T2$), 48 h ($T3$), and 72 h ($T4$) posteccentric exercise: (a) TAC: total antioxidant capacity; (b) GSH: reduced glutathione; (c) CARB: protein carbonyl levels; (d) TBARS: thiobarbituric acid-reactive substances; (e) CAT: catalase activity. *Significantly different compared to preeccentric exercise in low cORP group ($P < 0.05$).

assessing *in vivo* oxidative stress. Finally, like cORP, sORP measures the overall current redox balance, but this marker in most individuals did not change significantly after exercise and exhibited much less variation than cORP (Figure 2; Table 2).

Thus, the analysis based on the separation of the participants in two groups according to the cORP changes after exercise showed that in the low cORP group there was a significant increase in TBARS levels at 48 h and 72 h after exercise indicating an increase in lipid peroxidation (Figure 4). In our

previous studies as well as in other studies increase in lipid peroxidation after eccentric exercise has also been reported [13, 15]. In the high cORP group, TBARS levels did not change significantly at any time-point after exercise suggesting an absence of lipid oxidation and maybe of oxidative stress. TAC did not change significantly in any of the two cORP groups. Actually, it would be expected for TAC to follow the decrease or increase of cORP, since both markers assess the total antioxidant capacity. However, the lack of consistency between them is probably, as mentioned above, due to the different methodologies used for their estimation.

In conclusion, in the present study, ORP markers were used for the first time for assessing eccentric exercise-induced oxidative stress. The results suggested that especially cORP marker may be used for predicting oxidative stress induced by eccentric exercise. That is, high decrease in cORP values after exercise compared to before exercise may indicate induction of oxidative stress. On the other hand, high increase in cORP values after exercise may indicate no existence of oxidative stress after eccentric exercise. These conclusions may help to identify eagerly individuals affected more by eccentric exercise-induced oxidative stress, since cORP measurement is easily and fast performed. This would allow appropriate interventions (e.g., antioxidant supplementation) to be applied for avoiding detrimental effects on health or shortening the recovery period in those individuals affected by oxidative stress. However, more studies, especially with larger samples, are needed in order to confirm these findings.

Conflict of Interests

The authors declare that there is a financial conflict of interests.

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Research Article

Exercise Training and Calorie Restriction Influence the Metabolic Parameters in Ovariectomized Female Rats

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The estrogen deficiency after menopause leads to overweight or obesity, and physical exercise is one of the important modulators of this body weight gain. Female Wistar rats underwent ovariectomy surgery (OVX) or sham operation (SO). OVX and SO groups were randomized into new groups based on the voluntary physical activity (with or without running) and the type of diet for 12 weeks. Rats were fed standard chow (CTRL), high triglyceride diet (HT), or restricted diet (CR). The metabolic syndrome was assessed by measuring the body weight gain, the glucose sensitivity, and the levels of insulin, triglyceride, leptin, and aspartate aminotransferase transaminase (AST) and alanine aminotransferase (ALT). The exercise training combined with the CR resulted in improvements in the glucose tolerance and the insulin sensitivity. Plasma TG, AST, and ALT levels were significantly higher in OVX rats fed with HT but these high values were suppressed by exercise and CR. Compared to SO animals, estrogen deprivation with HT caused a significant increase in leptin level. Our data provide evidence that CR combined with voluntary physical exercise can be a very effective strategy to prevent the development of a metabolic syndrome induced by high calorie diet.

