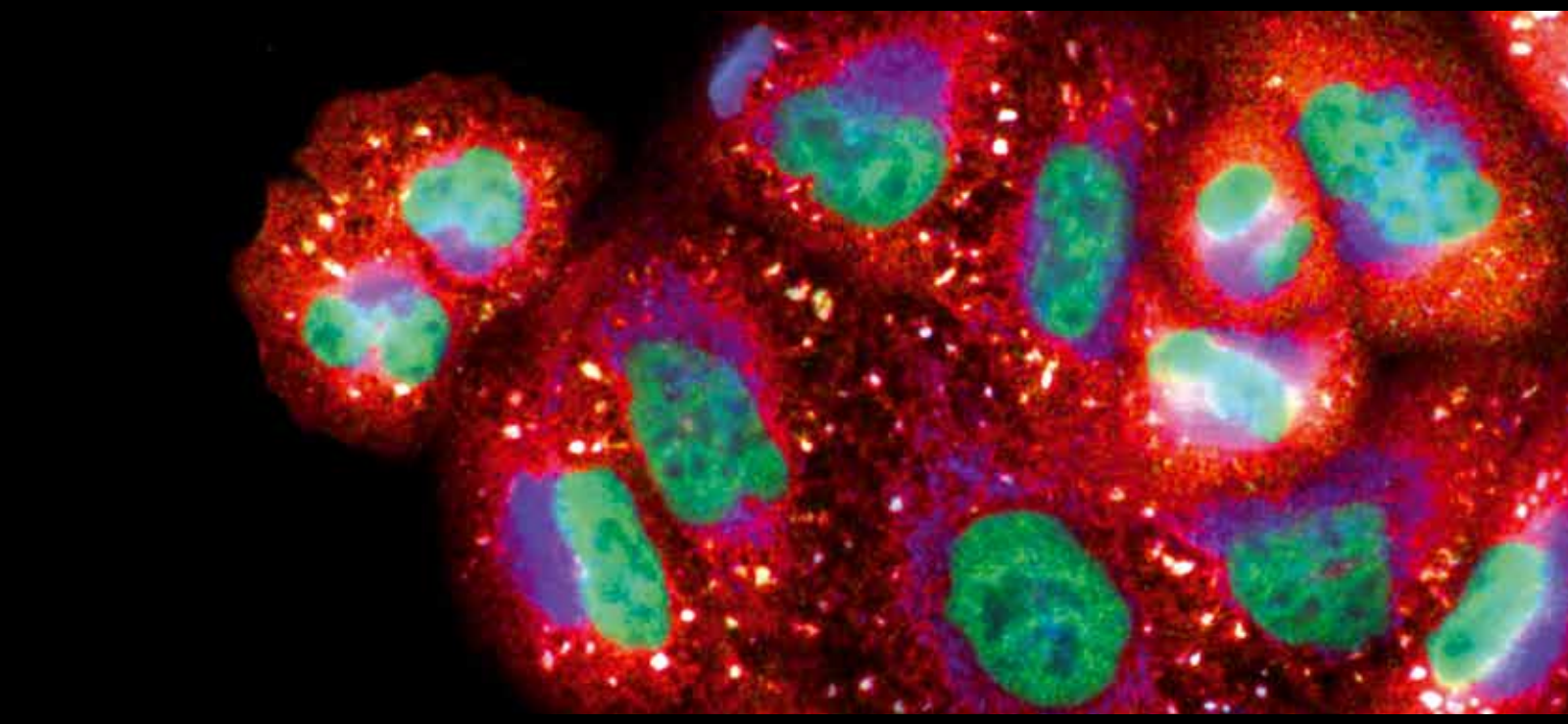
Oxidative Medicine and Cellular Longevity

Redox Biology of Exercise

Guest Editors: Michalis G. Nikolaidis, Chad M. Kerksick, Manfred Lamprecht, and Steven R. McAnulty



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Editorial Board

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2012, Article ID 407978, 3 pages
doi:10.1155/2012/407978

Editorial

Redox Biology of Exercise

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Received 26 August 2012; Accepted 26 August 2012

Redox biology is probably the most rapidly expanding field in biology. Indeed, the number of conferences, journals, and books devoted to redox biology is increasing and it is very often seen that major biology journals publish special issues on this area (e.g., [1–5]). This fact is probably due to the disclosure of the diverse roles reactive species have been found to serve, such as the control of the signaling of intracellular pathways [6], the mediation of enzyme activation [7], and the participation in antibiotic synthesis [8]. The significance of reactive species has been further underlined by the emerging links between cellular redox events and the etiology of many human diseases [9]. As a result of this progress in basic redox biology, the subfield of exercise redox biology has also markedly advanced.

Exercise is perhaps one of the most characteristic examples demonstrating that reactive species are not necessarily "harmful" entities, considering that the well-known benefits of regular exercise on muscle function and health are accompanied by repeated episodes of oxidative and nitrosative stress. In addition, an ongoing debate exists in the literature regarding the implications of antioxidant supplementation on physical performance and redox homeostasis. Considering that the redox biology of exercise is by nature multidisciplinary, this special issue is compiled of original and review articles combining chemical, analytical, biochemical, nutritional, physiological, and medical aspects relevant to reactive species biology. Reading through these papers the multiple facets of exercise redox biology are revealed.

The review article by E. C. Gomes et al. presents the current state of knowledge on the redox biology of exercise. It provides a comprehensive perspective on the contribution of various intracellular and extracellular sources and the identity of oxidants produced by exercising animals and humans. It also focuses on the possible role of these exercise-induced oxidants in important training adaptations such as angiogenesis, mitochondria biogenesis, and muscle hypertrophy. This article lays the groundwork for the other articles of the special issue that address oxidant effects on exercise performance and redox homeostasis and diseases. Specifically, H. Pan et al. indicated that electrical stimulation of skeletal muscle cells increased the production of reactive species as well as the mRNA and protein levels of interleukin-6. The authors hypothesized that reactive species generation induced by skeletal muscle contraction may be one of the factors regulating muscle-derived interleukin-6 production and release. Using a more physiological relevant methodology, S. Mrakic-Sposta et al. employed an electron paramagnetic resonance technique for the rapid and noninvasive measurement of reactive species concentration directly in fresh human peripheral blood. Using this innovative approach, they reported that short-term high-intensity exercise increased reactive species production whereas the resting levels of reactive species decreased following supplementation with the antioxidant cofactor α-lipoic acid.

Three papers investigated whether alterations in redox homeostasis can be monitored to assess the health and

fitness of the intensively training athlete. T. K. Tong et al. evaluated the impact of professional training on serum oxidant and antioxidant status in adolescent endurance runners and cyclists and compared it with that of untrained individuals. The authors reported that the resting blood redox homeostasis was well maintained in the adolescent athletes apparently due to the increase of antioxidants as a result of adaptations to chronic exercise. Similarly, C. A. Williams and A. O. Burk demonstrated that a three-day training event increased markers of antioxidant status in horses as a potential response to increased generation of reactive species during exercise. Finally, R. L. P. Ferraresso et al. using an innovative rat model showed that overtraining was associated with increased antioxidant enzyme activities and increased lipid peroxidation in blood and muscle. These data imply that monitoring of redox homeostasis in elite athletes may serve as a tool for overtraining diagnosis.

Six papers dealt with the effect of antioxidant supplementation on redox homeostasis and performance employing in vitro, in situ, in vivo, and even a combination of in vitro and in vivo approaches. R. J. Bloomer et al. reported that supplementation with coenzyme Q10 (an electron carrier in the electron transport chain) for four weeks affected neither exercise performance nor blood redox homeostasis in humans. On the other hand, A. E. Wagner et al. showed that combined supplementation of skeletal muscle cells with α-lipoic acid plus coenzyme Q10 improved energy homeostasis, stress response, and antioxidant defense mechanisms. Unless the additional supplementation of α-lipoic acid was responsible for these effects, the apparent contradiction between the two studies indicates that the potential antioxidant function of coenzyme Q10 in vivo cannot be safely extrapolated from in vitro tests. This may be due to the metabolic transformations and interactions that clearly affect the bioavailability and biological action of coenzyme Q10. To this end, A. S. Veskoukis et al. examined whether a polyphenol-rich grape pomace extract possesses in vitro antioxidant properties and whether the in vitro properties of the extract translate to an in vivo model when the extract was administered before exhaustive exercise to rats. The authors found that the polyphenol-rich extract possessed in vitro antioxidant activity which was not translated to in vivo antioxidant activity either at rest or after exercise (in fact, even some prooxidant effects were noted in vivo). In the light of these findings, it was suggested that the term "antioxidant" may be system related. Along the two poles of the in vitro-in vivo continuum, the study by A. Kyparos et al. employed an in situ model to investigate whether vitamin E can attenuate eccentric exercise-induced skeletal muscle injury. The authors found that vitamin E protected the soleus muscle from injury as indicated by the decreased fatigability at low-frequency stimulation and the almost complete recovery of single-twitch force immediately after fatigue. In an in vivo study in horses, E. D. Lamprecht and C. A. Williams reported that oral superoxide dismutase supplementation (encapsulated in a gliadin biopolymer to protect the enzyme against gastric proteolysis) did not affect the exercise-induced disturbances in redox homeostasis. Based on these studies, it is evident that antioxidant supplementation has discrepant effects on performance and redox homeostasis. This was also the major conclusion of the review article by M. G. Nikolaidis et al. regarding the effect of vitamin C and/or E supplementation on training and redox adaptations. Indeed, the relevant studies provided conflicting outcomes regarding the efficacy of vitamin C and E supplementation, mostly due to methodological differences in assessing redox status and training adaptations.

Lastly, two review articles analyzed the evidence of whether regular exercise can be used as a tool to combat two common and related lifestyle disease states: Type II Diabetes Mellitus and Metabolic Syndrome. Based on detailed analysis, E. T. de Lemos et al. supported that there are pathophysiological pathways that are associated with oxidative stress and inflammation in the development of Type II Diabetes Mellitus. The authors also asserted that regular exercise may act as a natural antioxidant and anti-inflammatory agent to prevent the serious complications of Type II Diabetes Mellitus. The Metabolic Syndrome is a clustering of obesity, diabetes, hyperlipidemia, and hypertension that affects roughly 20% of the population in Western industrialized countries. S. Golbidi et al. reviewed the relevant data and concluded that oxidative stress and the consequent inflammation induce insulin resistance (as supported by E. T. de Lemos et al. as well), which likely links the various components of the Metabolic Syndrome.

We hope that this compilation of research and review articles will stimulate further efforts to understand the biological importance and mechanisms of redox processes during exercise. Redox biology is at the heart of life sciences. This is because electron flow may be one of the most universal and fundamental approaches to biology [10]. Consequently, we believe that the field of exercise redox biology will be one of the key topics that will drive the exercise science in the future.

Acknowledgments

It is our pleasure to thank all authors, the referees, and the staff of Hindawi's Editorial Office for their invaluable work that made this special issue possible.

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Chad M. Kerksick
Manfred Lamprecht
Steven R. McAnulty

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2012, Article ID 465020, 10 pages
doi:10.1155/2012/465020

Research Article

Impact of Oral Ubiquinol on Blood Oxidative Stress and Exercise Performance

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Received 5 April 2012; Accepted 4 June 2012

Academic Editor: Steve R. McAnulty

Coenzyme Q10 (CoQ10) plays an important role in bioenergetic processes and has antioxidant activity. Fifteen exercise-trained individuals (10 men and 5 women; 30–65 years) received reduced CoQ10 (Kaneka QH ubiquinol; 300 mg per day) or a placebo for four weeks in a random order, double blind, cross-over design (3 week washout). After each four-week period, a graded exercise treadmill test and a repeated cycle sprint test were performed (separated by 48 hours). Blood samples were collected before and immediately following both exercise tests and analyzed for lactate, malondialdehyde, and hydrogen peroxide. Resting blood samples were analyzed for CoQ10 (ubiquinone and ubiquinol) profile before and after each treatment period. Treatment with CoQ10 resulted in a significant increase in total blood CoQ10 (138%; $P = 0.02$) and reduced blood CoQ10 (168%; $P = 0.02$), but did not improve exercise performance (with the exception of selected individuals) or impact oxidative stress. The relationship between the percentage change in total blood CoQ10 and the cycle sprint total work ($R^2 = 0.6009$) was noted to be moderate to strong. We conclude that treatment with CoQ10 in healthy, exercise-trained subjects increases total and reduced blood CoQ10, but this increase does not translate into improved exercise performance or decreased oxidative stress.

1. Background

Coenzyme Q10 (CoQ10), referred to as ubiquinol in its most active (~95%) and reduced form [1], has documented roles related to bioenergetics and antioxidant activity [2]. Muscle function and subsequent physical performance during high-stress conditions may be compromised due to a rise in reactive oxygen species (ROS) [3, 4], leading to an acute state of oxidative stress [5, 6]. This is particularly true in middle to older age individuals who may experience increased oxidative stress [7], and may have some degree of CoQ10 deficiency [8], as compared to their younger counterparts.

Although the use of supplemental antioxidants to combat exercise-induced oxidative stress has been questioned recently when delivered as vitamin E and vitamin C [9, 10], CoQ10 continues to be considered for this purpose. Specifically, it has been reported that supplementation with oral CoQ10 may minimize oxidative stress [11] and improve physical performance [12] in middle-aged adults. However, when investigating studies across a wide range of age groups, results have been mixed with some work documenting a performance benefit [13], and other work demonstrating little to no benefit following CoQ10 supplementation [14].

One possible explanation for the lack of evidence for CoQ10 to improve exercise performance may be the fact that traditional CoQ10 is poorly absorbed following oral ingestion [15]. This observation necessitates using high dosages of CoQ10 in order to obtain the desired physiological effects, something that has not been done in many of the published trials, with most daily dosages being less than or equal to 300 mg [16]. One newly developed form of CoQ10 (Kaneka QH ubiquinol) has been reported to have excellent bioavailability [17], with a noted 4.7-fold increase in plasma ubiquinol following single oral ingestion of CoQ10 at a dosage of 300 mg, and an approximate 10-fold increase following 28 days of treatment with CoQ10 at a daily dosage of 300 mg. Such an increase is far greater than what has been observed with traditional CoQ10 supplementation [18]. It is possible that such an improvement in bioavailability may translate into an improvement in physical performance, in

addition to attenuation in potential exercise-induced oxidative stress.

The present study tested the influence of a bioavailable form of CoQ10, delivered as a dietary supplement in a sample of exercise-trained men and women aged 30–65 years, on plasma CoQ10 levels and markers of oxidative stress before and after 28 days of treatment. In addition, markers of exercise-induced oxidative stress were measured before and after single bouts of aerobic and anaerobic exercise, both pre- and postintervention, and measures of exercise performance were determined. We hypothesized that treatment with CoQ10 would increase plasma levels of CoQ10 and result in lower exercise-induced oxidative stress and improved exercise performance.

2. Methods

2.1. Subjects and Screening. Seventeen individuals initially enrolled in this study, but only 15 (10 men and 5 women) successfully completed all aspects of this work. Subjects completed health history, drug and dietary supplement usage, and physical activity questionnaires prior to enrollment. Subjects were physically active, regularly participating in aerobic and/or anaerobic exercise training. All subjects were instructed to maintain their prestudy exercise training program throughout the course of the intervention period. Subjects were nonsmokers, did not report any history of cardiovascular or metabolic disorders, and did not use nutritional supplements that might impact the outcome measures (or were willing to stop their use before and throughout the study period. One subject stopped using creatine two weeks prior to beginning the study). Subjects descriptive data are as follows: age: 42.7 ± 10.4 yrs; body mass: 75.2 ± 16.3 kg; years of anaerobic exercise training: 11.2 ± 12.9; hours per week of anaerobic exercise: 1.8 ± 1.7; years of aerobic exercise training: 14.0 ± 13.7; hours per week of aerobic exercise: 5.3 ± 4.9. Prior to participation, each subject was informed of all procedures, potential risks, and benefits associated with the study through both verbal and written form in accordance with the approved procedures of the University Institutional Review Board for Human Subjects Research (021011-357). Subjects provided verbal and written informed consent prior to being admitted into the study.

Subjects' body weight was measured, and heart rate and blood pressure were recorded following a 10-minute period of quiet rest (all values noted to be within normal limits). A maximal graded exercise test (GXT) was conducted using a treadmill and a sprint test was performed using an electronically braked cycle ergometer. These tests were performed as part of a familiarization procedure to prepare subjects for the exercise performance testing as described below. The tests were performed on different days, separated by 48 hours to allow for adequate recovery, and the procedures used were identical to those described below.

At the conclusion of the familiarization tests, a full explanation of dietary data recording was provided to subjects, along with data collection forms. An overview of all study procedures was also provided. Subjects were then assigned their initial condition (CoQ10 supplement or placebo), instructed on when to take the capsules, and scheduled for their remaining laboratory visits.

2.2. Supplementation. The study design involved a random order, double-blind, crossover assignment to a CoQ10 supplement (Kaneka QH ubiquinol; Kaneka Nutrients, Pasadena, TX; containing 300 mg ubiquinol in a base of medium chain triglyceride oil, beeswax, soy lecithin, and ascorbyl palmitate) or placebo (medium chain triglyceride oil, beeswax, soy lecithin, and ascorbyl palmitate). Subjects ingested one capsule per day with breakfast, for a total of 30 days (28 days prior to the GXT and two-additional days leading up to the cycle sprint test—described below), with a 21-day wash out period between conditions. Prior work has indicated that a 28-day treatment period with 300 mg/day of supplemental Kaneka QH is more than adequate to elevate blood CoQ10 levels (~10-fold above baseline), while a 14-day washout period is sufficient for returning blood CoQ10 levels to near presupplementation values [17]. Moreover, dosages up to 900 mg/day for four weeks have been noted to be safe, without adverse events [19]. These findings are highlighted in the review by Hidaka and colleagues [20]. Both the supplement and placebo capsules were identical in appearance. For both conditions, capsules were distributed to subjects by research assistants in unlabeled bottles in amounts greater than needed for supplementation. Capsule counts upon bottle return allowed for estimation of compliance to intake.

2.3. Graded Exercise Test. Following each 28-day period of CoQ10 and placebo intake, subjects reported to the lab in the morning to perform a GXT on a treadmill, which allowed for the assessment of aerobic exercise performance. In an attempt to maintain consistency in testing, a script was read to each subject prior to performing the GXT. The protocol involved an increase in intensity every two minutes in the following manner: min 1-2, 3.0 mph, 0%; min 3-4, 3.5 mph, 0%; min 5-6, 4.0 mph, 0%; min 7-8, 4.5 mph, 0%; min 9-10, 5.0 mph, 0%; min 11-12, 5.0 mph, 5%; min 13-14, 5.5 mph, 5%; min 15-16, 5.5 mph, 7.5%; min 17-18, 6.0 mph, 7.5%; min 19-20, 6.0 mph, 10%; min 21-22, 6.5 mph, 10%; min 23-24, 6.5 mph, 12.5%; min 25-26, 7.0 mph, 12.5%; min 27-28, 7.0 mph, 15%; min 29-30, 7.5 mph, 15%. No subject exceeded 29 minutes of exercise testing. This identical protocol was administered following each of the 4-week supplementation periods. During the final 15 seconds of each stage of exercise, heart rate was recorded via heart rate monitors and the Borg (6–20) scale of exertion was used to allow subjects to indicate their level of perceived work. Total exercise time was recorded. Although subjects performed the GXT in the morning following an overnight fast, they were allowed to drink water *ad libitum* before and following the GXT.