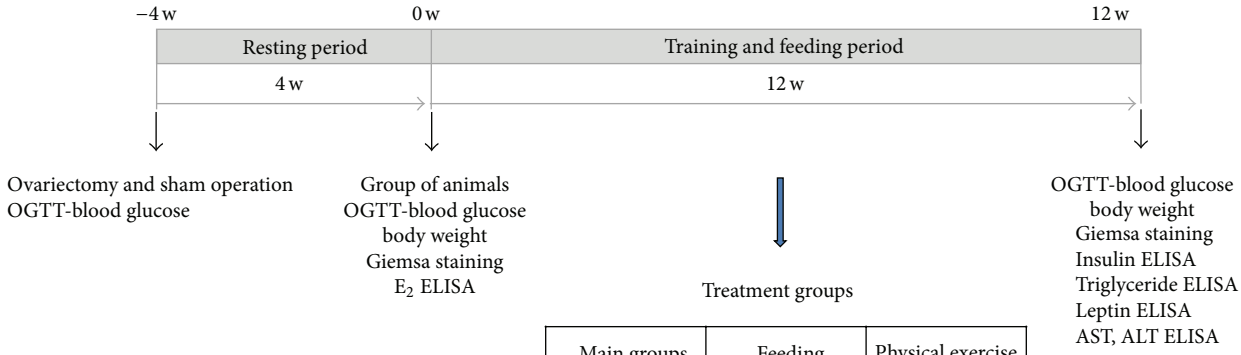
1. Introduction

The metabolic syndrome (MS), usually caused by high calorie diet and a lack of physical activity, covers a heterogeneous cluster of obesity-related diseases. This syndrome is common and its prevalence increases with the menopause and in an estrogen-deficiency state [1]. With the recent dramatic increase in life expectancy, many women are now spending a large part of their lives in a postmenopausal state, and an investigation of strategies to prevent or attenuate the deleterious effects associated with the ovarian hormone decrease is therefore necessary [2].

Ovariectomized (OVX) animals have been used as experimental models of obesity from a limited estrogen function. Estrogen depletion is associated with an increased visceral adipose tissue mass. The increased fat mass may be explained by changes in energy expenditure, because the menopausal transition leads to decreased energy expenditure and reduced fat oxidation [3]. Female aromatase knockout mice, which are unable to synthesize estrogen, display increased body weight and adipocyte hypertrophy, demonstrating the impact of an estrogen deficiency on fat accumulation [4].

Hormone replacement therapy (HRT) has been used to treat estrogen deficiency symptoms. However, there has subsequently been debate which concerns the negative overall risk-benefit ratio of HRT and alternative strategies for the treatment of menopausal and postmenopausal disorders [5]. Exercise training in animals decreases fat deposition, enhances insulin sensitivity, improves the glucose-stimulated insulin response, and increases the glucose transporter concentration [6]. However, voluntary physical exercise training combined with a normal or calorie-restricted or a high-fat phytoestrogen-free nutrition in OVX rats has not been investigated.

We set out to investigate the effects of exercise training combined with dietary restriction as a nonpharmacological intervention with the aim of controlling the effect of estrogen depletion, including obesity, hypertension, glucose tolerance, dyslipidemia, and systemic inflammation. The impact of OVX-induced obesity on metabolic profiles is a matter of



Treatment groups

Main groups	Feeding	Physical exercise
SO	CTRL	Nonrunning
		Running
	CR	Nonrunning
		Running
	HT	Nonrunning
		Running
OVX	CTRL	Nonrunning
		Running
	CR	Nonrunning
		Running
	HT	Nonrunning
		Running

Figure 1: Experimental design of the study. SO = sham-operated, OVX = ovariectomized, CTRL = standard chow, CR = calorie restriction, HT = high-triglyceride, w = weeks, E_2= estrogen, OGTT = oral glucose tolerance test, AST = aspartate aminotransferase transaminase, and ALT = alanine aminotransferase.

current interest. Thus, in the present study we tested whether 12 weeks of exercise training and/or calorie restriction (CR) would improve the metabolic parameters in OVX rats as compared with normal and high-fat diet-fed OVX rats, mimicking the food habits of humans living in western countries.

2. Methods

2.1. Animals and Experimental Design. This study was performed in accordance with the European Community guidelines on the care and use of laboratory animals and had been approved by the local Institutional Ethics Committee at the University of Szeged.

At 10 wk of age, female Wistar rats (Toxi-Coop Zrt., Hungary) were anesthetized and underwent ovariectomy surgery (OVX) or sham operation (SO). The OVX procedure was performed via a ventral abdominal midline small incision through which the ovaries were bilaterally clamped and removed. The uterine horns were tied and the uterus was left intact. After surgery, the animals were maintained under good conditions to allow them to recover. In the SO procedure, the ovaries were exteriorized to create similar stress, but were not clamped and removed. After a 4-week resting period to verify the OVX-induced menopause, the serum estrogen levels were checked via estrogen quantitative enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's directions (Quantikine rat Estrogen Elisa kit, R&D Systems Inc.). After the resting period, the OVX and SO groups were each subdivided into two new groups, based on the type of diet and recreational exercise. During a 12-week period (with or without training) the rats were fed standard chow (CTRL) subgroup, a high-triglyceride diet (HT) subgroup (60% standard chow with 40% fat), or 50% restricted food diet (CR, 50% of the daily standard chow consumption) subgroup. The daily food consumption was determined as the difference between the amount of chow supplied and the amount of chow remaining.

The running animals (R subgroup) were placed individually into cages fitted with a running-wheel and were allowed free access to the wheel for 24 h per day for 12 weeks. The exercising protocol, defined as a voluntary wheel-running model, was selected in an effort to isolate the effects of exercising from the additional stress associated with forced exercise protocols. Control (nonrunning) rats were placed in standard holding cages without a running-wheel for the same period. The experimental design of the study is presented in Figure 1.

Each rat was labeled and weighed weekly during the experimental period. Table 1 shows the changes in body weight during the 12-week training and feeding period.

Table 1: Body weights at the start and end of the training and feeding period.

Subgroup	Body weight (g) at the start of the training and feeding period (0 w)	Body weight (g) at the end of the training and feeding period (12 w)
SO CTRL	279 ± 5.5	354 ± 9.2
SO CTRL R	273 ± 9.9	331 ± 9.0
SO CR	283 ± 7.6	263 ± 5.3*
SO CR R	261 ± 6.1*#	234 ± 12.4*#
SO HT	282 ± 11.0	319 ± 11.3*
SO HT R	269 ± 6.1	325 ± 7.8*
OVX CTRL	300 ± 7.2*	349 ± 21.9
OVX CTRL R	321 ± 9.4*	378 ± 14.2
OVX CR	334 ± 10.1*	305 ± 9.3*
OVX CR R	312 ± 9.3*	253 ± 25.7*
OVX HT	309 ± 6.7*	377 ± 12.0
OVX HT R	326 ± 7.5*	393 ± 13.7*

Results are shown as means ± S.E.M.
Statistical significance: $^{*}P < 0.05$ relative to the SO CTRL group at the start and end of the training and feeding period, and $^{\#}P < 0.05$ is a significant difference between the running (R) and nonrunning groups.
SO: sham-operated, OVX: ovariectomized, CTRL: standard chow, CR: calorie restriction, HT: high-triglyceride, and R: running.

2.2. Oral Glucose Tolerance Test (OGTT). The blood glucose level was measured by means of the OGTT at the week-4, 0 and the end of the 12-week treatment period. The serum levels of insulin were measured by means of the OGTT at the end of the 12-week treatment period. After a 12 h fasting period tail blood was taken before the application of glucose by oral gavage (0.1 g/kg bw) and 30, 60, and 120 min afterwards. Blood samples were collected to determine insulin levels (measured by ELISA, Sunred Biological Technology Co., Shanghai). The blood glucose was analyzed via Accu Check Active strips. Only the 12-week data are presented.

2.3. Determination of Insulin, Triglyceride, Leptin, Aspartate Aminotransferase Transaminase (AST), and Alanine Aminotransferase (ALT). Blood samples were collected at the end of the 12-week treatment period. All samples were centrifuged and the serum was stored at −20°C until analysis. Serum levels of insulin and plasma levels of triglyceride, leptin, AST, and ALT were determined by ELISA according to the manufacturer's instructions (Sunred Biological Technology Co., Shanghai).

2.4. Statistical Analysis. The results reported in the table and figures are expressed as means ± S.E.M. Differences between groups were determined with Student's t-test and One Way ANOVA Analysis with Shapiro-Wilk normality test and Holm-Sidak post hoc method. P values less than 0.05 were considered significant.

3. Results

3.1. Body Weight. Table 1 lists the changes in body weight before and after the 12-week treatment period. As expected, at the start of the training and feeding period the OVX rats exhibited the highest body weight. After the 12-week treatment period, the weight of the OVX which was found to have rats further increased, whereas the CR alone (14%, $P < 0.05$) or in combination with physical exercise (28%, $P < 0.05$) resulted in a weight reduction.

3.2. Glucose Levels and Glucose Tolerance. Changes in glucose sensitivity, induced by OGTT after the 12-week treatment period, are presented at each time (0, 30, 60, and 120 min) in Figure 2(a). And the area under the curve for glucose in Figure 2(c) was determined. In both the SO and the OVX rats, wheel-running exercise resulted in an improvement in glucose tolerance. The data clearly demonstrate that voluntary exercise training associated with a CRled with the most effective improvement of the glucose sensitivity (in the SO rats at 60 min 28%, $P < 0.05$; in the OVX rats at 60 min 35%, $P < 0.05$).