2.4. Cycle Sprint Test. On a separate day from the GXT (within 48 hours), subjects returned to the lab in the morning to perform 5-repeated cycle sprints on an electronically

braked cycle ergometer (Lode Excaliber Sport; Groningen) using a fixed resistance based on body mass (torque factor: 0.7 Nm/kg). This test was allowed for the assessment of anaerobic exercise performance. Each sprint was 10 seconds in duration and two minutes of active recovery was allowed between each sprint. For each sprint, total work (kJ) and power output were calculated. Heart rate and perceived exertion were also measured following each sprint. While subjects performed the cycle sprint test in the morning following an overnight fast, they were allowed to drink water *ad libitum* before and following the test. As with the GXT, a script was read to each subject prior to performing the sprint test and the exact procedures for testing were used after each four-week period of supplementation. After the performance of the GXT and on the day between the maximal exercise tests, subjects continued with their assigned condition (CoQ10 or placebo), which provided a total of 30 days of treatment.

2.5. Blood Sampling. Venous blood samples (~20 mL) were collected from subjects' forearm via needle and Vacutainer before and following each period of supplementation with CoQ10 and placebo. Samples were collected at rest in a 10-hour fasted state, following a 10-minute period of quiet rest. Preintervention blood samples were analyzed for measures of oxidative stress, in addition to a CoQ10 profile. Following each intervention period, blood was collected before and immediately after each exercise test and analyzed for whole blood lactate, plasma malondialdehyde, and plasma hydrogen peroxide. Samples collected in tubes with EDTA were used for the analysis of whole blood lactate (Lactate Plus; Nova Biomedical, Waltham, MA) and the remaining blood was immediately centrifuged at 1500 g at 4°C to obtain plasma. Samples for analysis of CoQ10 profile were collected in sodium heparinized tubes and immediately centrifuged at 1500 g at 4°C to obtain plasma. Following centrifugation, the plasma was immediately stored in multiple aliquots at −70°C.

2.6. Biochemistry. Malondialdehyde was analyzed in plasma using a commercially available colorimetric assay (Northwest Life Science Specialties; Vancouver, WA), using a modified method described by Jentzsch et al. [21], which is suggested to improve the sensitivity of this assay. Despite the improved sensitivity, other lipid specific markers such as 8-isoprostanes and hexanoyl-lysine could have been considered in this work, as these assays have been suggested as more sensitive markers of lipid peroxidation as compared to malondialdehyde. Our failure to include these other markers could be considered a limitation of this work. For analysis of malondialdehyde, plasma samples were added to microcentrifuge reaction tubes with the addition of 1 M phosphoric acid and 2-thiobarbituric acid reagent. Samples and reagents were incubated for 60 minutes at 60°C. Following incubation and removal of the reaction tubes, the mixture was transferred to a microplate, and the absorbance was read using a spectrophotometer at both 535 and 572 nm. Quantification was performed with a calibration curve using tetramethoxypropane in a stabilizing buffer. Hydrogen peroxide was analyzed in plasma using the Amplex Red reagent method as described by the manufacturer (Molecular Probes; Invitrogen Detection Technologies, Eugene, OR). In the reaction mixture, hydrogen peroxide, in the presence of horseradish peroxidase, reacts with Amplex Red reagent to generate the red-fluorescence oxidation product, resorufin. Quantification was performed with a hydrogen peroxide calibration curve. Oxidative stress biomarker samples were analyzed in duplicate on first thaw. CoQ10 profile was analyzed in plasma using high-performance liquid chromatography in the Neuropharmacology and Clinical Laboratories of Cincinnati Children's Hospital Medical Center, using the procedures previously described in detail by Tang and colleagues [22].

2.7. Perceived Vigor. Subjects were asked to rate their perceived vigor during the past three weeks using a visual scale (where 10 = very energetic and 0 = very nonenergetic) at weeks 0, 4, 7, and 11. These times coincided with pre- and postintervention for both supplement and placebo.

2.8. Dietary Intake and Physical Activity. All subjects were instructed to maintain their normal diet, without attempts to increase or decrease antioxidant nutrient intake. Subjects completed a four-day food log surrounding the days of testing (2 days before the GXT, the day of the GXT, and the day after the GXT). All calorie containing foods and drinks were recorded. Nutritional records were analyzed for total calories, protein, carbohydrate, fat, and a variety of micronutrients (Food Processor SQL, version 9.9, ESHA Research, Salem, OR). Subjects were given specific instructions regarding abstinence from alcohol consumption during the 48 hours immediately prior to the test days. They were also instructed to maintain their normal physical activity, with the exception of refraining from strenuous physical activity during the 48 hours preceding each test day.

2.9. Statistical Analysis. For the analysis of resting blood measures and perceived vigor, outcome variables were analyzed using a 2 (condition) × 2 (pre-/postintervention) analysis of variance (ANOVA). For exercise performance data, variables were analyzed using a one-way ANOVA (for condition). For exercise bloodborne variables, data were analyzed using a 2 (condition) × 2 (time—pre-/post-exercise) ANOVA. Due to our unequal and small sample sizes for men and women, no formal attempt was made to compare the response between sexes; however, this was done in a cursory manner for the two-exercise-performance measures (GXT time and cycle total work). Tukey post hoc tests were used as needed. Dietary data were analyzed using a one-way ANOVA (for condition). All analyses were performed using JMP statistical software (version 4.0.3, SAS Institute, Cary, NC). Statistical significance was set at $P \leq 0.05$. The data are presented as mean ± SEM, except for subject descriptive characteristics (mean ± SD).

Table 1: Dietary intake of 15 exercise-trained men and women during the days surrounding treadmill and cycle testing following treatment with CoQ10 or placebo.

Variable	CoQ10	Placebo	*P* value
Kilocalories	2083 ± 188	2131 ± 181	0.85
Protein (g)	87 ± 8	86 ± 7	0.91
Protein (%)	17 ± 1	17 ± 1	0.63
Carbohydrate (g)	256 ± 25	255 ± 24	0.97
Carbohydrate (%)	48 ± 3	48 ± 3	0.95
Fiber (g)	18 ± 3	19 ± 2	0.86
Sugar (g)	92 ± 15	95 ± 14	0.89
Fat (g)	79 ± 10	84 ± 10	0.72
Fat (%)	34 ± 2	34 ± 2	0.90
Saturated Fat (g)	26 ± 4	28 ± 4	0.68
Monounsaturated fat (g)	15 ± 3	13 ± 3	0.45
Polyunsaturated fat (g)	7 ± 1	5 ± 1	0.19
Cholesterol (mg)	258 ± 45	287 ± 43	0.65
Vitamin C (mg)	56 ± 9	53 ± 8	0.80
Vitamin E (mg)	4 ± 0	3 ± 0	0.09
Vitamin A (RE)	385 ± 87	306 ± 83	0.52

Values are mean ± SEM.

3. Results

3.1. Compliance, Vigor, and Dietary Data. Of the initial 17-enrolled subjects, one man and one woman failed to complete all aspects of the study due to personal reasons with scheduling. The remaining 15 subjects successfully completed testing, with the exception of one woman not completing the GXT with both conditions, and one woman not completing the cycle sprint test with placebo. Regarding compliance to capsule intake, subjects were 93 ± 4% compliant to CoQ10 capsules and 98 ± 1% compliant to placebo capsules, with no statistical difference noted between conditions ($P = 0.20$). Regarding perceived vigor, no condition ($P = 0.24$), pre-/postintervention ($P = 0.92$), or interaction ($P = 0.79$) effect was noted, with values for CoQ10 (pre: 7.4 ± 0.5 and post: 7.3 ± 0.5) and placebo (pre: 6.8 ± 0.4 and post: 6.8 ± 0.5) near identical from pre- to postintervention. Regarding dietary intake, no difference was noted between conditions for any presented variable ($P > 0.05$). Dietary data are displayed in Table 1.

3.2. Exercise Test Data. No differences were noted between CoQ10 and placebo for exercise performance and related variables (heart rate and perceived exertion), with near identical mean values observed for all variables ($P > 0.05$). However, selected subjects did experience an improvement in GXT exercise time and cycle sprint test total work, as shown in Figure 1. Subjects appeared to work very hard during testing, as evidenced by a mean peak heart rate on the GXT of 180 beats per minute (above the age-predicted maximum) and a rating of perceived exertion value of close to 20/20. Total GXT time was over 22 minutes, which is considered excellent given the protocol used and the age of the subjects, and was not different ($P = 0.51$) for men (22.3 minutes) and women (21.0 minutes). Total work during the sprint cycle test was approximately 5000 kJ, and was greater ($P < 0.0001$) for men (5589 kJ) as compared to women (3514 kJ), which was expected based on the larger body mass of men. Despite these findings for GXT exercise time and total work during the sprint cycle test, no sex × condition interactions were noted for the GXT ($P = 0.88$) or sprint cycle test ($P = 0.72$). Data for the GXT are presented in Table 2(a), while data for the cycle sprint test are presented in Table 2(b). Individual subject data for total treadmill time and total work for the sprint cycle test are presented in Figure 1.

3.3. Resting Oxidative Stress and CoQ10 Profile. No differences were noted between CoQ10 supplementation and placebo for measures of oxidative stress at rest, before and after the 4-week intervention period ($P > 0.05$). However, treatment with CoQ10 resulted in a significant increase in total blood CoQ10 (138%; $P = 0.02$) and reduced blood CoQ10 (168%; $P = 0.02$) from pre- to postsupplementation. Due to the increase in total CoQ10 and the stable cholesterol values from pre- to postintervention, the CoQ10 : Cholesterol ratio was increased significantly ($P = 0.01$). Values for these parameters with placebo treatment remained relatively constant, with a slight decrease noted. No other variables within the CoQ10 profile were altered significantly ($P > 0.05$). Data are presented in Table 3. Although not a main focus of the present research, the relationship between the percentage change in total blood CoQ10 and exercise performance (placebo versus CoQ10 supplement) was noted for GXT time ($R^2 = 0.0744$; $P = 0.35$) and cycle sprint total work ($R^2 = 0.6009$; $P = 0.001$). Data are presented in Figure 2.

3.4. Exercise-Induced Oxidative Stress and Lactate. No differences were noted between CoQ10 and placebo for measures of oxidative stress in response to exercise ($P > 0.05$), with only a moderate oxidative stress observed in response to both the GXT and the cycle sprint test. With the GXT, the time effect approached significance for malondialdehyde ($P = 0.11$) and hydrogen peroxide ($P = 0.06$), while the same was true for malondialdehyde ($P = 0.09$) but not hydrogen peroxide ($P = 0.47$) with regard to the cycle sprint test. No condition or condition × time effects were noted for blood lactate in regard to either exercise test ($P > 0.05$). However, a time effect was noted for blood lactate for both exercise tests ($P < 0.0001$), with a significant increase from pre- to postexercise. Data are presented in Table 4.

4. Discussion

Findings from the present investigation indicate that supplementation of CoQ10 in healthy, exercise-trained men and women increases total and reduced blood CoQ10, in addition to CoQ10 : Cholesterol ratio. Neither an improvement in exercise performance is noted, nor does supplementation result in attenuation of resting or exercise-induced oxidative stress biomarkers. These data are specific to a sample of

Table 2: Heart rate, perceived exertion, and performance data of exercise-trained men and women during treadmill testing (a) and cycle sprints (b) following treatment with CoQ10 or placebo.

(a)

Variable*	CoQ10	Placebo	*P* value
Heart rate min 8 (bpm)	119.9 ± 6.4	122.2 ± 5.7	0.79
Heart rate min 14 (bpm)	148.2 ± 6.5	147.1 ± 5.0	0.87
Heart rate peak (bpm)	180.1 ± 3.5	180.5 ± 3.1	0.98
Perceived exertion min 8 (6–20 scale)	11.4 ± 0.4	11.9 ± 0.3	0.50
Perceived exertion min 14 (6–20 scale)	13.9 ± 0.6	14.3 ± 0.6	0.91
Perceived exertion peak (6–20 scale)	19.5 ± 0.2	19.7 ± 0.1	0.37
Total treadmill time (sec)	1324.5 ± 69.3	1317.3 ± 62.0	0.94

(b)

Variable**	CoQ10	Placebo	*P* value
Heart rate (bpm)	155.6 ± 3.6	156.4 ± 3.2	0.96
Perceived exertion (6–20 scale)	17.6 ± 0.3	16.9 ± 0.4	0.19
Peak power (watts)	875.0 ± 63.3	914.7 ± 57.2	0.65
Mean power (watts)	483.3 ± 35.2	511.0 ± 36.9	0.59
Total work (kJ)	4833.1 ± 351.6	5109.8 ± 368.9	0.59

Values are mean ± SEM.
*Heart rate and perceived exertion values taken during the final 15 seconds of minute 8 (stage 4) and minute 14 (stage 7) of treadmill testing; peak values for heart rate and perceived exertion taken at the end of the treadmill testing.**Values are averages taken over five-, 10-second cycle sprints.

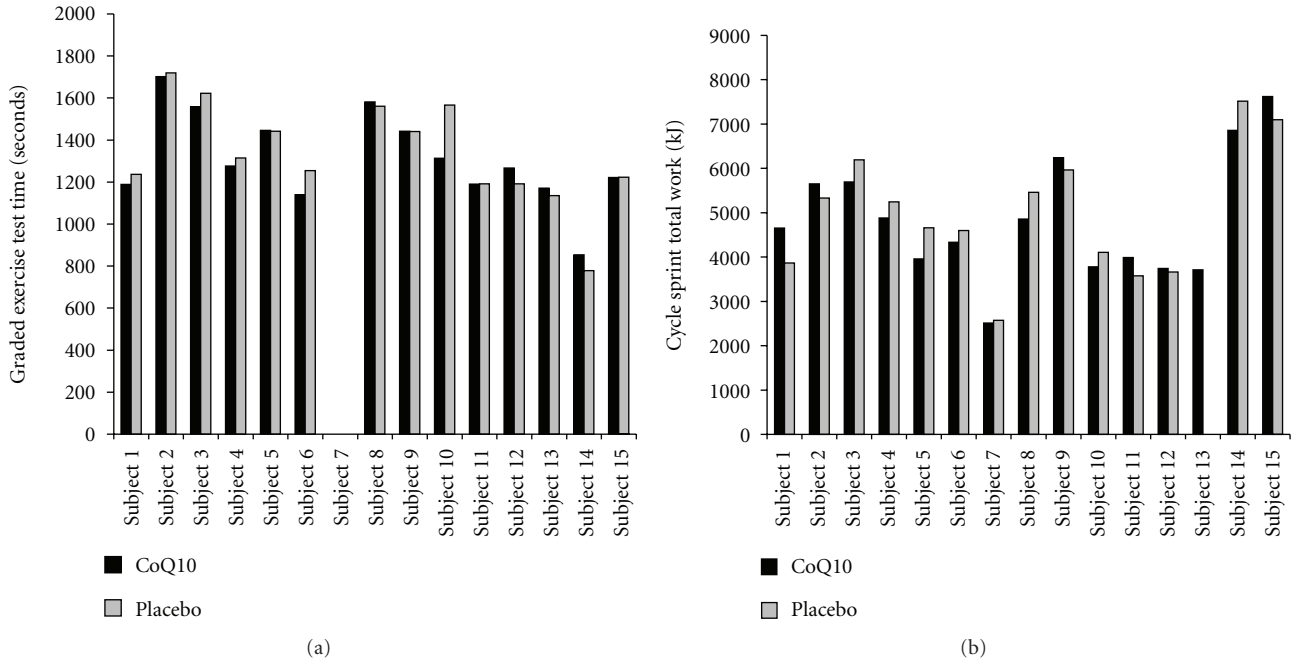

Figure 1: Treadmill time (a) and total work during cycle testing (b) of exercise-trained men and women following treatment with CoQ10 or placebo. Note: Subjects 7, 10, 11, 12, and 13 are women.

healthy, exercise-trained men and women supplementing CoQ10 at a daily dosage of 300 mg.

4.1. Plasma CoQ10. Treatment with CoQ10 resulted in a significant increase in plasma CoQ10 concentrations, which supports the prior work of Hosoe et al. [17], who found that the same dosage and time of administration increases plasma ubiquinol approximately 10-fold. The baseline plasma ubiquinol values in that study involving healthy subjects was 0.57 to 0.66 $\mu g \cdot mL^{-1}$, lower than what we observed in the present study. Although we did not observe such a magnitude of increase, our subjects did indeed experience a significant rise in total and reduced CoQ10 (Table 3). It is possible that the variance could be attributed

Table 3: Oxidative stress and Coenzyme Q10 blood profile (a) and statistical findings (b) of exercise-trained men and women before (Pre) and after (Post) treatment with CoQ10 or placebo.

(a)

Variable	CoQ10 Before	CoQ10 After	Placebo Before	Placebo After
Malondialdehyde (μmol·L^{-1})	0.87 ± 0.12	0.89 ± 0.11	0.92 ± 0.12	0.89 ± 0.12
Hydrogen Peroxide (μmol·L^{-1})	2.72 ± 0.34	3.23 ± 0.32	2.92 ± 0.35	3.36 ± 0.34
Total CoQ10 (μg·mL^{-1})	0.98 ± 0.10	2.33 ± 0.42	1.02 ± 0.11	0.99 ± 0.09
Reduced CoQ10 (μg·mL^{-1})	0.82 ± 0.08	2.13 ± 0.39	0.87 ± 0.09	0.84 ± 0.07
Reduced CoQ10 (%)	84.41 ± 2.10	90.62 ± 1.63	85.64 ± 2.38	85.96 ± 1.93
Oxidized CoQ10 (μg·mL^{-1})	0.17 ± 0.03	0.21 ± 0.04	0.15 ± 0.04	0.15 ± 0.03
Total Cholesterol (mg·dL^{-1})	199.33 ± 9.32	197.07 ± 9.55	198.80 ± 10.34	200.27 ± 12.25
CoQ10 : Cholesterol	0.48 ± 0.39	1.13 ± 0.15	0.51 ± 0.04	0.49 ± 0.04

Values are mean ± SEM.

(b)

Variable	Condition *P* value	Before/after *P* value	Condition × Before/after *P* value
Malondialdehyde	0.79	0.92	0.83
Hydrogen Peroxide	0.62	0.17	0.92
Total CoQ10	0.02*	0.04*	0.13
Reduced CoQ10	0.02*	0.03*	0.11
Reduced CoQ10%	0.30	0.10	0.42
Oxidized CoQ10	0.50	0.97	0.47
Total Cholesterol	0.62	0.55	0.96
CoQ10 : Cholesterol	0.01*	0.01*	0.00*

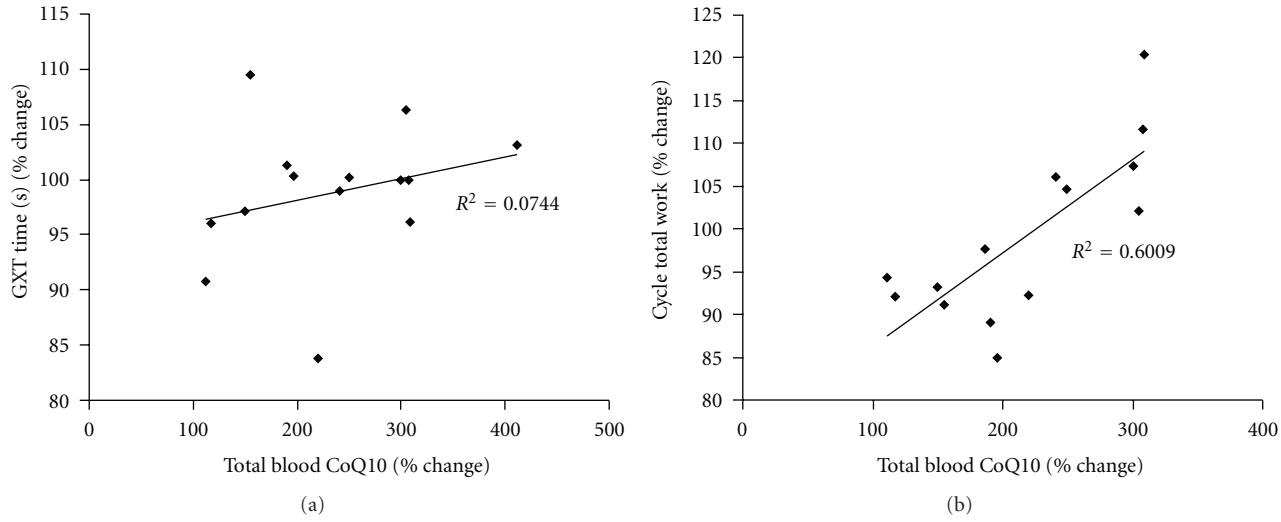

Figure 2: Relationship between the percentage change in GXT time (a) and cycle total work (b) and total blood CoQ10. Note: The percentage change value was calculated between placebo versus CoQ10.

to a difference in formulation, and that a greater magnitude of increase in plasma CoQ10 could result in more favorable findings for our other outcome measures, as described below. Based on the role of CoQ10 in regulating metabolic function and the impact of cholesterol on vascular health, our finding of an increase in CoQ10 : Cholesterol ratio (i.e., normalization of CoQ10 for cholesterol concentrations) may have implications for improved cardiovascular and metabolic health, as indicated previously for conditions such as advanced congestive heart failure.

4.2. Resting Oxidative Stress. CoQ10 supplementation has been reported to lower biomarkers of oxidative stress when

Table 4: Oxidative stress and blood lactate of exercise-trained men and women before (pre) and after (post) treadmill and cycle exercise, following treatment with CoQ10 or placebo.

Time	Malondialdehyde (μmol·L^{-1})	Hydrogen Peroxide (μmol·L^{-1})	Lactate* (mmol·L^{-1})
Treadmill (before) *CoQ10*	0.89 ± 0.11	3.23 ± 0.32	1.25 ± 0.56
Treadmill (after) *CoQ10*	0.93 ± 0.11	4.61 ± 0.53	8.83 ± 0.58
Treadmill (before) *Placebo*	0.89 ± 0.12	3.36 ± 0.34	1.33 ± 0.56
Treadmill (after) *Placebo*	1.05 ± 0.10	4.14 ± 0.49	8.36 ± 0.53
Cycle (before) *CoQ10*	0.81 ± 0.10	3.54 ± 0.53	1.12 ± 0.53
Cycle (after) *CoQ10*	0.98 ± 0.10	3.86 ± 0.52	10.36 ± 0.51
Cycle (before) *Placebo*	0.92 ± 0.10	3.58 ± 0.59	1.18 ± 0.57
Cycle (after) *Placebo*	1.08 ± 0.10	4.10 ± 0.59	10.06 ± 0.57

Values are mean ± SEM.
Treadmill:
No condition ($P = 0.95$), time ($P = 0.11$), or condition × time ($P = 0.23$) effect for MDA.
No condition ($P = 0.41$), time ($P = 0.06$), or condition × time ($P = 0.94$) effect for H_2O_2.
No condition ($P = 0.75$) or condition × time ($P = 0.61$) effect for lactate; *Time effect ($P < 0.0001$) for lactate.
Cycle:
No condition ($P = 0.30$), time ($P = 0.09$), or condition × time ($P = 0.94$) effect for MDA.
No condition ($P = 0.82$), time ($P = 0.47$), or condition × time ($P = 0.87$) effect for H_2O_2.
No condition ($P = 0.84$) or condition × time ($P = 0.73$) effect for lactate; Time effect ($P < 0.0001$) for lactate.

measured at rest [11, 23]. For example, Niklowitz et al. investigated the changes in 8-hydroxydeoxy-guanosine (8-OHdG) after 28 days of CoQ10 supplementation at 3 mg/kg/day [11]. The authors reported a delayed formation of 8-OHdG over the course of study. Also investigating the role of CoQ10 on oxidative stress biomarkers, Weber and colleagues supplemented healthy subjects with 90 mg/day of CoQ10 and reported a decrease in TBARS after two weeks of treatment [23]. In the present study, it is possible that our lack of a difference between pre- and postsupplementation of CoQ10 for oxidative stress biomarkers was due to the fact that all measures were relatively low to begin with, as we have recently reported for active men and women [24]. Despite any potential antioxidant effect of the CoQ10, there may have been little need to further decrease the already low-resting levels of these oxidative stress biomarkers. This is particularly true when considering that a mild degree of ROS generation (and oxidative stress) appears a vital component of normal physiological functioning [25]. Additionally, the baseline total CoQ10 levels of our subjects were relatively high and could have impacted the benefit of the supplementation. Perhaps the inclusion of diseased and/or older subjects would have allowed us to detect a significant effect of CoQ10 treatment.

4.3. Exercise-Induced Oxidative Stress. Aside from resting conditions, several investigations have been conducted focused on attenuating exercise-induced oxidative stress via use of antioxidant treatment. Pertaining to this line of research, results from these studies are largely mixed [6, 26, 27], and are likely dependent on the type, dosage, and time frame of treatment of the antioxidant(s), the tissue sampled, the exercise protocol used to induce oxidative stress, the time of measurement, the assays used, and the test subjects recruited (i.e., trained versus untrained, old versus young, and healthy versus diseased), among other variables [28]. With such variance across study designs, a clear statement regarding the effectiveness of antioxidant supplements to minimize oxidative stress resulting from exercise is difficult to compose.

The above also appears true for CoQ10 supplementation, with at least two studies involving prophylactic treatment noting attenuation in exercise-induced oxidative stress biomarkers [29, 30]. Specifically, Tauler and colleagues investigated three months of supplementation involving an antioxidant cocktail, including 100 mg of CoQ10, following a 60-minute soccer match [29]. A noted decrease in plasma oxidative stress was observed with antioxidant supplementation—although details as to which components of the antioxidant cocktail were responsible for the findings are not available. Gül and coworkers noted a partial prevention in exercise-induced lipid peroxidation in a sample of sedentary men following repeated cycle sprints when subjects first supplemented with 100 mg of CoQ10 for eight weeks [30]. In contrast, Braun and coworkers reported no difference in exercise-induced oxidative stress following eight weeks of supplementation with 100 mg per day of CoQ10 in male cyclists [31]. Others note minimal or no difference in exercise-induced oxidative stress following CoQ10 treatment [32]. As with the findings of the present study, it is possible that the inclusion of active subjects who often do not experience a significant increase in oxidative stress in response to acute exercise bouts [6], likely contributed to the findings of no effect. That is, there simply was too small of an increase noted, leaving little room for attenuation. Similar findings have been noted previously with regard to exercise and CoQ10 supplementation in both younger and older individuals [33].

4.4. Physical Performance. With regards to antioxidant supplementation and physical performance, a lack of benefit has been noted in much prior work [34, 35]. However, CoQ10

offers more than simply antioxidant effects, as this nutrient is involved in bioenergetic processes [2]. Due to this, the use of CoQ10 as a potential ergogenic aid has been studied for the past several years, and to many individuals, this potential effect appears to be of greater overall importance as compared to the antioxidant effect of this agent. While some studies have noted favorable findings with regards to measures of physical performance [13, 16, 36], many have not—with few or no differences observed between placebo and CoQ10 supplementation groups [14, 31, 33, 37]. In studies in which no effect has been noted, it is possible that the dosage of CoQ10 was too low [32, 37, 38], in particular considered the relatively poor bioavailability of traditional CoQ10 [1].

In the present study, a sufficient dosage of CoQ10 was provided in order to increase plasma CoQ10 status significantly (Table 3). Despite this elevation, no significant performance benefit was noted for either aerobic or anaerobic exercise tests (Table 2). However, selected individuals appeared to respond to CoQ10 treatment, in particular with regard to the cycle sprint test. Moreover, the relationship between the percentage change in total blood CoQ10 and exercise performance (in particular the cycle sprint total work; $R^2 = 0.6009$) was noted to be moderate to strong. These findings provide some indication that higher levels of total blood CoQ10 appear related to higher cycle sprint exercise performance.

Regarding those individuals who appeared to respond to CoQ10 treatment, no discernable pattern of response was noted based on sex or age. For example, of the four subjects who were noted to experience an improvement in treadmill time on the GXT, three were men (aged 30, 40, and 45 years) and one was a woman (aged 44 years). Of the six subjects who were noted to experience an improvement in total work performed during the cycle sprint test, five were men (aged 35, 36, 44, 54, and 57 years) and one was a woman (aged 32 years). Moreover, no noted differences were observed for these "responders" with regard to oxidative stress biomarkers, when compared to those who did not respond to supplementation. These findings are presented in Figure 1 for both total treadmill time (a) and total work during the cycle sprint test (b). This pattern does not shed any light on a given population who may respond best to treatment with CoQ10, but highlights what has been observed for almost all nutritional supplements—some individuals response to treatment and some do not. Additional work with a larger sample of younger and older men and women, in particular of various fitness backgrounds and the use of a variety of physical tests, may more fully elucidate the potential for this nutrient to provide an ergogenic effect.

When considering the above, the results for CoQ10 supplementation as pertaining to both antioxidant effect and physical performance are mixed, with findings mainly influenced by the subjects tested and the type of exercise employed. Our results clearly demonstrate the effect of supplemental CoQ10 on elevating blood levels of CoQ10, while failing to exhibit an antioxidant or ergogenic effect in a sample of exercise-trained subjects. Of course, this statement is specific to the outcome measures included within the present research design. Our results in no way eliminate the possibility for benefit of CoQ10 supplementation on outcomes not measured here.

5. Conclusion

The findings presented here indicate that supplementation of CoQ10 in healthy, exercise-trained men and women increases total and reduced blood CoQ10, in addition to CoQ10 : Cholesterol ratio. However, no change in resting or exercise-induced measures of oxidative stress is noted, nor is aerobic or anaerobic exercise performance improved following four weeks of supplementation, with the exception of selected individuals. It is possible that more robust effects may be noted within a sample of sedentary and/or older individuals, as well as in those with known disease. In addition, the active subjects in this study had a baseline total CoQ10 value of close to $1.0\,\mu g \cdot mL^{-1}$, which is higher than the $0.6–0.8\,\mu g \cdot mL^{-1}$ observed in typical populations, and thus may have contributed to a mitigated response to supplementation. While we used an exercise stressor in the present study in an attempt to increase ROS production and to test the antioxidant potential of CoQ10, other more potent ROS generators such as the ingestion of high-fat feedings may better determine the antioxidant potential of this supplement. Future research is needed to provide answers to the above questions. Lastly, as ubiquinol has demonstrated a distinct effect on gene expression, including activation of genes involved in lipid and lipoprotein metabolism, and countering expression of inflammation (effects not observed with ubiquinone), further research is necessary to relate the benefits of elevated blood values of total and reduced CoQ10 to exercise performance.

Conflict of Interests

Financial support for this work was provided by Kaneka Nutrients (Pasdena, TX). The investigators and the University of Memphis have no direct or indirect interest in the tested product (Kaneka QH) or in Kaneka Nutrients.

Acknowledgment

Funding for this work was provided by Kaneka Nutrients (Pasadena, TX).

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2012, Article ID 741545, 15 pages
doi:10.1155/2012/741545

Review Article

Regular Physical Exercise as a Strategy to Improve Antioxidant and Anti-Inflammatory Status: Benefits in Type 2 Diabetes Mellitus

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Received 20 April 2012; Revised 28 June 2012; Accepted 11 July 2012

Academic Editor: Chad M. Kerksick

Over the last 30 years the combination of both a sedentary lifestyle and excessive food availability has led to a significant increase in the prevalence of obesity and aggravation of rates of metabolic syndrome and type 2 diabetes mellitus (T2DM). Several lines of scientific evidence have been demonstrating that a low level of physical activity and decreased daily energy expenditure leads to the accumulation of visceral fat and, consequently, the activation of the oxidative stress/inflammation cascade, which underlies the development of insulin resistant T2DM and evolution of micro, and macrovascular complications. This paper focuses on the pathophysiological pathways associated with the involvement of oxidative stress and inflammation in the development of T2DM and the impact of regular physical exercise (training) as a natural antioxidant and anti-inflammatory strategy to prevent evolution of T2DM and its serious complications.

1. Introduction

Oxidation is viewed in general as a chemical process whereby electrons are removed from molecules, generating highly reactive free radicals, which include reactive oxygen species (ROS), such as superoxide and hydroperoxyl, and reactive nitrogen species (RNS) [1]. Reactive species arise as natural byproducts of aerobic metabolism at rest and play a role in several signalling cascades of distinct physiological processes, including phagocytosis, vasorelaxation, and neutrophil function [2, 3]. Excessive levels of ROS or reduction of the antioxidant defenses, such as superoxide dismutases (SODs), heme oxygenase-1 (HO-1), NAD(P)H quinone oxidoreductase-1 (NQO-1), catalase or thioredoxin, causes oxidative stress, as has been repeatedly described for a number of various diseases, including type 2 diabetes mellitus (T2DM) [4–12].

Recent work has indicated that chronic inflammation, together with oxidative stress, is an important pathophysiological factor in the development of T2DM, in particular through the effects of proinflammatory cytokines, such as C-reactive protein (CRP), tumor necrosis factor alpha (TNF-α), interleukin (IL)-6 and IL-1β, among others [8, 13, 14]. On the contrary, anti-inflammatory cytokines, such as adiponectin and IL-10, seem to be protective against those pathological conditions, namely, by inhibiting TNF-α action on adhesion of endothelial cell, reducing nuclear factor (NF)-κB activation, and delaying macrophage foam-cell development [15–17]. Inflammatory cytokines are potent stimulators for the production of reactive species by macrophages and monocytes, thus inducing oxidative stress [18]. Additionally, and simultaneously, an increase in oxidative stress derived-inflammation has been hypothesized to be a major

mechanism in the pathogenesis and progression of T2DM [19].

Regular exercise (with or without caloric restriction) has been increasingly viewed as an effective therapeutic strategy for the management of T2DM [20]. Indeed, aerobic and regular exercise improves metabolic status and insulin sensitivity, reducing the risk of cardiovascular disease [21]. Data from the literature suggest that acute exercise increases oxidative stress, in contrast to chronic exercise, in which adaptation to the stimulus decreases oxidative stress. Therefore, it appears that acute exercise induces the generation of ROS whereas exercise training maintains redox homeostasis [22–24]. Concurrently, while a single bout of acute exercise is accompanied by a proinflammatory response that in many aspects is similar to those induced by infection and sepsis [25, 26], regular exercise has anti-inflammatory effects [27, 28]. Therefore, the objectives should be defined for each case, in a way that does not impair or overstimulate an immune response. Although there are clear benefits of exercise practice in diabetic patients, a detailed comprehension of the molecular basis underlying these helpful effects remains incomplete.

Based on the current literature, as well as on our knowledge concerning the effects of exercise training in an obese animal model of T2DM, the Zucker Diabetic Fatty (ZDF) rats, this paper will briefly review, firstly, the key pathophysiological aspects of the disease, focusing on the involvement of oxidative stress and inflammation and then the use of regular physical exercise of moderate intensity (training) as a strategy to improve antioxidant and anti-inflammatory status in T2DM.

2. Oxidative Stress and Inflammation in Type 2 Diabetes Mellitus

2.1. Oxidative Stress and T2DM. Increasing evidences link free radicals and oxidative stress to the pathogenesis of T2DM and development of complications [12, 29–32]. Several studies, both in animal models of diabetes and in diabetic patients, have shown that elevated extra- and intracellular glucose concentrations result in oxidative stress and contribute to the development and progression of diabetes and related complications [33–37].

Major sources of oxidative stress during diabetes include glucose autooxidation, overproduction of ROS by mitochondria, nonenzymatic glycation, and the polyol pathway [38, 39]. In the latter, aldose reductase converts glucose into sorbitol with NADPH as a coenzyme; in diabetic conditions, increased flux through the polyol pathway enhances oxidative stress due to increased consumption of NADPH by aldose reductase. Since NADPH is required for generation of endogenous antioxidant glutathione (GSH), reduced NADPH availability depletes GSH, leading to greater oxidative stress [40, 41] (Figure 1). Other mechanism through which diabetes can increase oxidative stress involves electron transport in mitochondria. It has been proposed that high intracellular glucose levels increase the transfer of electrons through the electron transport chain in mitochondria during oxidative respiration, generating ROS [40, 42]. Furthermore, changes caused by diabetes alter the redox balance and affect redox-sensitive proteins, such as protein kinase C-epsilon, which enhances mitochondrial ROS production. Additionally, advanced glycation end-products (AGEs) generated under conditions of hyperglycemia stimulate NADPH oxidase that, in turn, can induce production of ROS (Figure 1). In a surprising development, augmented Wnt signaling stimulates mitochondrial biogenesis that can lead to increased ROS levels in mitochondria and greater oxidative damage [43]. Increased mitochondrial ROS is harmful by several reasons, including the damages caused on mitochondrial components, such as DNA, membrane proteins and lipids; opening of the mitochondrial permeability transition pore (MPTP) [44], thus releasing proapoptotic proteins from the mitochondria, such as cytochrome c, that stimulate cell death. ROS generated in the mitochondrial respiratory chain have been proposed as secondary messengers for activation of NF-κB by TNF-α and IL-1 [42] (Figure 1). Although most data demonstrate mitochondria ROS overproduction (first of all superoxide) in diabetes and diabetic complications, some studies suggested that there are other key sources responsible for ROS overproduction (oxidative stress) in diabetes, such as glucose-stimulated superoxide formation catalyzed by NADPH oxidase [45, 46], or insulin (that stimulate superoxide formation catalyzed by NADPH oxidase) or even superoxide production catalyzed by xanthine oxidase [47, 48]. Other studies have referred the role of lipoxygenases as producers of reactive radicals during enzymatic reactions [49, 50]. Lipoxygenase products, especially 12(S)-HETE and 15(S)-HETE, are involved in the pathogenesis of several diseases, including diabetes, where they have proatherogenic effects and mediate the actions of growth factors and proinflammatory cytokines [49, 50].

Nonmitochondrial sources of ROS also include cyclooxygenase (COX) enzymes, which catalyze the synthesis of various prostaglandins. Pro-inflammatory cytokines seem to induce COX2 expression through NADPH oxidase stimulation and ROS production. Elevated levels of glucose are able to induce endothelium-derived vasoconstrictor prostanoids [51], suggesting a role for COX2 in diabetic vasculopathies. Further evidence supporting a role for oxidative stress in the induction of COX expression is the fact that expression of COX enzymes is normalized by glycemic control [52], and also by inhibition of oxidative phosphorylation, protein kinase C, NF-κB [42] or by mutation of the NFκB binding elements at the COX2 promoter site [53].