3.3. Serum Insulin Levels. Serum insulin measurements at the end of the 12-week treatment period revealed an augmented insulin level in the OVX rats as compared with the SO CTRL subgroup. The HT diet alone significantly increased the insulin level in the SO group (67%, $P < 0.05$). The CR improved the insulin level in both the OVX and SO groups (59%, $P < 0.05$). The strongest reductions of the insulin level were observed in the SO and the OVX animals which participated in the CR and running. However, the 12 weeks of exercise caused a more significant reduction in 60 min (in the SO CR R rats 76%, $P < 0.05$; in the OVX CR R rats 40%, $P < 0.05$). Data are presented in Figure 2(b).

3.4. Plasma Triglyceride (TG) Levels. Figure 3 shows the plasma levels of TG measured by ELISA. The highest TG levels were observed in both the SO (42%, $P < 0.05$) and the OVX rats (45%, $P < 0.05$) fed with the HT diet and were significantly higher as compared with the SO CTRL subgroup. However, the combined effects of the 12-week exercise and the CR decreasing the TG levels only in the OVX animals (33%, $P < 0.05$) and the exercise training alone were also effective in the OVX HT subgroup (44%, $P < 0.05$).

3.5. Plasma Leptin Concentrations. The plasma leptin levels were measured by ELISA in each group. As compared with the SO animals, estrogen deprivation caused a significant increase in leptin level. The CR combined with 12 weeks of exercise decreased the leptin values similarly in both the SO and the OVX rats (28%, $P < 0.05$). The exercise training was effective only in the OVX HT subgroup (26%, $P < 0.05$). Data are presented in Figure 4.

3.6. Plasma AST and ALT Levels. The concentrations of AST and ALT showed that both were augmented in the OVX group HT diet which caused increases in the enzyme levels