Another source of ROS is the cytochrome P450 monooxygenases, a large category of enzymes involved in the metabolism and detoxification of endogenous and exogenous compounds [54]. Diabetes affects the different isoforms of the cytochrome P450 system and seems to be responsible for adverse hepatic events associated with T2DM [54]. For example, there is an increased expression of CYP2E1 in T2DM [55] and in ob/ob mice and male fatty Zucker rat [56]. Due to a low degree of coupling between enzyme turnover and substrate binding, CYP2E1 has an unusually high capacity of generating free radicals, which are thought to result in lipid peroxidation, thus contributing to liver disease,

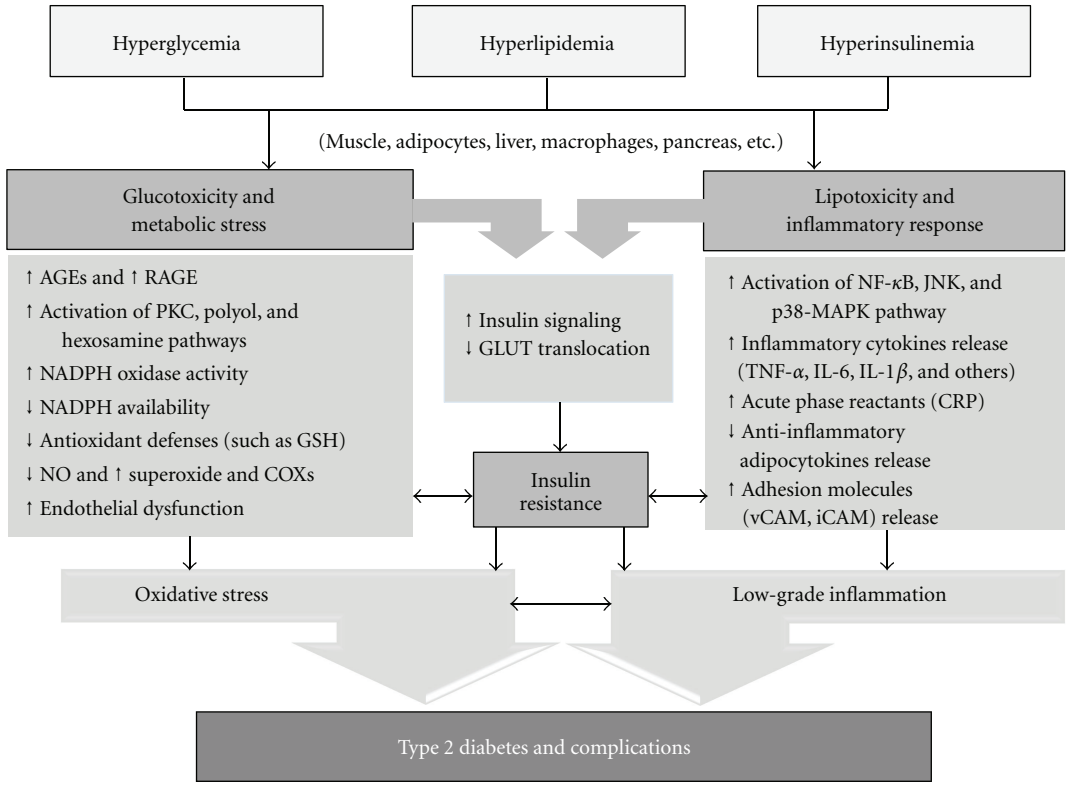

Figure 1: Schematic illustration of some of the key pathophysiological aspects involved in the development of T2DM, focusing on the involvement of oxidative stress and inflammation and underlying cellular/molecular mechanisms. AGEs, advanced glycation end-products; COXs, cyclooxygenases; CRP, C-reactive protein; GLUT, glucose transporter; GSH, endogenous antioxidant glutathione; iCAM, intracellular adhesion molecule-1; IL-1β, interleukin 1β; IL-6, interleukin 6; JNK, Jun N-terminal kinase; p38-MAPK, mitogen-activated protein kinase; NADPH, nicotinamide adenine dinucleotide phosphate; NF-KB, factor nuclear kappa B; NO, nitric oxide; PKC, protein kinase C; RAGE, receptor of advanced glycation end-products; TNF-α, tumor necrosis factor alpha; vCAM, vascular cell adhesion molecule.

including nonalcoholic steatohepatitis (NASH), which is closely associated with T2DM [57].

ROS activate a number of stress-sensitive kinases, whose downstream effects mediate insulin resistance. Therefore, activation of these kinases upregulates and activates nuclear factor kappa B (NFκB) and activator protein-1 (AP-1), which subsequently (i) activate c-Jun N-terminal kinase (JNK) and inhibit NFκB kinase-β (IKK), (ii) transcriptionally upregulate pro-inflammatory cytokine genes, and (iii) increase the synthesis of acute phase reactants (Figure 1).

2.2. Inflammation in T2DM. Obesity, as a result of inactivity and/or overeating, plays a key role in the development of insulin resistance and pancreatic beta-cell dysfunction. Increased triglycerides (TGs) stores, especially in visceral or deep subcutaneous adipose tissues, lead to large adipocytes which are resistant to insulin-evoked lipolysis suppression, then resulting in increased release of free fatty acids (FFAs) and glycerol. This "dyslipidaemic phenotype of diabetes," characterized by increased content of TGs and oxidized low density lipoproteins (ox-LDL), together with decreased levels of high density lipoproteins (HDL), is responsible for the lipotoxicity profile of diabetes (Figure 1). Lipotoxicity has been used to describe the deleterious effect of tissue fat accumulation on glucose metabolism and includes the notion that increased plasma FFA/intramyocellular levels of toxic lipid metabolites (such as long-chain fatty acyl CoAs, diacylglycerol and ceramides) play a role in the pathogenesis of muscle/liver insulin resistance [58].

Additionally, fat cells produce adipocytokines, interacting with several tissues such as muscle, liver, and arterial tissue where they exert deleterious effects on metabolism and vascular function. The adipose tissue of obese and T2DM individuals is infiltrated by mononuclear cells and is in a state of chronic inflammation [59]. The adipocytes and infiltrated macrophages secrete proinflammatory/prothrombotic cytokines, such as the TNF-α, interleukin-6 (IL-6), resistin, adipsin, acylation-stimulating protein (ASP), plasminogen activator inhibitor 1 (PAI-1) and angiotensinogen, that promote atherogenesis and cause insulin resistance. Adipocytes also produce adiponectin, a potent insulin-sensitizing and antiatherogenic cytokine, now included in a vast group of substances named adipocytokines. Low adiponectin levels have been correlated with visceral obesity and whole-body insulin sensitivity [60]. This fat cell hormone acts as an

insulin sensitizer, inhibiting TGs formation in liver and stimulating fatty acid oxidation in muscle through 5′ adenosine monophosphate-activated protein kinase (AMPK) and peroxisome proliferators activated receptor alpha (PPAR-α) [61]. Despite their apparent importance in the insulin resistance syndrome, the aforementioned adipocytokines are just examples of a family of adipocyte-derived factors that modulate insulin resistance and systemic inflammation. Besides new adipocytokines, also certain myokines appear to affect insulin sensitivity and inflammatory responses. As such, the list of insulin (de)sensitizing proteins and cytokines is still far from complete. The secretion of cytokines depends not only on the amount of adipose tissue but also of its location visceral or intra-abdominal fat being more harmful than subcutaneous fat. The pro-inflammatory effects of cytokines occur via signaling cascades involving NF-κB and JNKs pathways [62, 63]. The increase of pro-inflammatory cytokines, associated with the dyslipidemic profile in T2DM, modulates the function and survival of pancreatic beta-cells. Several studies showed that exposure of beta-cells to high levels of saturated fatty acids and lipoproteins leads to their death. This effect is accelerated by hyperglycemia, demonstrating that lipotoxicity and glucotoxicity, in concert, determinate beta-cell failure [64–67] (Figure 1).

Inflammation has long been considered as a major risk factor in diabetes and associated with development and progression of diabetic complications [68]. Hyperglycemia-induced oxidative stress promotes inflammation through increased endothelial cell damage, microvascular permeability, and increased release of pro-inflammatory cytokines, including TNF-α, IL-6, and IL-1β, ultimately leading to decreased insulin sensitivity and evolution of diabetic complications [69, 70] (Figure 1).

2.3. The Oxidative-Inflammatory Cascade in T2DM. The above considerations direct us to consider a tight interaction between inflammation and oxidative stress that may be referred as the oxidative-inflammatory cascade (OIC) in T2DM. According to Lamb and Goldstein (2008), the OIC is a delicate balance modulated by mediators of the immune and metabolic systems and maintained through a positive feedback loop [1]. Within this cascade, ROS from the immune system, adipose tissue, and mitochondria mediate/activate stress-sensitive kinases, such as JNK, protein kinase C (PKC) isoforms, mitogen-activated protein kinase (p38-MAPK) and inhibitor of kappa B kinase (IKK-b). These kinases activate the expression of pro-inflammatory mediators, such as TNF-α, IL-6, and monocyte chemoattractant protein-1 (MCP-1). The action of TNF-α, MCP-1, and IL-6, locally and/or systemically, further induces the production of ROS, thus potentiating the positive feedback loop [71] (Figure 1).

The vascular dysfunction accompanies T2DM and it seems to be caused by the ROS-dependent adhesion molecules, such as intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), which facilitate the attraction, adhesion, and infiltration of white blood cells into sites of inflammation and the formation of vascular dysfunction [72, 73]. The OIC-activated kinases are mainly responsible for the development of insulin resistance [74–76], beta cell dysfunction [77–79] and vascular dysfunction [80–82]. Therefore, modulation of OIC mechanisms involved in metabolic and immune processes can improve glucose metabolism, insulin resistance, vascular function and, consequently, delay the development of T2DM (Figure 1).

3. Antioxidant and Anti-Inflammatory Effect of Exercise Training in T2DM

A sedentary lifestyle is a risk factor for T2DM, with several clinical studies illustrating a reduction of mortality and morbidity in physically active individuals compared to sedentary individuals [83–85]. Exercise or physical activity may contribute to ameliorate insulin resistance by improving insulin action and vascular function (via increased nitric oxide (NO) bioavailability) as well as by increasing ROS-detoxification and decreasing ROS generation [86–89]. Even though the data obtained from animal studies cannot be directly extrapolated to humans, animal models of T2DM can offer excellent opportunities to evaluate experimental conditions and to assess tissues that cannot be tested in humans. Therefore, experimental studies have been contributing to improve the knowledge about the endocrine, metabolic, and morphological changes underlying the pathogenic mechanisms of the disease, as well as about the effectiveness of therapeutic options. In the following topics, we will review the benefits of regular aerobic exercise practice on antioxidant defenses and on inflammatory markers of T2DM, based on the information already available in the literature, from both clinical and experimental studies, as well as based on our experiments using the ZDF rat as a model of obese T2DM.

In order to avoid repeating the information throughout the text, the physical exercise program presented in our studies, which will be mentioned during the paper, was a regular and moderate intensity aerobic exercise (defined as training), consisting of 12 weeks (1 h/day, 3 times/week) of swimming program, voluntary, for both diabetic ZDF *fa/fa* rats and lean (ZDF +/+) animals, between 8 and 20 weeks of age [90–92]. The animals were maintained under controlled temperature (22°C), humidity (60%), and lighting (12 h of light) conditions, given a rodent maintenance chow (A-04 Panlab, Barcelona, Spain) adjusted to their respective weights (100 mg/g of weight) and distilled water *ad libitum*. They perform their exercise in a cylindrical tank, 120 cm in diameter and 80 cm in height, containing water with a controlled temperature (30–32°C); the animals were placed in the tank every day at the same hour (09.00–10.00 h) under the supervision of the same person; the swimming period was initially for 15 min/d and was gradually increased such that the rats were able to perform exercise for 60 min/d, which was achieved in 1 wk; after 1 wk of this training period, the rats were made to swim for 1 h, three times a week; at the end of each exercise session, the animals were dried and kept in a warm environment; the sedentary rats were kept in the container where the swimming sessions were held for

a period of 60 min to ensure that these control rats underwent the same amount of stress as the test animals that performed exercise. To minimize the acute effects of the exercise, exercised animals were sacrificed 48 h after the end of the last training session. The night before sacrifice, food was removed from the animals cages.

3.1. Exercise Training as a Natural Antioxidant in T2DM

3.1.1. Exercise and Oxidative Stress. In order to maintain homeostasis, cells have developed highly complex enzymatic and nonenzymatic antioxidant systems which, working synergistically, can protect the body against free radical-induced damage. Enzymatic antioxidants include GLPx, CAT, SOD, HO-1, NAD(P)H quinone oxidoreductase-1 (NQO-1), and thioredoxin [93]. Nonenzymatic antioxidants include vitamins E and C, thiol antioxidants (glutathione, thioredoxin), among others [94]. In brief, SOD promotes the dismutation of the superoxide radical to form hydrogen peroxide (H_2O_2) and oxygen; glutathione peroxidase (GPx) uses GSH as a reducing equivalent to reduce H_2O_2, thus generating oxidized glutathione and water; catalase converts H_2O_2 to water and oxygen; GSH can remove oxygen radicals directly and assist in the recycling of vitamins C and E; peroxiredoxin III, which is a member of a newly identified family of peroxidases, is localizated within the mitochondria and seems to be a critical regulator of mitochondrial H_2O_2 concentrations, which promotes apoptosis in cooperation with other mediators of apoptotic signaling [95, 96]. All of these antioxidants are able to combine with ROS, generating less reactive species. Since production of ROS is a result of normal aerobic metabolism, under physiological conditions they are efficiently removed by cellular antioxidant systems.

Several studies have shown that chronic exercise training positively alters the oxidative homeostasis of cells and tissues by decreasing the basal levels of oxidative damage and increasing resistance to oxidative stress [97–101]. In fact, regular exercise causes adaptations in the antioxidant capacity, protecting cells against the harmful effects of oxidative stress, thus preventing cellular damage [102]. In healthy elderly men, after habitual physical activity, an enhancement of intrinsic antioxidant potential, and a reduction in lipid peroxidation occurs [103]. Adaptation to oxidative stress in trained individuals is clearly evidenced by a decrease in DNA damage, by sustained levels of protein oxidation and by an increment of resistance against chronic administration of hydrogen peroxide [103]. Training is also able to alter the metabolism of purines, reducing the availability of substrate for xanthine oxidase (XO) in the trained muscle and plasma content of hypoxanthine and uric acid [104]. Previous research has shown that exercise and physical activity upregulate antioxidant defences, which is the case of SODs in the cardiovascular systems [105, 106]. Furthermore, the "nuclear factor erythroid 2-related factor 2 (Nrf2)" has recently been described as an important transcription factor against oxidative stress in health and during diabetes [107]. The ability of exercise to induce ROS activates Nrf2, which increase the expression of antioxidant enzymes, such as GPx, GST, and HO-1. However, there are no clear evidences concerning the putative influence of exercise training in Nrf2 signaling [108–110]. The importance of HO-1 in the antioxidant defense system occurs from an induction of ferritin synthesis, which diminishes the cellular pool of free iron and also from the enhancement of bilirubin levels, which are potent antioxidants [111, 112].

The above-reported protection happens under conditions of moderate exercise, while exhaustive exercise can clearly be damaging. During acute or extenuating conditions, exercise enhances the body's hemodynamic and metabolic responses [113]. An immediate effect of exercise is the increased maximal oxygen consumption ($\dot{V}O_2$ max) and metabolic activity, due to an increase in muscle contraction as a result of physical activity [114]. This condition leads to an imbalance between free radicals and antioxidants, as the increased consumption of oxygen for respiration generates increased amounts of ROS, mainly through leakage of electrons from the mitochondrial electron transport chain and the oxidation of xantine by xantine oxidase [115]. Despite the paradox that exhaustive exercise might induce ROS formation, mild oxidative stress produced by regular exercise appears to be able to reduce oxidative damage, as above described. The adaptive response, however, does not only depend on the degree of stress but also on preexisting conditions, as well as age, of the exercising subject.

3.1.2. Exercise and Oxidative Stress in T2DM. As commented above, in T2DM exercise decreases ROS generation, ameliorates insulin resistance, and improves vascular function [116]. Our group has demonstrated in diabetic ZDF animals submitted to a 12-week swimming training protocol (3 h/week they will perform at a metabolic rate of 2-3 METs with a $\dot{V}O_2$ ranging from 46 to 63 $mL \cdot min^{-1} \cdot kg$ which means a moderate intensity exercise ~45–65% of $\dot{V}O_2$ max) an amelioration of insulin resistance and diabetic dysmetabolism. A decrease in systolic and mean blood pressure and in heart rate, alongside a diminishment of differential pressure, was also observed. The reduction of blood pressure suggests an improvement of vascular arterial compliance, with reduction in cardiac work and left ventricular hypertrophy amelioration [92, 117, 118]. The regular exercise was able to prevent serum oxidative stress, viewed by the reduction of lipid peroxidation, evaluated by malondialdehyde (MDA) levels, and by the increment of serum total antioxidant status (TAS) and SOD activity (Figures 2(a) and 2(b)), thus reinforcing the antioxidant action of training. Furthermore, the reduction of serum 3-nitrotyrosine (3-NT) levels in the trained diabetic rats suggests a decrease in peroxynitrite contents (Figure 2(c)). Our results were in agreement with others that have reported increased NO production in subjects who practiced chronic exercise, coincident with decrease in blood pressure and platelet activation [119]. Most of the clinical and experimental studies have reported beneficial effects of regular physical activity in increasing NO bioavailability and in reducing oxidative stress [120–122]. Physical activity increases eNOS expression and/or eNOS Ser phosphorylation [123], leading to a reduction of ROS generation, as well as to a beneficial influence on gene expression of antioxidant