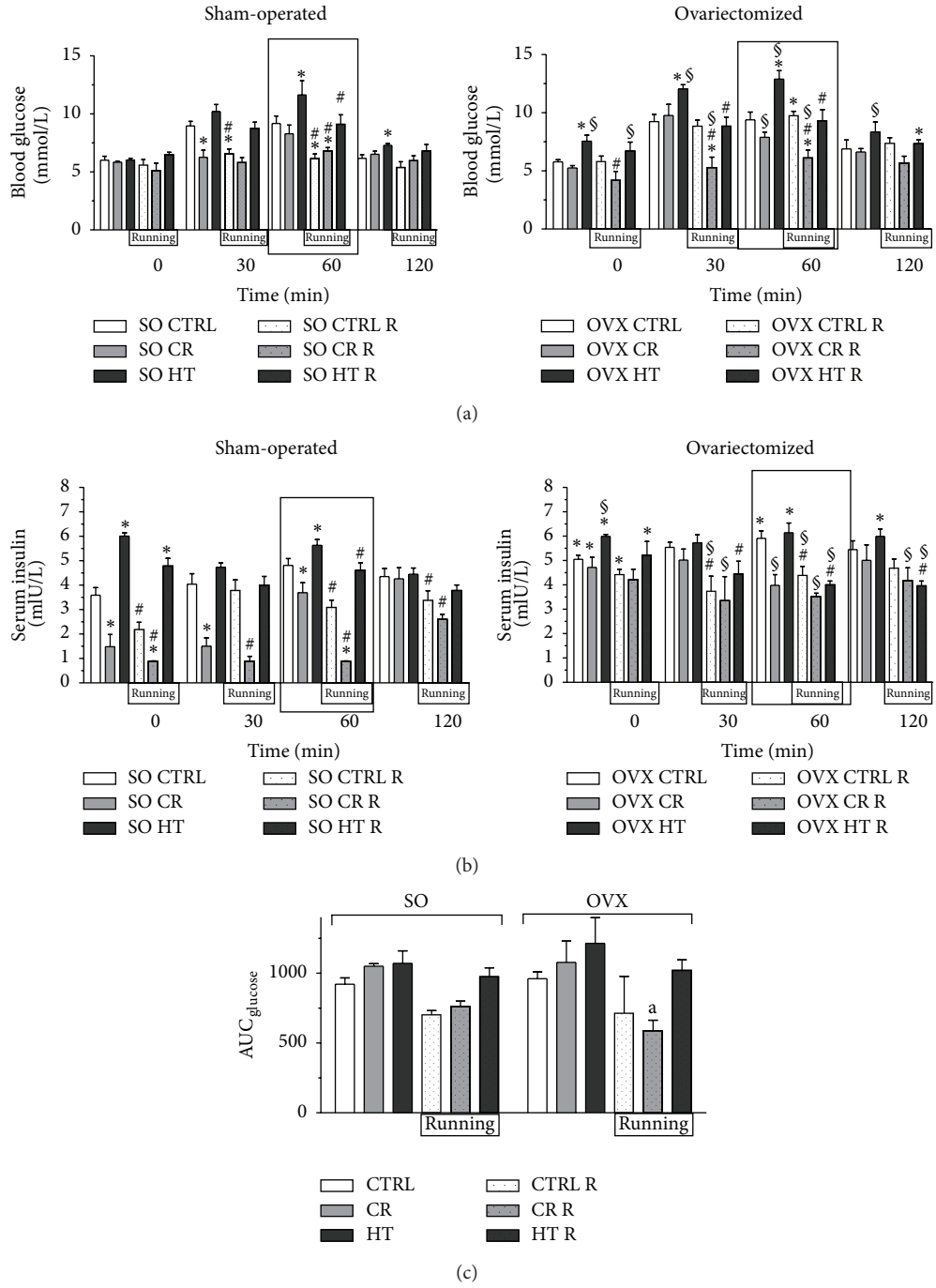


Figure 2: (a): Effects of 12-week wheel-running exercise and nutrition on the level of blood glucose (expressed in mmol/L) before (0 min) and after the oral glucose tolerance test (OGTT). Data are shown as means ± S.E.M. $n = 10$–12. Statistical significance: $^{*}P < 0.05$ relative to the SO CTRL group at 0, 30, 60, and 120 min, and $^{\#}P < 0.05$ is a significant difference between the running (R) and nonrunning groups at 0, 30, 60, and 120 min, $^{\S}P < 0.05$ relative to the OVX CTRL group at 0, 30, 60, and 120 min. SO = sham-operated, OVX = ovariectomized, CTRL = standard chow, CR = calorie restriction, HT = high-triglyceride. The most significance differences are highlighted in box. (b): Effects of 12-week wheel-running exercise and nutrition on the serum levels of insulin (expressed in mIU/L) before (0 min) and after the oral glucose tolerance test (OGTT). Means ± S.E.M. $n = 10$. Statistical significance: $^{*}P < 0.05$ relative to the SO CTRL group at 0, 30, 60, and 120 min, and $^{\#}P < 0.05$ is a significant difference between the running (R) and nonrunning groups at 0, 30, 60, and 120 min, $^{\S}P < 0.05$ relative to the OVX CTRL group at 0, 30, 60, and 120 min. The most significance differences are highlighted in box. SO = sham-operated, OVX = ovariectomized, CTRL = standard chow, CR = calorie restriction, HT = high-triglyceride. (c): The effects of 12-week wheel-running exercise and nutrition on the areas under the curve (AUC) for glucose after the OGTT. Data are shown as means ± S.E.M. $n = 5$. Statistical significance: $^{a}P < 0.05$ relative to the OVX HT group. SO = sham-operated, OVX = ovariectomized, CTRL = standard chow, CR = calorie restriction, HT = high-triglyceride, and AUC = area under curve.

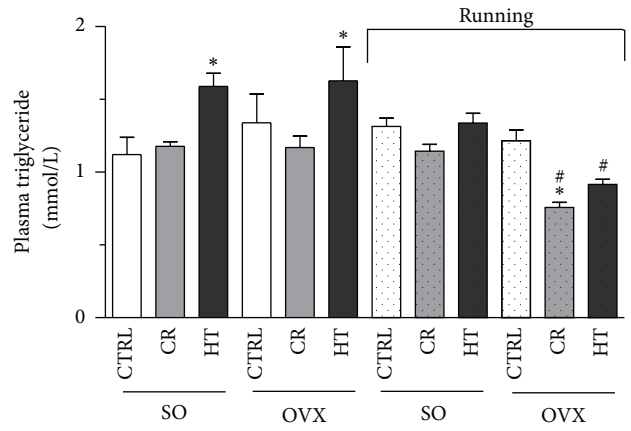


Figure 3: Effects of 12-week wheel-running exercise and nutrition on the plasma levels of triglyceride (expressed in mmol/L). Results are shown as means ± S.E.M. $n = 12$. Statistical significance: $^{*}P < 0.05$ relative to the SO CTRL group, and $^{\#}P < 0.05$ is the significant difference between the running (R) and nonrunning groups. SO = sham-operated, OVX = ovariectomized, CTRL = standard chow, CR = calorie restriction, and HT = high-triglyceride.