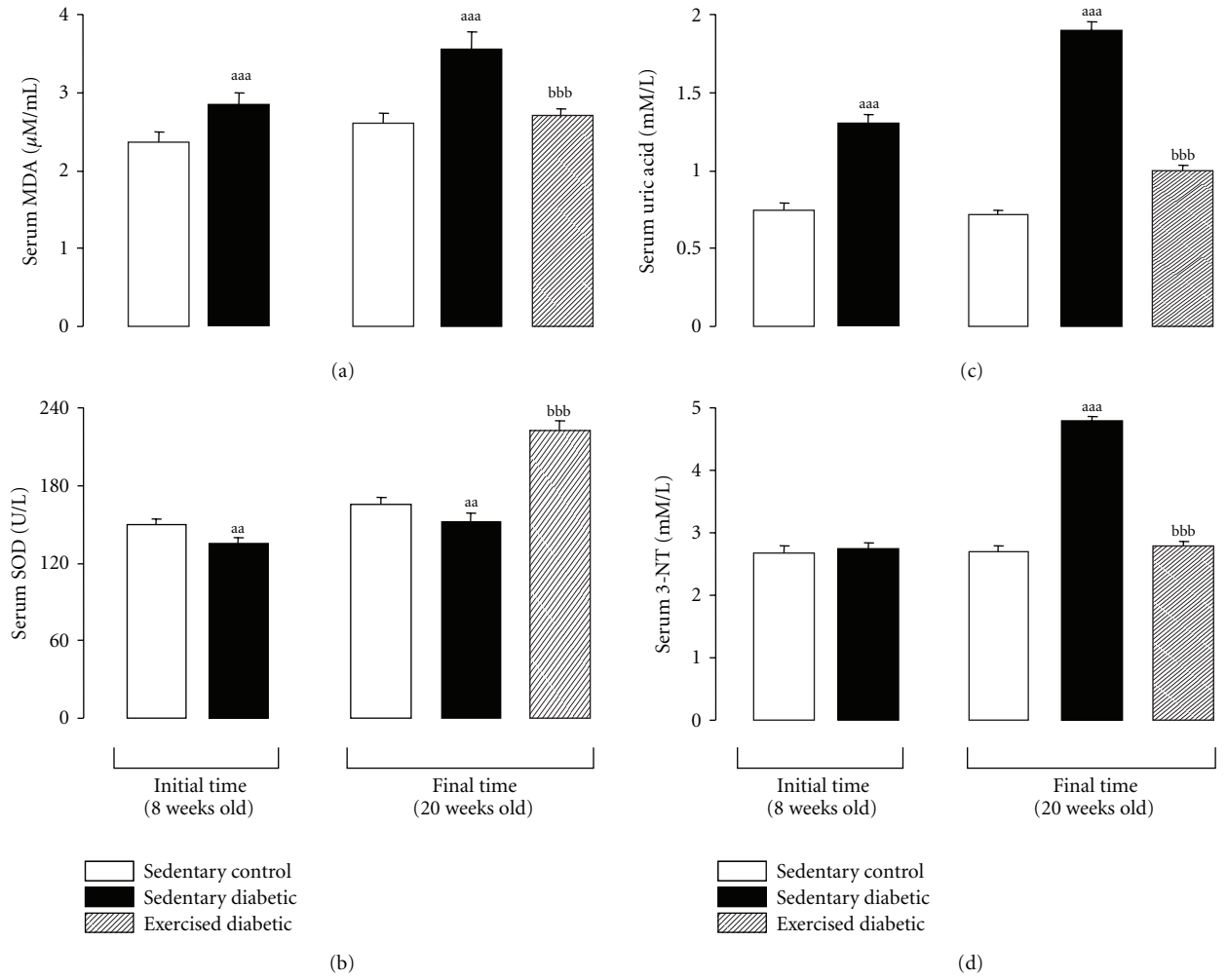

Figure 2: Evolution of serum MDA (a), SOD (b), uric acid (c), and 3-NT (d) levels between the initial time (8 weeks old) and the final time (20 weeks old) in sedentary control and diabetic rats and in diabetic exercised rats. Data are means ± sem of eight values (rats) per group. Significant differences between sedentary diabetic and sedentary control rats: $^{aa}P < 0.01$ and $^{aaa}P < 0.001$. Significant differences between exercised diabetic and sedentary diabetic rats: $^{bbb}P < 0.001$. MDA, malondialdehyde; SOD, superoxide dismutase; 3-NT, 3-nitrotyrosine.

enzymes, which promotes protective adaptations [124]. The upregulation of antioxidant defenses in animal models of T2DM was also observed by Nishida et al. [104], which also reported increased Cu/Zn-SOD protein production as a results of low-intensity exercise, in contrast with increased Mn-SOD after moderate intensity exercise [104]. Nevertheless, additional work is needed to assess the importance and physiological roles of this preferential upregulation in SODs by exercise in diabetes.

Although antioxidant properties have been attributed to uric acid, high levels of uric acid are strongly associated with the development of hypertension, visceral obesity, insulin resistance, dyslipidaemia, T2DM, kidney disease, and cardiovascular events [125, 126]. Several studies suggest that, under certain concentrations, uric acid might have antioxidant activity, preventing lipid peroxidation; nevertheless, its association with chronic disease highlights the uric acid oxidant-antioxidant paradox [127]. Ideally, exercise training should be able to reduce pro-inflammatory levels of uric acid to antioxidant and protective levels. The results of de Lemos et al. [91] in ZDF rats submitted to 12 weeks of swimming training showed a decreased serum uric acid, to levels near those of the control rats (Figure 2(d)). Studies from other authors have reported that a six-month moderate intensity (50–70% HR_{max}) aerobic exercise is able to decrease lipid peroxidation, as well as to increase GSH and catalase activity in T2DM and obese individuals [128, 129]. A similar study in obese individuals reported attenuation in exercise-induced lipid peroxidation following 24 weeks of a moderate intensity resistance training [130]. More recently, Oliveira et al. [131] compared the effects of 12 weeks training with 3 different types of exercise (aerobic training, strength training and combined training) on T2DM male and female human subjects, demonstrating that the aerobic training program provided important upregulation in antioxidant enzymes and increased NO bioavailability, which may help in minimizing oxidative stress and the development of the chronic complications of diabetes.

3.2. Exercise Training as a Natural Anti-Inflammatory in T2DM

3.2.1. Exercise and Inflammation. The effects of regular or chronic exercise on basal levels of inflammatory markers have been used to recommend exercise as an anti-inflammatory therapy. According to Kasapis and Thompson, a single session of exercise triggers an increase in pro-inflammatory cytokines release, associated with leukocytosis and increased plasma concentration of CRP [132]. This pro-inflammatory response to acute exercise is accompanied by a sudden increase in oxidative stress, followed by adaptive mechanisms against inflammation [133]. Moreover, a longitudinal study showed that regular training induces a reduction in CRP levels, suggesting an inflammatory action, visible in several conditions, including T2DM, insulin resistance, and other cardiovascular/cardiometabolic diseases. Regular exercise is associated with decreased contents of CRP, IL-6, and TNF-α and, simultaneously, increase of anti-inflammatory substances, such as IL-4 and IL-10 [108], reinforcing the anti-inflammatory nature of exercise [134, 135].

Cytokines are released not only from mononuclear cells but also from muscle cells. Starkie et al. showed that physical exercise directly inhibits endotoxin-induced TNF-α production in humans, most likely through IL-6 release from exercising muscle [136]. Typically, IL-6 is the first cytokine present in circulation after exercise practice, followed by an increase in IL-1ra and IL-10 [137]. The ubiquitous role of IL-6 and the hypothesis of an exercise-induced anti-inflammatory IL-6 release were recently reviewed [138, 139]. Therefore, IL-6, a multifactorial cytokine, regulates cellular and humoral responses and plays a pivotal role in inflammation, being associated with several pathological conditions, including T2DM, and thus emerging as an independent early predictor for T2DM and as a marker of low-grade inflammation [138, 139]. However, what is even more interesting concerning IL-6, as Fisman and Tenenbaum [138] commented, are the putative beneficial effects played as an anti-inflammatory factor, which is particularly evident in insulin sensitivity during exercise [138]. Therefore, a marked increase in circulating levels of IL-6 after exercise without muscle damage has been a remarkably consistent finding. The magnitude by which plasma IL-6 increases is related to exercise duration, intensity of effort, muscle mass involved in the mechanical work and endurance capacity [140]. IL-6 has been indicated as the strongest candidate for the humoral factor released after exercise, working in a hormone-like fashion, in which it is released by the muscle, now viewed as an endocrine organ, for influencing other organs [139]. Although this hypothesis requires further clarification, the role of IL-6 as both the "good" and the "bad," depending on the circumstances, as commented by Fisman and Tenenbaum [138], opens a new angle on the way interleukins act, and in particular concerning the effects of exercise in insulin resistance and diabetes. In this anti-inflammatory environment, IL-6 inhibits TNF-α production, as previously reported in animals [141]. Furthermore, exercise also suppresses secretion of TNF-α by pathways independent of IL-6, as shown by the results obtained with knockout mice for IL-6 submitted to exercise [142]. Consistent with the improvement in inflammatory status, exercise also interferes with circulating adiponectin levels in T2DM.

The anti-inflammatory nature of exercise training has been associated to a reduced cardiovascular disease, particularly due the training-evoked increased expression of antioxidant and anti-inflammatory mediators in the vascular wall, which could directly inhibit atherosclerosis development [143]. The available information concerning the effects of physical exercise on adiponectin levels is scarce and divergent [144]. Several studies showed that chronic exercise (programs of 6 weeks to 6 months) did not induce changes in adiponectin levels [145]. Kriketos et al. also reported, after 2-3 sessions of moderate exercise, a remarkable increase in adiponectin levels (260%) that remained elevated for 10 weeks, without body weight modifications [146]. The systematic review performed by Simpson and Singh [144], considering literature searches in databases conducted from ten years and including 8 randomized controlled trials, concluded that exercise of varying prescription increase serum adiponectin in 38% of the trials, demonstrating small-to-moderate effect sizes [144]. Nevertheless, the same study showed inconsistent data in the literature for increasing adiponectin levels after short-term exposure to robust aerobic or resistance training of moderate-to-high intensities, reinforcing the need for more studies reporting reliable findings concerning a clear relationship between changes in adiponectin contents and exercise mode, intensity, and frequency [144]. However it has been shown that muscle Adipo R-1 is elevated in response to physical exercise [147], which elevates metabolic signal transduction of adiponectin, then improving oxidative metabolism. Therefore, the regulation of these adipocytokines, including adiponectin, is likely to contribute to the prevention of T2DM by chronic exercise.

3.2.2. Exercise and Inflammation in T2DM. The protective effect of exercise against chronic inflammation associated diseases may, to some extent, be ascribed to an anti-inflammatory activity. Several studies show that markers of inflammation are reduced following longer term behavioral changes involving reduced energy intake and increased physical activity [134]. The data mentioned herein highlighted the idea that the beneficial effect of exercise seems to be related to its ability to decrease inflammatory cytokines levels and/or increase anti-inflammatory ones, which might be also true for pathological conditions, such as T2DM. The results of the studies conducted by de Lemos et al., above mentioned [90, 117, 118], clearly demonstrated the anti-inflammatory capacity of swimming exercise training in diabetic ZDF rat. Actually, training was able to prevent the increase of pro-inflammatory cytokines and CRP observed in the diabetic rats. Those findings were in the line of those of Martin-Cordero et al., which found that obese Zucker rats, a model of metabolic syndrome, present an impairment of pro-inflammatory cytokines (TNF-α, IL-6, IL-1beta and interferon gamma(IFN-γ)) release by macrophages, an effect that was improved by habitual physical activity [148, 149]. de Lemos et al. [91] also found an increment of serum

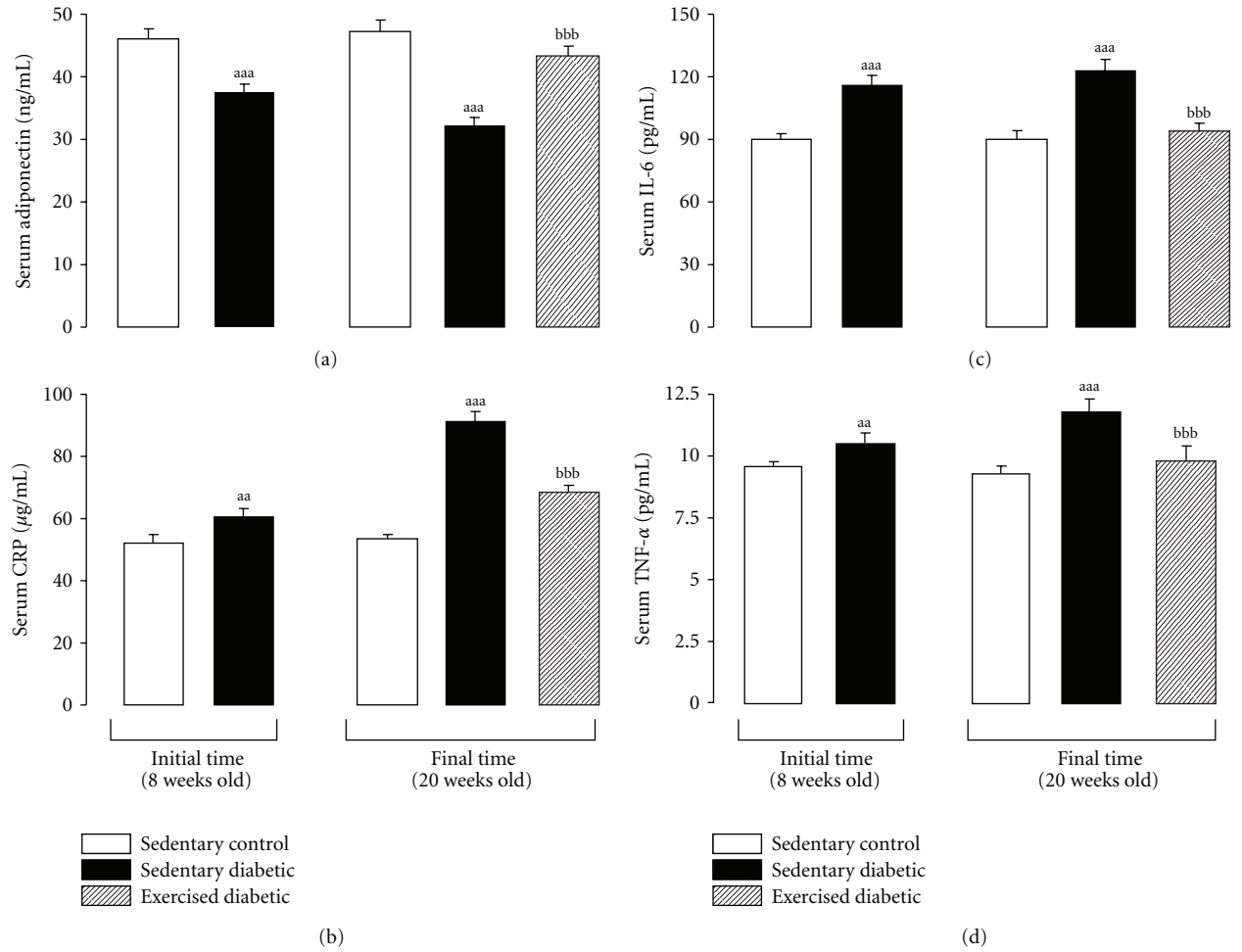

Figure 3: Evolution of serum adiponectin (a), CRP (b), IL-6 (c), and TNF-α (d) levels between the initial time (8 weeks old) and the final time (20 weeks old) in sedentary control and diabetic rats and in diabetic exercised rats. Data are means ± sem of eight values (rats) per group. Significant differences between sedentary diabetic and sedentary control rats: $^{aa}P < 0.01$ and $^{aaa}P < 0.001$. Significant differences between exercised diabetic and sedentary diabetic rats: $^{bbb}P < 0.001$. CRP, C-reactive protein; IL-6, interleukin 6; TNF-α, tumour necrosis factor alpha.

adiponectin in trained obese diabetic ZDF (*fa/fa*) rats to levels near those found in the control lean rats (Figure 3(a)) [91]. Adiponectin anti-inflammatory actions have been associated with an improvement of cardiometabolic profile, which might be due, at least in part, to regulatory actions on other factors, including on CRP, IL-6, and TNF-α levels [150], which was also demonstrated in our study using the ZDF rat submitted to swimming exercise training (Figures 3(b), 3(c), and 3(d)). Considering that the adiponectin measurement was performed 48 hours after the last training session, the results may suggest an extension of the anti-inflammatory effect obtained by a single bout of exercise.

Pancreatic islets from type 2 diabetic patients present amyloid deposits, fibrosis, and increased cell death, which are associated with the inflammatory response [151]. T2DM is also characterized by hyperglycemia, dyslipidemia, increased circulating inflammatory factors and cellular stress, which are critical in precipitating islet inflammation *in vivo*. Chronic exposure of beta-cell to these mediators induces excessive production of ROS and activation of caspases, which inhibit insulin secretion and promote apoptosis of pancreatic beta-cells [152]. The impact of islet-derived inflammatory factors and islet inflammation on beta-cell function and mass may be either beneficial or deleterious. Therefore, depending on their roles in regulating pancreatic beta-cell function, some cytokines are protective while others can be detrimental. Actually, chronic exposure of islets to IL-1β, IFN-γ, TNF-α, and resistin inhibits insulin secretion and induces beta-cells apoptosis [153]. Other cytokines, such as adiponectin and visfatin, exert protective effects on pancreatic beta-cell function. In addition to circulating cytokines, islets also produce a variety of cytokines in response to physiologic and pathologic stimuli, and these locally-produced cytokines play important roles in regulation of pancreatic beta-cell function as well [153]. To maintain the normal pancreatic beta-cell function, the deleterious and protective cytokines need to be balanced. The abnormal control of cytokine profile in islets and in plasma is associated with pancreatic beta-cell

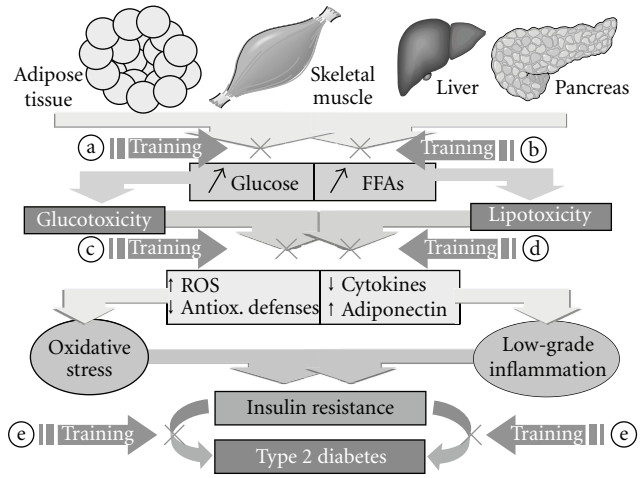

Figure 4: Schematic illustration of the proposed effects of regular physical exercise (training) in type 2 diabetes: exercise training exerts antihyperglycaemic (a), antidyslipidaemic (b), antioxidant (c), and anti-inflammatory (d) effects and thus prevents/delays the development of T2DM (E). FFAs, free fatty acids; ROS, reactive oxygen species.

dysfunction and T2DM [12]. All those emerging evidences reinforce the paradigm that islet inflammation is involved in the regulation of beta-cell function and survival in T2DM. Few studies have previously reported the putative beneficial effects of exercise training on pancreas, *per se*. Studies in Otsuka Long Evans Tokushima Fatty (OLETF), Goto-Kakizaki (GK), Zucker fatty (ZF), and ZDF rats have shown improvements in whole-body insulin sensitivity and preservation of beta-cell mass after exercise training [154, 155]. Insulin sensitivity improvements by exercise may confer an indirect beneficial effect on beta-cells by decreasing insulin demand and minimizing beta-cell exhaustion, at the same time ameliorating hyperglycemia-mediated loss in beta-cell function [156]; however, a direct effect on pancreatic function could not be excluded. Although almost every study has demonstrated beta-cell mass preservation with exercise training, none of them focused on inflammation. The recognition that islet inflammation is a key factor in TD2M pathogenesis has highlighted the concern regarding the protection of pancreatic islets and endocrine function. Therefore, restoring the normal cytokine profile in endocrine pancreas and plasma may hold great promise for more efficient beta-cell dysfunction treatment and T2DM management. de Lemos et al. [92] has demonstrated, using the ZDF rat as animal model of obese T2DM, that exercise training was able to prevent accumulation of pro-inflammatory cytokines (IL-6 and TNF-α) on endocrine pancreas. A decrease in pancreas immunostaining of both cytokines was observed, suggesting a protective effect of regular physical exercise against local inflammation.

4. Conclusions and Perspectives

Type 2 diabetes, cardiovascular diseases, colon cancer, breast cancer, and dementia constitute a cluster of diseases that defines "a diseasome of physical inactivity" [157], thus being of crucial importance to understand the mechanisms underlying the deleterious effects of physical inactivity and the beneficial actions of exercise training.

Oxidative stress, as well as inflammation, plays a critical role in the pathogenesis and progression of diabetes and diabetic-associated morbility. There are multiple sources of ROS production in diabetes, including those of mitochondrial and nonmitochondrial origins. Low-grade chronic inflammation is characterized by augmented systemic levels of some cytokines and CRP. The increased production of ROS and a concomitant decline of antioxidant defense mechanisms lead to the activation of adipose tissue and mitochondria mediate/activate stress-sensitive kinases. These kinases activate the expression of pro-inflammatory mediators that further induce the production of ROS and potentiate the positive feedback loop.

Emerging evidence suggests that exercise training activates the expression of cellular antioxidant systems, but is also able to produce ROS, which are by no means detrimental. Instead, they are required for normal force production in skeletal muscle, for the development of training-induced adaptation in endurance performance, as well as for the induction of endogenous defense systems [158–160]. Regular exercise is associated with lower levels of CRP, IL-6, and TNF-α and, simultaneously, with increases in anti-inflammatory substances, such as adiponectin, IL-4 and IL-10. Therefore, regular and moderate exercise training can have antioxidant and anti-inflammatory systemic protective effects in type 2 diabetes (Figure 4). The health-beneficial effects of exercise-induced myokines and heat shock protein are also gaining increased recognition.

Considering the data now reviewed, the evidences of beneficial effects of regular exercise may contribute to a growing awareness of potential risks by sedentary populations and public authorities and to a reinforcement of exercise prescription as adjuvant to drug therapy for treatment/attenuation of T2DM and its serious complications. However, further research is required to better understand the effects

of exercise on inflammatory pathways and on the oxidative stress cascade. Furthermore, it will also be pivotal the proper establishment of type, duration, and intensity of training recommended in order to maximize the benefits of exercise training for the different subgroups of T2DM patients.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2012, Article ID 707941, 11 pages
doi:10.1155/2012/707941

Review Article

Does Vitamin C and E Supplementation Impair the Favorable Adaptations of Regular Exercise?

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Received 1 April 2012; Revised 18 June 2012; Accepted 20 June 2012

Academic Editor: Felipe Dal-Pizzol

The detrimental outcomes associated with unregulated and excessive production of free radicals remains a physiological concern that has implications to health, medicine and performance. Available evidence suggests that physiological adaptations to exercise training can enhance the body's ability to quench free radicals and circumstantial evidence exists to suggest that key vitamins and nutrients may provide additional support to mitigate the untoward effects associated with increased free radical production. However, controversy has risen regarding the potential outcomes associated with vitamins C and E, two popular antioxidant nutrients. Recent evidence has been put forth suggesting that exogenous administration of these antioxidants may be harmful to performance making interpretations regarding the efficacy of antioxidants challenging. The available studies that employed both animal and human models provided conflicting outcomes regarding the efficacy of vitamin C and E supplementation, at least partly due to methodological differences in assessing oxidative stress and training adaptations. Based on the contradictory evidence regarding the effects of higher intakes of vitamin C and/or E on exercise performance and redox homeostasis, a permanent intake of non-physiological dosages of vitamin C and/or E cannot be recommended to healthy, exercising individuals.

1. Introduction

The antioxidant vitamins C (ascorbic acid) and E (α, β, δ, and γ tocopherols and tocotrienols) are involved in protecting cellular organelles from oxidative damage [1, 2]. Exercise can increase free radical production by 2- to 4-fold [3] and produce changes in redox status which may exert oxidative stress on muscles and other tissues leading to alteration of lipids, proteins, and genetic material [4]. Short-term exercise results in temporary increases in concentrations of oxidized products [5], but habitual exercise may result in an augmented endogenous antioxidant system and a reduction in oxidized products [4, 6, 7]. Supporting endogenous antioxidant defense systems with additional oral doses of antioxidants has received much attention as a strategy to reduce oxidative stress, decrease muscle damage, and improve exercise performance.

Indeed, a significant number of athletes, including elite athletes, consume vitamin supplements seeking beneficial effects on performance [8]. However, recently, there is a growing evidence of the negative effects of antioxidant supplementation on exercise performance in both animal and human studies [9, 10]. In light of the results of these prolific studies [9, 10], it was concluded that vitamin C and/or E interfere with the adaptive responses to endurance exercise training. More studies followed trying to delineate the possible effects of antioxidant supplementation on adaptations in exercise performance and/or redox homeostasis [11–17]. In this context, we performed the present literature analysis to evaluate whether antioxidant vitamin C and/or E supplementation affect the favorable adaptations of exercise.

2. Criteria for Study Inclusion and Methodological Issues

There are too many studies to properly analyze the effect of antioxidants other than vitamins C and E (e.g., [18–25]). We have chosen to focus only on vitamins C and E for three reasons. First, because vitamins C and E are by far the two most well-characterized antioxidants. Second, because both vitamins C and E exert their antioxidant action via a common mechanism, that is by donating a hydrogen atom to a free radical [26]. Third, because there is a well-described dependency between these two antioxidants, since vitamin C recycles vitamin E via the tocopheroxyl radical [27]. Finally, it is important to consider that the number of antioxidants, aside from vitamins C and E is huge and include many ill-characterized molecules with debatable antioxidant function [28].

Eleven studies, consisting of both rat and human work, were selected for analysis (Tables 1 and 2). No restrictions were made regarding the type of exercise used (e.g., endurance or resistance). In addition, a relevant study that employed a nonphysiological exercise model, namely, in situ muscle stimulation [13], is also included, mainly because it provides unlimited access to skeletal muscle and offers mechanistic insights for the data derived from physiological models.

In order for a study to be included in the present analysis the following criteria should have been met:

(1) some manner of simultaneous implementation of chronic exercise and vitamin C and/or vitamin E supplementation for more than three weeks. This time period was judged sufficient to allow exercise training adaptations to appear;

(2) recruitment of untrained or moderately trained individuals to allow for adequate physiological training adaptations;

(3) use of appropriate control group(s);

(4) no use of other antioxidant/nutrient supplementation (e.g., carotene, lipoic acid) except for vitamin C, vitamin E, or a combination of the two;

(5) measurement of unambiguous biochemical (e.g., activity of mitochondrial enzymes or insulin sensitivity) and/or physiological endpoints of chronic exercise (e.g., VO_2 max or muscle strength).

3. Effect of Vitamin C and/or E Supplementation on Adaptations to Chronic Exercise

Asha Devi et al. [29, 30] examined the effect of vitamin E (α-tocopherol; 50 IU/kg bw/day) supplementation on the adaptations induced by 12 weeks of endurance swimming in young (4 or 8 months), middle-aged (12 months), and old rats (22 months). The untrained animals (3 males per group) were orally supplemented with vitamin E and swam for 8.5 weeks. The levels of vitamin E in the left and right ventricles of the heart of supplemented rats increased 17–44% at the end of the study. In general, the levels of lipid peroxidation in the heart (determined through thiobarbituric acid reactive substances; TBARS) were lower in the animals who received the daily dosage of vitamin E. Overall, catalase activity increased in the vitamin-E-supplemented hearts except for the very old animals (i.e., 22 months old), where no differences appeared between the supplemented and the nonsupplemented animals. Similarly, superoxide dismutase (SOD) activity in the heart was generally higher in the supplemented animals than the nonsupplemented ones. Swimming training improved the blood lipid profile of rats (i.e., elevated HDL cholesterol and lower LDL cholesterol), and this was generally more evident in the vitamin-E-supplemented rats. Finally, vitamin-E-supplemented rats exhibited higher-endurance capacity from 21 to 31% compared to the untrained counterparts. The findings of this study suggest that vitamin-E-supplementation may induce an ergogenic effect and promote favorable adaptations to blood lipid profile.

Higashida et al. [11] studied the effect of combined vitamin C (750 mg/kg bw/day) and vitamin E (α-tocopherol; 150 mg/kg bw/day) supplementation on the training-induced adaptive responses of muscle mitochondria and insulin sensitivity in untrained rats (3 males in the placebo groups aged 3 months and 6 males in the supplemented groups aged 3 months). Two studies were performed whereby a short-term study provided vitamins C and E for nine days and the animals swam for the last three days of the vitamin treatment. A longer study provided vitamin supplementation for eight weeks and in conjunction had the animals swim for the last three weeks of supplementation. No measurement of vitamins was performed in plasma or tissues. The last acute exercise session resulted in an 80% increase in plasma TBARS, a response that was prevented in the rats given vitamin C and vitamin E. Noteworthy, there was no effect of antioxidant supplementation on the resting levels of lipid peroxidation. Based on this finding, it is probable that the resting levels of many oxidative stress markers (including TBARS) can give much less or no information compared to the ones modified by an acute-exercise session. In other words, it may be easier to find an effect of antioxidant supplementation on redox status after exercise than at rest. This may render exercise as a convenient model to study redox status homeostasis. In general, both the short and the long exercise regimes induced similar adaptations. Briefly, swimming increased the protein levels of copper zinc SOD (CuZnSOD), manganese SOD (MnSOD) (only after the short-term study), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), and seven mitochondrial proteins (cytochrome oxidase I, cytochrome oxidase IV, citric synthase, ATP synthase, succinate-ubiquinone oxidoreductase, NADH-ubiquinone oxidoreductase, and long-chain acyl-CoA dehydrogenase). Exercise-induced upregulation of these proteins was unaffected by the vitamin supplementation. In addition, glucose transporter 4 (GLUT4) expression and insulin responsiveness (determined by measuring the glucose analog 2-deoxyglucose in the extracellular space and intramuscularly in an in vitro assay) increased to about the same extent in both control and vitamin-supplemented

Table 1: Basic study design characteristics of the studies investigated the effect of vitamin C and/or vitamin E supplementation on training adaptations.

Study	Species	Number, sex, and age per group	Training state	Vitamin supplementation	Exercise protocol
Asha Devi et al. [29, 30]	Rat	3 M (4, 8, 12, 22 m)	Untrained	Vit E (50 IU/kg bw/day)	Swimming (30 min, 5 sessions/week for 8.5 weeks)
Gomez-Cabrera et al. [9]	Human	5 M (29 y) or 9 M (31 y)	Untrained	Vit C (1000 mg/day)	Cycling (65−80% VO_2 max, 3 sessions/week for 8 weeks)
Gomez-Cabrera et al. [9]	Rat	6 M (3 m)	Untrained	Vit C (500 mg/kg bw/day)	Running (75% VO_2 max, 5 sessions/week for 3 or 6 weeks)
Higashida et al. [11]	Rat	3 M (3 m) or 6 M (3 m)	Untrained	Vit C (750 mg/kg bw/day) and vit E (150 mg/kg bw/day)	Swimming (6 h, 6 sessions/week for 3 days or 3 weeks)
Ristow et al. [10]	Human	10 M (26 y)	Untrained or moderately trained	Vit C (1000 mg/day) and vit E (400 IU/day)	Circuit training (65 min, 5 sessions/week for 4 weeks)
Roberts et al. [12]	Human	7 M (23) or 8 M (21)	Moderately trained	Vit C (1000 mg/day)	Interval running (50−90% VO_2 max, 50 min, 4 sessions/week for 4 weeks)
Ryan et al. [13]	Rat	7 M (3 or 30 m)	Untrained	Vit C (20 g/kg bw/day) and vit E (30 g/kg bw/day)	Maximal stretch-shortening contractions (80 contractions, 3 sessions/week for 4.5 weeks)
Theodorou et al. [14]	Human	14 M (26 y)	Moderately trained	Vit C (1000 mg/day) and vit E (400 IU/day)	Resistance training (75 maximal eccentric actions, 2 sessions/week for 4 months)
Yfanti et al. [15–17]	Human	10 M (31 y) or 11 M (29 y)	Moderately trained	Vit C (500 mg/day) and vit E (400 IU/day)	Cycling (40−85% VO_2 max, 5 sessions/week for 12 weeks)

Abbreviation: m: months; M: males; y: years.

Table 2: Redox and physiological variables measured in the studies investigated the effect of vitamin C and/or vitamin E supplementation on training adaptations.

Study	Vitamin	Redox biomarker	Training endpoint
Asha Devi et al. [29, 30]	Vit E (heart)	TBARS, catalase, SOD (heart)	Swimming time, plasma lipid profile
Gomez-Cabrera et al. [9]	Vit C (plasma)	—	VO_2 max
Gomez-Cabrera et al. [9]	Vit C (plasma)	MnSOD, GPx (muscle)	Running time, cytochrome c
Higashida et al. [11]	—	TBARS, MnSOD, CuZnSOD (muscle)	Mitochondrial proteins, GLUT4, glucose transport activity
Ristow et al. [10]	—	TBARS (plasma and muscle), MnSOD, CuZnSOD, GPx1, catalase (muscle)	Insulin sensitivity
Roberts et al. [12]	—	—	Various performance tests, substrate metabolism
Ryan et al. [13]	—	Hydrogen peroxide, 8-OHdG, MDA, 4-hydroxyalkenals, GSH, GSSG, MnSOD, CuZnSOD, GPx, catalase (muscle)	Muscle hypertrophy, muscle function
Theodorou et al. [14]	Vit C, vit E (plasma)	TBARS, protein carbonyls, GSH, GSSG, catalase, uric acid, albumin, bilirubin, TAC (plasma or erythrocytes)	Muscle performance, muscle damage
Yfanti et al. [15–17]	Vit C, vit E (plasma)	MDA, protein carbonyls, MnSOD, CuZnSOD, GPx1, catalase, (muscle)	Body composition, VO_2 max, power, insulin sensitivity, GLUT4 (muscle), glycogen (muscle), CS (muscle), HAD (muscle), lipid profile (plasma)

Abbreviations: CS: citric synthase; CuZnSOD: copper zinc superoxide dismutase; GLUT4: glucose transporter type 4; GPx: glutathione peroxidase; GSH: glutathione; GSSG: glutathione disulfide; HAD: β-hydroxyacyl-CoA dehydrogenase; MDA: malondialdehyde; MnSOD: manganese superoxide dismutase; 8-OHdG: 8-hydroxy-2′-deoxyguanosine; SOD: superoxide dismutase; TAC: total antioxidant capacity; TBARS: thiobarbituric acid reactive substances.

groups. No physiological training endpoint was measured. However, based on the fact that vitamins C and E did not affect the adaptive increases in mitochondria as a result of training, it is highly probable that no changes in endurance would have taken place. Based on the findings of this study, vitamin C and E supplementation does not have an inhibitory or a promoting effect on the adaptive responses related to mitochondria and glucose metabolism of skeletal muscle to chronic exercise.

Ryan and colleagues [13] explored the effects of chronic electrical stimulation along with vitamin C and vitamin E supplementation on redox homeostasis and muscle function in young (7 males per group aged 3 months) and old rats (7 males per group aged 30 months). The untrained rats were subjected to 80 maximal concentric-eccentric actions per session, three times per week for 4.5 weeks. Vitamin C (20 g/kg bw/day) and vitamin E (α-tocopheryl acetate; 30 g/kg bw/day) supplementation began one week before the first electrical stimulation session. The level of vitamins C and E in plasma or tissue was not determined. The researchers measured hydrogen peroxide (precursor of hydroxyl radical in the presence of ferrous iron), total and oxidized glutathione (major redox couple), 8-hydroxy-2′-deoxyguanosine (8-OHdG; biomarker of oxidized DNA), malondialdehyde (MDA; biomarker of lipid peroxidation) and 4-hydroxyalkenals (biomarker of lipid peroxidation), in muscle. Moreover, they measured the mRNA, protein and activity levels of catalase, CuZnSOD, MnSOD, and glutathione peroxidase (GPx) in muscle. Due to the large number of redox biomarkers determined and the three independent variables tested (i.e., nutrition: supplementation versus no supplementation; exercise: training versus no training; aging: young versus old), it is difficult to reconcile the effects of electrical stimulation and/or vitamin supplementation on redox homeostasis. In general, the levels of hydrogen peroxide, total glutathione, MDA, and 8-OHdG increased in skeletal muscle from young and aged animals after electrical stimulation indicating the presence of oxidative stress. These increases were attenuated or blocked in the vitamin-C- and E-supplemented animals demonstrating the antioxidant effects of vitamins C and E. In general, the activity of the antioxidant enzymes GPx, catalase, CuZnSOD, and MnSOD either increased or were not affected by electrical stimulation. The effect of vitamin supplementation on the activity of antioxidant enzymes was not uniform. Similarly, the evidence of a disparity among mRNA abundance, protein abundance, and enzyme activity was presented, indicating that it is difficult to predict the antioxidant enzyme activity from mRNA and/or protein data. Electrical stimulation increased the mass of tibialis anterior (the muscle subjected to the stimulation), and this effect appeared in both control and supplemented animals. Vitamin supplementation did not affect force, concentric work, or eccentric work of either resting or stimulated muscles in young rats. In contrast, the positive work of skeletal muscle in aged animals increased only in the vitamin supplemented rats, demonstrating an ergogenic effect of vitamins C and E in this group of animals. In summary, vitamin C and E supplementation generally reduced oxidative stress induced by electrical stimulation and improved concentric work in old animals but not in young.

Gomez-Cabrera et al. [9] investigated the effect of vitamin C supplementation on molecular and physiological adaptations which took place after running training in both animals and humans. In the animal study (6 males per group, 3 months old), untrained rats ran on a treadmill for either three or six weeks while being administered vitamin C orally (500 mg/kg bw/day). Vitamin C supplementation resulted in a threefold increase in plasma vitamin C concentration when compared to non-vitamin-C-supplemented animals. Vitamin C supplementation hindered the increase in the mRNA levels of MnSOD and GPx, the protein levels of PGC-1α, and the mRNA and protein levels of nuclear respiratory factor 1 (NRF-1) and mitochondrial transcription factor A (mTFA) induced by training. In addition, vitamin C supplementation blunted the increase of cytochrome c (i.e., a biomarker of mitochondrial biogenesis) in the trained animals. In addition, vitamin C administration largely hampered the increase in endurance capacity which occurred as a result of training. In fact, the authors reported that control rats increased running endurance by 187% whereas vitamin-C-supplemented rats increased by just 26%. In the human study (5 males in the placebo group aged 29 years and 9 males in the supplemented group aged 31 years), untrained participants cycled for eight weeks while being orally administered vitamin C (1000 mg/day). Vitamin C supplementation stimulated a 3.8-fold greater increase in plasma vitamin C concentration when compared to the non-vitamin-C-supplemented participants. No biomarkers of redox homeostasis or mitochondrial biogenesis were measured in the human study. The effects of the training protocol on VO_2 max, were dependent on whether the animals received vitamin C or not. For example, the non-supplemented humans experienced a 22% increase ($P < 0.05$) in VO_2 max whereas the participants who received vitamin C showed a nonsignificant ($P > 0.05$) increase of 11% in VO_2 max. It is worth mentioning that in both the animal and human studies a vitamin-only-supplemented sedentary control group was not included. As a result, it is not possible to discern the isolated effects of vitamin C on the measured variables. Gomez-Cabrera et al. [9] were the only researchers who studied the adaptations to vitamin C or E supplementation in two species, rats and humans, concurrently (in fact, this is the only comparative study reviewed in the present article). Comparative exercise studies are indispensable in providing a criterion as to how safe it is to extrapolate findings from animals to humans, which is frequently done by researchers in an effort to compare their findings in animals with those in humans. Gomez-Cabrera et al. [9] found that vitamin C and E supplementation caused similar changes in the adaptations to chronic exercise of both species. To our point of view, though, the major setback in translational redox biology research is the fact that, contrary to the common belief, findings from animal experiments cannot be automatically translated to humans. In fact, to the best of our knowledge, no study has ever investigated whether using laboratory animals is a valid experimental model of human redox biology in vivo. The major conclusion

of this study is that supplementation with vitamin C markedly lowers the training efficiency in both animals and humans and hinders the training-induced upregulation of antioxidant enzymes in animals.