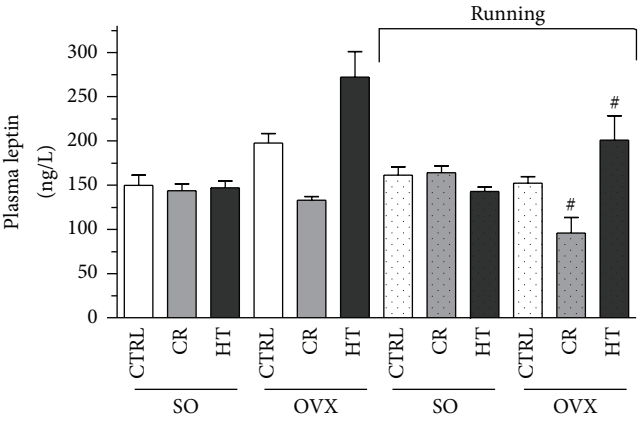


Figure 4: Effects of 12-week wheel-running exercise and nutrition on the plasma levels of leptin (expressed in ng/L). Means ± S.E.M. $n = 10$. Statistical significance: $^{*}P < 0.05$ relative to the SO CTRL group, and $^{\#}P < 0.05$ is the significant difference between the running (R) and nonrunning groups. SO = sham-operated, OVX = ovariectomized, CTRL = standard chow, CR = caloric restriction, and HT = high-triglyceride.

(AST: 41%, $P < 0.05$; ALT: 23%, $P < 0.05$). It emerged that 12 weeks of voluntary exercise or CR were effective, but the most marked reduction was observed from the combination of exercise and CR. Data are shown in Figure 5.

4. Discussion

Epidemiological, clinical, and molecular studies have shown that estrogen and estrogen receptors play an important role in metabolic homeostasis [7] through influencing fat metabolism, regulating the activity of molecules involved in orexigenic action, and regulating the neuronal activity of energy homeostasis [8], and the loss of estrogen may have profound effects on the glucose homeostasis and the body composition both in menopause women [9] and in rodents [4, 10].

The estrogen deficiency after menopause leads to becoming overweight or obesity, and physical exercise is one of the important modulators of this body weight gain [11]. The chronic consumption of a HT diet in rats induced MS, as evidenced by visceral obesity, hyperglycemia, dyslipidemia, an endothelial dysfunction, and hypertension [12]. In the present study, we set out to investigate the combined effects of physical activity and nutrition on estrogen-depleted OVX rats. We observed that the combination of the dietary control with exercise training was more effective in reducing the body weight, improving the leptin regulatory processes, and restoring the insulin, glucose, triglyceride, AST, and ALT levels in the plasma.

It is widely accepted that the prevalent lifestyle model of western societies, characterized by limited physical activity, an excessive calorie intake, and repetitive behavioral patterns contributes to dysregulation of the otherwise homeostatic control of body weight. In addition to physical activity, the reduction in energy intake may play a key factor in the modulation of life span. Extension of maximal life span is currently possible in animal models with CR. CR appears to prolong life by reducing reactive oxygen species (ROS)-mediated oxidative damage. Cornelius et al. discuss the role of CR on longevity processes by activating vitagenes which are involved in preserving cellular homeostasis during stressful conditions. They illustrate a complex network in which CR and hormetic CR-mimetics compounds by activating vitagenes can enhance defensive systems involved in bioenergetics and stress resistance homeostasis. Beside the anatomical and Mendelian paradigms, this approach may help to facilitate healthy lifestyle [13, 14].

Our experimental findings agree with those from other investigations in that the combination of exercise and diet modification has good effects on the metabolic parameters [15, 16]. As the augmented metabolic parameters correlated positively with cardiovascular disease-induced metabolic disorders [15, 17–21], our experimental findings are strongly supportive of the benefit of exercise as a means of reducing the metabolic parameters and hence the cardiovascular risk.