Ristow et al. [10] evaluated the effects of a combination of vitamin C and vitamin E (α-tocopherol) on antioxidant enzymes and insulin sensitivity in the muscle of untrained and moderately trained individuals (10 males per group aged 26 years). Human participants were supplemented with vitamins C (1000 mg/day) and E (400 IU/day) and exercised for 45 minutes in a circuit fashion for four weeks. Skeletal muscle biopsies were obtained before and after the training intervention. Although, the level of vitamins C and E in plasma or tissue was not determined, the group that was supplemented prevented the increase in lipid peroxidation (measured through TBARS) in skeletal muscle after a specific short-term exercise regimen lasting three days. Exercise increased the expression of reactive species-sensitive transcriptional regulators of insulin sensitivity (i.e., peroxisome-proliferator-activated receptor gamma (PPARγ) and PPAR coactivators, PGC-1α and PGC-1β) and up-regulated antioxidant enzymes (i.e., CuZnSOD, MnSOD, and GPx1) in both trained and untrained individuals. In addition, exercise training increased insulin sensitivity as measured by glucose infusion rate during a hyperinsulinemic euglycemic clamp. Particularly, regarding the insulin sensitivity, it is of note that the euglycemic-hyperinsulinemic clamps and the muscle biopsies were performed seven days after the end of the training. The improvement in insulin sensitivity in response to training reverses rapidly after cessation of training and is no longer present after seven days [31]. Therefore, the finding of the persistent increase in insulin sensitivity in the control group is hard to explain. It is noteworthy that all of the above effects of exercise training were blocked or reduced by the vitamin C and E supplementation. Despite the fact that VO_2 max was measured before the initiation of the training, it was not measured at the end of it. Consequently, no conclusion can be drawn whether vitamin C and E supplementation also hindered the anticipated improvements in endurance capacity. In addition, a vitamin only sedentary control group was not included. In summary, vitamin C and E supplementation prevented the induction of antioxidant enzymes and insulin sensitivity after chronic exercise in both trained and untrained humans.

Roberts et al. [12] determined the effects of four weeks of vitamin C (1000 mg/day) supplementation on the training-induced improvements in exercise performance of moderately trained humans (7 males in the placebo group aged 23 years and 8 males in the supplemented group aged 21 years). A high-intensity interval running protocol lasting four weeks was implemented. The level of vitamin C before and after the supplementation was not determined. Training improved both VO_2 max and performance as measured by several physical fitness tests. In addition and as expected, training increased fat oxidation rate and decreased carbohydrate oxidation rate during acute exercise. Neither of the physical performance tests nor substrate metabolism during exercise were affected by vitamin C supplementation. In conclusion, vitamin C supplementation during four weeks of interval training did not affect training-induced improvements in exercise performance of humans.

Theodorou et al. [14] investigated the effects of a combination of vitamin C and vitamin E (α-tocopherol) along with an eccentric resistance training on muscle performance and blood redox homeostasis in moderately trained humans (14 males per group aged 26 years). This is the only study that used a resistance exercise protocol instead of an endurance protocol. In fact, an eccentric exercise model was employed to induce redox homeostasis alterations, which is characterized by long (lasting for many days after exercise) and extensive increases in oxidative stress and induce resistance training adaptations [32–34]. This type of exercise was selected for increasing the statistical power to find a significant effect if one existed. In a double-blinded fashion, men received either a daily oral supplement of both vitamin C (1000 mg/day) and vitamin E (400 IU/day) or a placebo for 11 weeks. The subjects performed an eccentric-exercise session two times per week for four weeks. Before and after the training protocol, the subjects underwent one session of acute-eccentric exercise to explore the short-term effects of eccentric exercise in both trained and untrained individuals. The vitamin-supplemented group had 36% higher plasma vitamin C concentration and 33% higher plasma vitamin E concentration than the nonvitamin supplemented counterparts. The results failed to support any effect of vitamin supplementation. Acute and chronic eccentric exercise similarly modified muscle damage, muscle performance, blood redox biomarkers, and hemolysis in both the supplemented and the nonsupplemented group. This occurred despite the fact that acute and chronic eccentric exercise induced marked changes in muscle damage, performance, and redox homeostasis.

In a large study published in a series of three articles, Yfanti et al. [15–17] investigated the effect of combined vitamin C and vitamin E (α-tocopherol) supplementation along with endurance training on redox homeostasis, training adaptation biomarkers (i.e., muscle glycogen and muscle mitochondrial enzymes), blood lipid profiles, insulin sensitivity, body composition, and endurance performance. Using a double-blinded placebo-controlled design, moderately trained young men received either oral supplementation with vitamin C and E (500 mg/day and 400 IU/day, respectively; 11 males aged 29 years) or placebo (10 males aged 31 years) before and during 12 weeks of bicycle training at a frequency of five days per week. Muscle biopsies along with blood samples were collected before and after training. The vitamin-supplemented group exhibited 37% higher plasma vitamin C concentration and 47% higher plasma vitamin E concentration than the non-vitamin-supplemented group. Lipid peroxidation (assessed through TBARS) in skeletal muscle increased after both acute and chronic exercise irrespective of vitamin supplementation. However, when several postexercise time points were combined by calculating the area under the curve, a significant increase in muscle TBARS appeared only in the vitamin C and E group and not in the placebo group. Therefore, it could be inferred that the level of lipid peroxidation in blood plasma was higher overall in the vitamin-supplemented group. These two findings

indicate prooxidant effects of vitamin supplementation. In general, training had no impact on the mRNA levels or the protein levels of catalase, CuZnSOD, and GPx1. On the other hand, vitamin C and E supplementation increased the mRNA levels of CuZnSOD and GPx1. Unexpectedly, vitamin supplementation did not affect the protein levels of these enzymes. This disparity between mRNA and protein abundance agrees with the findings reported by Ryan et al. [13] and indicates the difficulty of predicting antioxidant enzyme responses at the protein level from mRNA data. This study also measured three well-established biochemical markers of endurance training adaptation, namely, glycogen concentration and the activity of citric synthase and β-hydroxyacyl-CoA dehydrogenase in skeletal muscle. As expected, endurance training increased the content of both glycogen and the mitochondrial enzymes and this effect appeared in both vitamin- and non-vitamin-supplemented rats. Regarding the blood lipid profile, endurance training increased the HDL cholesterol similarly in the two groups, while no effects appeared in the other blood lipids. Insulin-stimulated glucose uptake (using a euglycemic-hyperinsulinemic clamp) increased similarly in both the vitamin and the placebo group in response to training. In the same way, no differences appeared between the vitamin and the placebo group in the protein content of several insulin cascade molecules. Body composition (i.e., fat mass and fat-free mass determined using dual energy X-ray absorptiometry) were not affected by the training protocol or the vitamin supplementation. Finally, exercise performance (i.e., VO_2 max, maximal power output and workload at lactate threshold) increased in both groups independently of vitamin supplementation. In conclusion, the work of Yfanti et al. [15–17] indicates that vitamin C and E supplementation, despite some effects on redox homeostasis, did not affect the biochemical and physiological phenotype of individuals trained strenuously in an endurance fashion for 12 weeks.

4. Discussion

The studies included in our paper reveal conflicting results regarding the effects of vitamin C and E supplementation on adaptation to chronic exercise. Two publications demonstrated ergolytic (i.e., negative) effects [9, 10], six papers yielded no effect [11, 12, 14–17], and two studies reported an ergogenic outcome [13, 29]. Of the two negative papers, one involved a human component and both provided an animal aspect to the investigation. The rat study was conducted with a very high dose of vitamin C (500 mg/kg body weight/day), which translates to 30–40 grams per day of vitamin C when converted to human body weight [9]. The human studies were conducted with reasonable and otherwise practical dosages: 1000 mg/day of vitamin C and/or additionally 400 IU of RRR-alpha-tocopherol over three to eight weeks [9, 10]. It is noteworthy that supplementation with just vitamin C is not comparable to supplementation with a combination of vitamins C and E, even when concentrations of vitamin C are similar. In this respect, added vitamin E might change the impact on redox homeostasis due to its antioxidant and also prooxidant properties [35].

Of the six manuscripts that demonstrated no influence of vitamin C and E supplementation on various adaptations, two were rat studies with the remainder being performed with human subjects. Again, dosing issues were prevalent in the rat studies [9, 11, 13, 29, 30] whereby dosages of vitamins were approximately 0.5–59 grams per day of vitamin C and 0.15–88 grams per day of vitamin E. Although the redox biology between rats and humans is apparently similar and animal studies are often essential before investigating humans, the dosages employed in the rat studies make it difficult to interpret biological relevance for human redox biology.

The human studies showing no effects [14–17] were conducted with reasonable dosages of vitamins C (500–1,000 mg/day) and E (400 IU) over 4–16 weeks. Moreover, Theodorou et al. [14] and Yfanti et al. [15–17] also used the same stereoisomers of alpha-tocopherol, the natural RRR form of vitamin E, and similar surrogate markers of redox homeostasis (protein carbonyls, catalase). In contrast, they tested distinct training regimens and used different outcome measurements to determine their overall outcomes (resistance versus endurance training, muscle performance versus VO_2 max, etc.). In comparison to the Ristow paper [10] that showed a negative effect of supplementation, these studies used the same type and dosage of vitamin E but utilized completely different training regimens.

Two studies revealed ergogenic effects of vitamin E supplementation or a combination of vitamins E and C [13, 29], both conducted with rats. All of the rat studies under review used high dosages of vitamin E although Asha Devi et al. [29, 30] used the lowest with about 3,500 IU of vitamin E per day over 8.5 weeks when converted to human body weight. The two studies are not comparable to human investigations regarding dosage, training regimen, and outcome measures and therefore, it is difficult to translate these results to humans.

The discrepancy among studies described above regarding the influence of vitamin C and/or E supplementation on adaptations in redox homeostasis also applies to the effects of antioxidant supplementation on alterations in redox homeostasis in response to acute exercise. In fact, most of the relevant studies have reported that vitamin C and/or E supplementation does not affect redox status [14, 18, 36–40], less studies have reported that attenuates oxidative stress [41–45] and there are even reports indicating a pro-oxidant effect [46, 47]. We believe that this "agreement on disagreement" between responses and adaptations to exercise indicates the inherent complexity of redox biochemistry and signifies the difficulty in providing unidirectional predictions after supplementation with antioxidant agents.

Another important methodological issue of the pertinent studies is that all of them employed untrained or recreationally trained individuals (i.e., VO_2 max 50–58 mL/kg/min [10, 15–17]; Theodorou et al. [14] did not report the physical capacity of the participants). This limitation has important practical applications because most of the individuals taking antioxidant supplements are actually well trained or even professional athletes. This is particularly relevant for the antioxidant supplementation field, since it has been

repeatedly reported that strenuous bouts of exercise do not result in a significant increase in blood oxidative stress in trained men [48, 49]. This is probably because participation in a regular exercise program is associated with a chronic upregulation in antioxidant defense [14, 50], which may serve to provide protection against the exercise-induced increase in free radicals. Taking these facts into account, it is possible that the possible negative effects of antioxidant supplementation on performance and redox status of well-trained individuals may be miniscule or absent considering that there is little room for improvement. Besides, none of the pertinent studies have employed females as participants. This is also an important limitation considering that, for example, lower amounts of exercise-induced lipid peroxidation may be produced in females due to increased antioxidant potential as a result of elevated estrogens levels [51].

It is worth mentioning that the properties of specific antioxidants do not necessarily translate to other antioxidants. For example, despite the fact that some studies have reported that vitamin C [9] or combined vitamin C and E supplementation [10] attenuated or prevented the exercise adaptations this does not mean in any way that all types of antioxidants induce negative effects. Indeed, the disparity noted among the vitamin C and E studies and analyzed in the present review can also be seen with other antioxidants. For example, supplementation with N-acetylcysteine or ubiquinone-10 reported to attenuate the adaptive responses to exercise [22, 52], spirulina or quercetin to enhance the adaptive responses to exercise [53, 54] as well as green tea extract or coffee berries to exert no effect on adaptations to exercise [55, 56]. There is a tendency to include molecules with heterogeneous properties, such as those presented previously, under the general heading of "antioxidants." This approach might lead to erroneous interpretations, because these molecules have different mechanisms of action and the antioxidant activity is only one of their functions, if any [57]. Therefore, one should avoid extrapolating the results from one antioxidant to another.

Why is there so much divergence among studies addressing the effect of vitamin C and/or E supplementation on exercise adaptations? We believe that the main reason is the near uniqueness of each study in terms of type of exercise (aerobic or anaerobic), species (rat or humans), age (young or old), tissue (blood or muscle), oxidant biomarker, and training endpoints examined. Other factors that could explain some of the diversity of results include nutrition, subject characteristics, exercise characteristics, and experimental error arising from the complexity of the techniques employed in redox status analysis. Finally, the lack of consensus may be partially explained by the biological variability of redox biochemistry. Thus, it is desirable to develop and achieve standardization and agreement on key influencing factors which investigators should employ when designing studies in this area of biology.

Except for the methodological factors that may be responsible for the lack of a consensus among the studies, an appropriate conceptual framework may also be needed to integrate the disparate exercise adaptations to antioxidant supplementation. Hormesis is a dose-response phenomenon characterized by either a U-shaped or an inverted U-shaped dose response depending on the endpoint measured [58, 59]. In hormesis, dose response is characterized by low-dose stimulation and high-dose inhibition, leading to the biphasic, hormetic dose-response curve [58, 59]. The conceptual framework provided by hormesis can potentially reconcile differences that emerge among relevant studies regarding the effect of antioxidant supplementation on exercise adaptations. The hormetic concept predicts that the effects of antioxidant supplementation on muscle performance and redox homeostasis are dependent on the antioxidant dose. In fact, the limited evidence indicates that too much generation of reactive species may be harmful while the modest generation may be beneficial [60]. Based on this evidence, it is plausible to assume that the divergence regarding the effects of antioxidant supplementation on exercise adaptations and redox homeostasis may partly be explained by the different degrees of reactive species decomposition. Despite the promising first data and the appealing nature of the hormesis concept, the establishment of deviation from linearity in dose-response relationships for exercise-induced alterations in redox homeostasis requires studies specifically designed to locate and describe the possible hormetic effects of exercise.

Collectively, it becomes challenging to draw clear conclusions surrounding the impact of vitamins C and E on adaptations to chronic exercise. While the selected hypotheses from these studies may seem clear and ripe for comparisons, too many differences exist among the employed methodological approaches to derive definitive conclusions regarding the influence of vitamin C and E supplementation on adaptation to chronic exercise. Therefore, we suggest that investigating colleagues of this work direct stronger attention on the following key factors:

(1) selection and detailed description of the appropriate training stimulus,

(2) determination of valid and reliable redox biomarkers such as isoprostanes and glutathione using appropriate techniques and assays,

(3) measurement of biomarkers in the same body fluids and cell materials,

(4) collection of specimens at multiple time points after exercise,

(5) application of antioxidant supplementation on already well-trained individuals,

(6) assessment of performance through valid and reliable testing procedures,

(7) assessment of training adaptations through the use of valid and reliable biochemical and physiological markers,

(8) administration of a specific dosage of vitamins C and E at a specified time during the training period,

(9) assessment of dietary intake of redox-active nutrients like antioxidants,

(10) evaluation and reporting of hydration status.

5. Conclusion and Future Perspectives

Recent studies that employed both animal and human models provide conflicting and confusing outcomes regarding the efficacy of vitamin C and E supplementation on training adaptations. A close examination of this literature reveals many situations in which methodological differences are likely contributors to the widely varied outcomes. In consideration of these shortcomings, this paper was developed to highlight key differences in the literature and state the need to develop a more widespread systematic approach to investigations in this area. Recommendations regarding key methodological considerations are provided, and it is our hope that future researchers can use this paper as a reference or an area of basis upon which to build future studies which result in consistent and meaningful outcomes that will facilitate and optimize the findings in this important area of exercise biology. Based on the conflicting evidence regarding the effects of higher intakes of vitamin C and/or E on exercise performance and redox homeostasis, a permanent intake of nonphysiological dosages of vitamin C and/or E should not be recommended to healthy individuals. This must not be confused with a high intake of fruit and vegetables, which is considered safe and beneficial.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2012, Article ID 920932, 15 pages
doi:10.1155/2012/920932

Research Article

Biomarkers of Antioxidant Status, Inflammation, and Cartilage Metabolism Are Affected by Acute Intense Exercise but Not Superoxide Dismutase Supplementation in Horses

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Received 17 March 2012; Accepted 6 May 2012

Academic Editor: Manfred Lamprecht

Objectives were to evaluate effects of (1) repetitive arthrocentesis on biomarkers of inflammation (prostaglandin E_2, PGE_2) and aggrecan synthesis (chondroitin sulfate-846; CS) in synovial fluid (SF); (2) exercise and superoxide dismutase (SOD) supplementation on biomarkers of inflammation, antioxidant status, and aggrecan synthesis, in horses. *Preliminary trial*. Standardbreds underwent four arthrocentesis procedures within 48 h and exhibited elevated CS and no changes in PGE_2. *Exercise trial*. this randomized crossover design used twelve Standardbred mares which received either treatment (3000 IU d^{-1} oral SOD powder) or placebo (cellulose powder) for 6 wks which culminated with them running a repeated sprint exercise test (RSET). Samples were collected before (PRE), during (PEAK), and following exercise (POST). Exercise resulted in increased ($P < 0.05$) antioxidant defenses including erythrocyte SOD, total glutathione, glutathione peroxidase, gene transcripts for interferon-gamma, interleukin-10, and interleukin-1β in blood, and decreased plasma nitric oxide. Exercise increased ($P < 0.05$) SF CS and adjusted-PGE_2, and higher ($P < 0.05$) CS and PGE_2 were found in hock versus carpus joints. No treatment effects were detected. Results suggest normal adaptive responses likely due to exercise-induced tissue microdamage and oxidative stress. Additional research is needed to identify benefit(s) of SOD supplementation in horses.