An improved insulin sensitivity is a hallmark outcome of exercise training: importantly, endurance training can restore the insulin response in obese, insulin-resistant rodents, and humans [22, 23]. Insulin resistance is a common condition in obesity, type 2 diabetes, dyslipidemia, and hypertension and there is experimental evidence that insulin resistance and hyperinsulinemia precede the development of obesity and other MS factors [24]. Exercise training may be one of the preventive and therapeutic strategies against impaired leptin and insulin signal transduction in the hypothalamus of obese individuals and associated with the markedly increased phosphorylation or activity of various proteins involved in leptin and insulin signal transduction [25, 26]. Riant et al. observed impaired glucose tolerance and insulin resistance in OVX mice fed with high fat diet [27]. We have shown that rats fed with a HT diet display hyperinsulinemia and hyperglycemia,

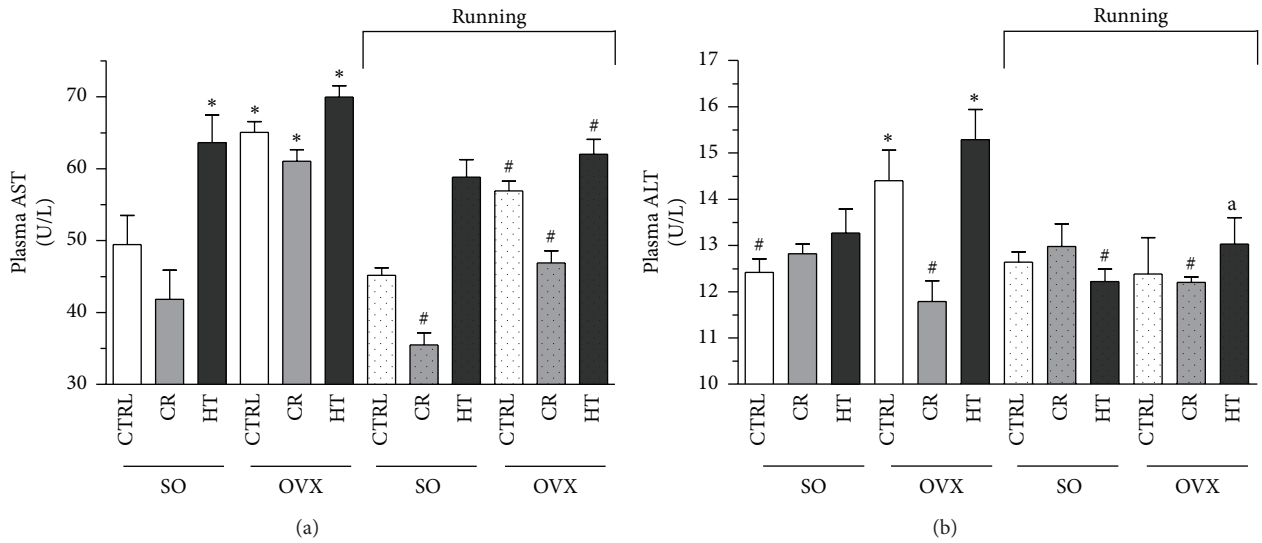


Figure 5: (a): Effects of 12-week wheel-running exercise and nutrition on the plasma levels of AST (expressed in U/L). Values are means ± S.E.M. Statistical significance: $^{*}P < 0.05$ relative to the SO CTRL group, and $^{\#}P < 0.05$ is the significant difference between the running (R) and nonrunning groups. SO = sham-operated, OVX = ovariectomized, CTRL = standard chow, CR = calorie restriction, HT = high-triglyceride. (b): Effects of 12-week wheel-running exercise and nutrition on the plasma levels of ALT (expressed in U/L). Means ± S.E.M. Statistical significance: $^{*}P < 0.05$ relative to the SO CTRL group, and $^{\#}P < 0.05$ is the significant difference between the running (R) and nonrunning groups. SO = sham-operated, OVX = ovariectomized, CTRL = standard chow, CR = calorie restriction, and HT = high-triglyceride.

which can be improved by physical exercise combined with dietary restriction.

The HT diet seems to influence the glucose metabolism in the exposed animals. The serum levels of insulin underwent significant changes in the OVX rats as compared with the SO rats, which could be taken as an indication that they were progressively developing an insulin-resistant condition.

It is well established that an increase in plasma triglyceride level elevates the risk of the development of atherosclerosis and dramatic clinical events such as acute myocardial infarction and stroke. Furthermore, lowering the plasma triglyceride content can reverse the progression of atherosclerosis and prevent cardiovascular events [28]. As an essential component of the metabolic syndrome, the blood triglyceride level was also investigated in this study. Plasma triglyceride levels were significantly higher in ovariectomized rats fed with HT but these high values were suppressed by exercise training. The exercise training associated with CR, especially, was the most effective therapy in OVX rats. The beneficial effects of estrogens on the blood lipid profile have long been known [29]. Physical activity is likewise well known to act on the blood lipid profiles in a positive manner [30]. Our study revealed significant effects of their combination and we believe that the combination may similarly result in additive effects with respect to the prevention of arteriosclerosis. We earlier demonstrated significant differences in hemeoxygenase and nitrogen monoxide synthase activities and cardiovascular parameters between male and female rats and revealed the anti-inflammatory effects of voluntary exercise training; in this respect, related investigations on the cardiovascular tissue of these animals are ongoing [31, 32].

Leptin hormone, secreted in the periphery by fat cells, plays an important role in the endocrine system; it signals the status of the body's energy stores and downregulates feeding behavior, regulating the appetite and energy expenditure [33]. Leptin is required for energy stores to be sensed in the central nervous system and is therefore essential for functions such as the normal energy homeostasis and reproduction [34]. Leptin is an adipocytokine that is mainly expressed in adipose tissue [34]. It is able to resist insulin secretion and exhibits a positive correlation with the body fat content [35].

Kang et al. reported that the plasma leptin level was significantly increased in a high-fat diet group as compared to a high-fat diet combined with training group and suggested that the effect of leptin sensitivity in the periphery may primarily relate to combined dietary control and exercise training more than to dietary control alone [16]. Short-term and long-term calorie restriction without exercise training has been shown to reduce plasma leptin levels dramatically in obese humans and rats [36–38]. Components other than physical exercise can play a role in the regulation of feeding behavior and the related mechanisms in developing mice [39]. The reduction in fat deposition with training in OVX rats raises the question of whether the level of leptin is improved in these rats.

Nonalcoholic fatty liver disease (NAFLD) is emerging as an acknowledged component of the MS. Markers of this condition, such as elevations in the serum concentrations of AST and ALT, may be considered reliable predictors of the development of the MS [40]. OVX resulted in higher body weights and augmented the AST and ALT levels as compared with those of the SO rats. Cameron et al. demonstrated that endurance exercise normalized the plasma AST and ALT levels in a diet-induced MS rat model [12]. Other studies have revealed the presence of NAFLD in rats that consumed a high-fat diet (71% of the energy intake from fat) for 3 weeks.

Similarly, our data indicated high plasma levels of ALT and AST [41, 42]. Plasma AST and ALT levels were significantly higher in ovariectomized rats fed with HT but these high values were suppressed by exercise training. We found that both 12 weeks of exercise or CR alone was effective, but the most marked reduction was observed from the combination of exercise and CR.

After a 12-week HT diet, the OVX rats in the present study exhibited some symptoms of the MS. OVX nonrunning rats exhibited a significantly higher final body weight, and augmented AST, ALT, triglyceride, and leptin levels. These effects were prevented, in part, by exercise or a CR, but most effectively by a combination of the two. This indicates that the prevention of a weight gain may be a result not only of exercise training but also of metabolic changes under the control of estradiol. This indicates that exercise training has a strong influence in lowering the body fat accumulation following a decrease in estrogen levels.

In summary, the results of the present short-term investigations indicate that combined therapy involving a CR and exercise training can exert a positive influence on parameters related to the lipid metabolism in OVX rats. It is our conclusion that our data provide evidence that a CR combined with physical activity can be a very effective strategy for prevention of the development of an MS induced by high calorie diet.

Conflict of Interests

The authors declare that there is no conflict of interest in relation to this work.

Authors' Contribution

All authors participated in the design and interpretation of the study, in the analysis of the data, and in the drafting and review of the paper. Anikó Pósa and Renáta Szabó equally contributed to this work.

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