1. Introduction

Equine athletes suffer from challenges of the immune system and inflammation related to exercise [1–4]. Intense physical activity has been shown to induce subclinical tissue damage and a subsequent immune response involving the upregulation of inflammatory mediators termed cytokines. Exercise-induced increases in cytokine transcripts including tumor necrosis factor alpha (TNFα), interleukin-1 beta (IL-1β), interferon gamma (IFNγ), interleukin-6 (IL-6), and interleukin-10 (IL-10) are comparable to that of an acute phase immune response and have been demonstrated in horses [5, 6]. Furthermore, inflammatory processes have also been associated with redox imbalances favoring pro-oxidants, resulting in oxidative stress [7]. Exercise-induced increases in the generation of reactive oxygen species (ROS), including, free radicals have also been demonstrated in horses [6, 8–12]. Over exaggerated or sustained responses to intense exhaustive exercise may set the stage for a chronic inflammatory and/or an immunosuppressed state and a predisposition to infection, poor physical performance, and/or onset of chronic diseases [13, 14].

Specifically, degenerative joint disease is a major concern to the equine industry. Joint discomfort in horses is the most common cause of lameness, diminished athletic performance and quality of life, economic burden, and animal loss [15–18]. Due to high rates of morbidity and mortality related to arthropathies, biomarkers indicative of early changes related to the development of joint disease have been identified, providing an opportunity to detect and mitigate the disease process early on. Prostaglandin E_2 (PGE_2) is a prostanoid important for the maintenance of local homeostasis, mediation of inflammation, and sensitization to pain [19, 20]. Elevated PGE_2 concentration in

synovial fluid is considered an indicator of synovitis [21, 22] and a predictor of equine joint disease [2]. The CS-846 epitope (CS) is found on fragments of newly synthesized aggrecan that have been released from the cartilage matrix [23] and can serve as a biomarker for cartilage metabolism, specifically aggrecan synthesis, in equine joints [21, 22, 24].

Identification of nutritional interventions which are effective in supporting resilience to exercise-induced inflammation and oxidative stress is needed. Most nutritional supplements are intended to decrease onset, severity or progression of disease symptoms, but lack controlled research. With verification of safety and efficacy in the target specie, nutritional interventions may provide practical and less cost-prohibitive options compared to other medical or pharmacological therapies [25, 26]. Furthermore, the use of orally available antioxidant enzymes may have an advantage over nonenzymatic supplements, because they catalyze the detoxification of their ROS substrates in a disproportionate manner, instead of getting stoichiometrically consumed like exogenous antioxidant substrates [26].

Superoxide dismutases (SOD) are enzymatic antioxidant defenses which catalyze the dismutation of superoxide ions into oxygen and hydrogen peroxide. Studies reporting the successful use of antioxidant enzymes as dietary interventions are increasing. Furthermore, there are several reports indicating that supplementation with exogenous SOD is effective in reducing proinflammatory cytokines and inhibiting neutrophil infiltration to sites of tissue damage in several inflammation models [27, 28]. Exogenous SOD has been used in various forms, as an oral supplement [29–31], topical treatment [32], and injectable therapy [33, 34]. Effectiveness in modifying systemic inflammatory responses, antioxidant status, and chondroprotective effects in diarthrodial joints, following oral supplementation in horses, remains to be investigated. Therefore, the purpose of this study was to evaluate the effect(s) of oral SOD supplementation in horses following intense exhaustive exercise. Objectives of the current study were as follows: (1) to evaluate the effects of repeated arthrocentesis within a 48 h time frame on markers of inflammation and cartilage metabolism in synovial fluid of healthy horses at rest and (2) to evaluate the effects of a single bout of intense exhaustive treadmill exercise and exogenous SOD supplementation on biomarkers of inflammation, antioxidant status, and anabolic cartilage metabolism in healthy horses.

2. Materials and Methods

2.1. Subjects. A sample of 12 healthy, unfit Standardbred mares aged 8 ± 1 yr, weighing 513.8 ± 16.7 kg, with lameness scores of 0.3 ± 0.1 (on a scale of 0–5, with 5 being non-weight bearing; [35]), body condition scores of 5.3 ± 0.1 (on a scale of 1–9, with 1 being emaciated and 9 being morbidly obese; [36]), and per cent body fat at 20.7 ± 1.8% [37] were used in this study. Mares were housed in New Jersey at the Rutgers University Equine Exercise Physiology Laboratory on 2-acre exercise lots and were provided *ad libitum* access to moderate quality grass hay, water, and salt. They also received 1 kg of a 10% crude protein sweet feed (Nutrena Vitality, Cargill Inc., Minneapolis, MN, USA) twice daily to meet maintenance requirements. All animal procedures described herein have been approved by the Rutgers University Institutional Animal Care and Use Committee. Aspects of the experimental design were approved retrospectively.

2.2. Preliminary Trial. The objective of this preliminary trial was to evaluate effects of repeated arthrocentesis within a 48 h time frame on a marker of inflammation and anabolic cartilage metabolism in synovial fluid of healthy horses at rest. Synovial fluid samples were collected from 6 randomly selected mares out of the sample population previously described. Aseptic arthrocentesis was performed on the same radiocarpal joint space in each horse on four occasions within a 48 h time frame at the following intervals-initial joint tap (T_1), second joint tap 24 h following the initial tap (T_2), third tap 26 h following the initial tap (T_3), and a fourth tap 48 h following the initial tap (T_4). Mares were mildly sedated with 0.3–0.5 mL of detomidine hydrochloride (10 mg mL^{-1}) intravenously, 10 min prior to each arthrocentesis procedure. The synovial fluid was initially collected into sterile syringes using sterile 20 gauge one inch hypodermic needles, and then immediately transferred to prechilled 10 mL tubes containing ethylenediaminetetraacetic acid (EDTA) and placed on ice. Samples were centrifuged for 20 min at 1500 ×g to remove any cellular debris, aliquoted and stored at −80°C for later analysis of PGE_2 (R&D Systems Parameter High-Sensitivity PGE_2 Assay, Minneapolis, MN, USA; Intra-assay CV = 18.0% Interassay CV = 13.9%; [38]) and CS (Aggrecan CS-846 Epitope ELISA, IBEX Pharmaceuticals Inc., Montreal, Quebec, Canada; Intra-assay CV = 3.1%; Interassay CV = 8.1%; [24]) according to manufacturer's instructions.

2.3. Exercise Trial

Design/Supplement. This study was conducted as a placebo controlled randomized crossover design to evaluate the effects of a single bout of intense exhaustive treadmill exercise and exogenous SOD supplementation on biomarkers of inflammation, antioxidant status, and anabolic cartilage metabolism in healthy horses. Mares were randomly assigned to either a treatment group (TRT) or placebo group (CON). The TRT group received 3 g/d (3000 IU of enzyme activity) of a proprietary oral formulation of SOD powder extracted from cantaloupe melon and chemically combined with wheat gliadin (vegetal hydrophobic biopolymer). The SOD supplement was verified prior to the study, to provide a minimum of 1000 IU of enzyme activity per gram of powder, using a nitro blue tetrazolium reduction method previously described [39]. The CON group received 3 g/d of a microcrystalline cellulose powder. Both TRT and CON groups received their supplement top dressed on their morning grain ration and all investigators were blind to the treatments. The initial phase of the study consisted of a 42 d supplementation period which concluded with

a repeated sprint exercise test (RSET), previously determined to influence biomarkers of inflammation and antioxidant status in horses [6]. Mares then completed a 42 d washout period, the experimental groups were crossed over and an identical 42 d supplementation period and RSET were completed. Before each phase of the study, standardized methods were used to evaluate each mare for body condition [36], orthopedic soundness [35], and per cent body fat [37].

Exercise Protocol. The RSET lasted an average of 18.4 ± 0.7 min and began with 2.5 min of walking at 1.5 m s^{-1} followed by 4 min of trotting at 4 m s^{-1}. Following warm up, mares completed 2 min sprints at 7, 8, 9, and 10 m s^{-1} with 2 min of walk at 1.5 m s^{-1} between each sprint [40, 41]. Mares ran to fatigue or until the test was completed, at which time they spent 1 min walking at 1.5 m s^{-1}. Fatigue was defined as an inability of the mares to keep up with the treadmill despite humane encouragement.

2.4. Sampling and Analysis.

Synovial Fluid. Samples were taken 24 h before exercise (PRE), 30 min, 2 h, and 24 h after exercise (POST). Samples were collected via aseptic arthrocentesis from one radiocarpal and one tibiotarsal joint space as described above for the preliminary trial, and placed into prechilled 10 mL tubes containing EDTA and immediately placed back on ice. The SF was centrifuged for 20 min at 1500 ×g to remove any cellular debris, aliquoted and stored at −80°C for later analysis of CS and PGE_2 using commercial ELISA kits, as described above.

Venous Blood. Samples were collected relative to the RSET, before exercise (PRE), at completion of the sprint portion of the RSET (PEAK), 30 min, 2, 4, 24, and 36 h after exercise (POST). Blood was collected using an indwelling jugular catheter and placed into prechilled 10 mL tubes containing sodium heparin, another 10 mL tube containing EDTA, and 10 mL serum separator tubes containing clot activator and gel for serum separation (Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA). Heparinized tubes were placed back on ice and immediately analyzed for packed cell volume (Hct) using microhematocrit technique (CritSpin S-120, Iris Sample Processing, Westwood, MA, USA) and were centrifuged for 10 min at 1500 ×g at 4°C. The plasma fraction was then analyzed for total protein (TP) using digital refractometry (Palm Abbe Veterinary Refractometer, MISCO Inc., Cleveland, Ohio, USA). Serum tubes remained at room temperature for approximately 1 h to allow for blood clotting and were centrifuged for 20 min at 1500 ×g at 10°C. Plasma and serum fractions were aliquoted and frozen at −80°C for later analysis. Blood components were analyzed for plasma lactate (LA; YSI Sport 1500, YSI Inc. Life Sciences, Yellow Springs, Ohio, USA; intra-assay CV = 0.79%; interassay CV = 0.72%), serum creatine kinase (CK; VetTest CK Slides; VetTest 8008 analyzer, IDEXX Laboratories Inc., Westbrook, MA, USA; intra-assay CV = 1.39%; interassay CV = 1.29%), plasma total nitrite as an indicator of nitric oxide (NO; BioAssay Systems, Hayward, CA, USA; intra-assay CV = 4.7%; interassay CV = 7.96%; [42]), and serum CS (as described above for the preliminary trial). All assays were run according to manufacturer's instructions.

Erythrocyte Lysate. For assays utilizing erythrocyte lysate, 500 μL whole blood from a sodium heparin collection tube was transferred to a microcentrifuge tube and was centrifuged at 2500 ×g for 5 min at 4°C. The plasma supernatant was discarded and the remaining erythrocytes were washed with 500 μL of sterile 0.9% sodium chloride solution, thoroughly vortexed and centrifuged a second time as described above. The saline supernatant was carefully removed and discarded from the sample, and the remaining erythrocytes were lysed with 1 mL of ice-cold distilled deionized water. Erythrocyte lysate was aliquoted and stored at −80°C within 20 min of collection, for later analysis of SOD activity (SOD Assay Kit-WST, Dojindo Molecular Technologies Inc., Rockville, MD, USA; interassay CV = 13.7%; [43]), hemoglobin concentration (QuantiChrom 92 Hemoglobin Assay Kit, BioAssay Systems, Hayward, CA, USA; [44]), glutathione peroxidase activity (GPx; OxisResearch, Foster City, CA, USA; intraassay CV = 0.99%; interassay CV = 10.8%; [45]), and total glutathione (GSH-T; OxisResearch, Foster City, CA, USA; intraassay CV = 1.1%; interassay CV = 1.2%; [46]), all according to manufacturer's instructions.

RNA Collection. Blood was also collected by means of jugular puncture into PAXgene blood RNA collection tubes (Qiagen/Becton Dickinson, Valencia, CA, USA) containing quaternary amine surfactants at PRE, 30 min, 2 h, and 24 h POST sample times. Total RNA was collected from 2.5 mL of whole blood according to manufacturer's instructions and the RNA was quantified using a spectrophotometer (Biophotometer, Eppendorf, Westbury, NY, USA). The RNA purity and quantity in each extraction were found to be sufficient for gene expression analysis and amplification efficiencies were between 1.8 and 2.2. A detailed description of RNA preparation, reverse transcription, and amplification was described previously [47]. Relative quantification (2-ΔΔCT method) was used to analyze the changes in gene expression [48, 49]. Cytokine (target) gene expression was normalized to that of the endogenous control gene beta-glucuronidase (β-GUS) and fold changes in target gene expression were calculated relative to a calibrator sample (mean resting baseline) within each data set. Amplification efficiency variation was taken into account and corrected for using the LinReg 7.0 software [50]. The following cytokine transcripts were measured: tumor necrosis factor alpha (TNFα; pro-inflammatory), interleukin-1 beta (IL-1β; pro-inflammatory), interferon gamma (IFNγ; immunomodulatory), interleukin-6 (IL-6; multifunctional), and interleukin-10 (IL-10; anti-inflammatory). Equine cytokine primer and probe sequences and PCR amplicon fragment sizes were previously published [6].

Statistical Analysis. For the preliminary trial, data are presented as the mean ± SE and analyzed using a MIXED model

Table 1: Preliminary trial. synovial fluid concentrations of chondroitin sulfate-846 (CS) and prostaglandin E_2 (PGE_2) during 4 repeated arthrocentesis procedures in a 48 h time frame at the following intervals: initial joint tap (T_1), 24 h following T_1 (T_2), 26 h after T_1 (T_3), and 48 h following T_1 (T_4), in carpus joints. Data are presented as mean ± SE. Means without a common superscript differ ($P < 0.05$).

Variable	T_1	T_2	T_3	T_4
CS, ng mL^{-1}	5214.7 ± 717.5^{a}	5842.9 ± 784.9^{ab}	5225.1 ± 679.3^{a}	6643.5 ± 1247^{b}
PGE_2, pg mL^{-1}	802.8 ± 249.2	1119.2 ± 279.1	579.2 ± 171.7	1188.9 ± 245.6

Table 2: Exercise trial. hematocrit (Hct), plasma total protein (TP), plasma lactate (LA), and serum creatine kinase (CK) concentrations before (Pre), during (Peak), 30 min, 2 h, 4 h, 24 h, and 36 h after exercise (Post). Data are presented as means ± SE; different superscripts denote differences within rows at $P < 0.05$.

Variable	Pre	Peak	30 min POST	2 h POST	4 h POST	24 h POST	36 h POST
Hct, %	36.5 ± 0.9^{a}	54.1 ± 1.2^{b}	45.2 ± 1.2^{c}	40.4 ± 1.1^{d}	35.4 ± 1.1^{a}	40.1 ± 1.2^{d}	40.1 ± 1.0^{d}
TP, g dL^{-1}	6.3 ± 0.1^{a}	7.5 ± 0.2^{b}	6.5 ± 0.1^{c}	6.3 ± 0.1^{a}	6.2 ± 0.1^{a}	6.6 ± 0.1^{c}	6.6 ± 0.1^{c}
LA, mmol L^{-1}	0.7 ± 0.1^{a}	23.4 ± 2.1^{b}	14.0 ± 2.1^{c}	2.2 ± 0.3^{a}	1.0 ± 0.1^{a}	0.7 ± 0.1^{a}	0.8 ± 0.1^{a}
CK, U L^{-1}	205.6 ± 15.0^{a}	276.7 ± 21.9^{b}	255.8 ± 20.6^{c}	254.3 ± 21.2^{c}	255.7 ± 22.3^{c}	206.3 ± 14.3^{a}	228.4 ± 18.1^{d}

ANOVA with repeated measures in SAS to evaluate effects of repeated arthrocentesis sampling. Significant main effects were further analyzed using Tukey-Kramer post hoc analysis to further elucidate significant changes as inferred when $P < 0.05$.

For the exercise trial, cytokine data are presented as relative mRNA transcripts (RMT) or the mean fold changes in target gene expression normalized to an endogenous control gene (β-GUS) and relative to that of a calibrator sample (resting, presupplementation baseline) in response to intense exercise ± SE. All other data are summarized as the mean ± SE. Data were analyzed using a MIXED model ANOVA with repeated measures in SAS 9.1 to evaluate effects of SOD supplementation (TRT versus CON), acute exercise (as sample time), joint space (as carpus and hock; for synovial fluid parameters), and interactions. Horse was nested within treatment as the subject, and sample time was designated as the repeated effect. The Satterthwaite approximation of standard errors was utilized to account for any unequal variances. Significant main effects were further analyzed using Tukey-Kramer post hoc analysis to further elucidate significant changes as inferred when $P < 0.05$. Pearson's product moment correlation was used to test for associations between the variables measured and only significant associations were reported.

3. Results

3.1. Preliminary Trial. Effect of repeated arthrocentesis was detected for CS ($P = 0.04$; Table 1) where synovial fluid concentrations were higher ($P < 0.05$) at (T_4) when compared to (T_1) and (T_3). No differences ($P > 0.05$) in concentrations of CS were detected between (T_4) and (T_2). Differences between sampling times for PGE_2 ($P > 0.05$; Table 1) were not detected.

3.2. Exercise Trial

Response to Exercise. There were no TRT effects detected for any parameter measured ($P > 0.05$). A main effect of exercise ($P < 0.0001$) was detected for Hct, plasma TP, plasma LA, and serum CK, where maximal values occurred at PEAK during exercise (Table 2).

Biomarkers of Antioxidant Status. The RSET did influence SOD activity ($P = 0.002$), GPx activity ($P < 0.0001$), GSH-T ($P < 0.0001$), and NO ($P = 0.0002$). Erythrocyte SOD activity increased from PRE to PEAK ($P = 0.001$) followed by a decrease ($P = 0.006$) to preexercise activity at 30 min POST (Figure 1(a)). Erythrocyte GPx activity was higher ($P < 0.01$; Figure 1(b)) at PEAK when compared to other time points, and returned to preexercise activity by 30 min POST. Similar to GPx, erythrocyte GSH-T was also highest ($P < 0.001$; Figure 1(c)) at PEAK compared to other samples times, and gradually returned to preexercise values by 4 h POST. Plasma NO decreased ($P = 0.002$; Figure 1(d)) from 30 min POST to 24 h POST and returned to PRE values at 36 h POST.

Biomarkers of Systemic Inflammatory Response. The RSET influenced IFNγ ($P = 0.007$), IL-1β ($P = 0.0002$), and IL-10 ($P = 0.007$) transcripts; however, there was no effect of sample time on IL-6 or TNFα gene expression ($P > 0.05$). Interferon gamma transcripts increased ($P = 0.03$; Table 3) from PRE to PEAK and decreased ($P = 0.01$) to preexercise levels at 2 h POST. Gene expression for IL-1β was higher at 2 h POST ($P < 0.005$; Table 3) when compared to PRE, 30 min and 24 h POST samples. Transcripts for IL-10 also tended to increase ($P = 0.08$; Table 3) from PRE to PEAK and then decreased ($P = 0.005$) from PEAK to 24 h POST returning to pre-exercise values. Correlations were detected and can be found in Table 4.

Biomarkers of Joint Health. For CS concentrations, there were main effects of exercise ($P = 0.009$) in serum samples, as well as main effects of exercise ($P < 0.0001$) and joint ($P < 0.0001$) for CS in synovial fluid. Further data analysis revealed lower ($P < 0.05$) serum concentrations of CS at 30 min and 2 h POST samples when compared to 24 h POST.

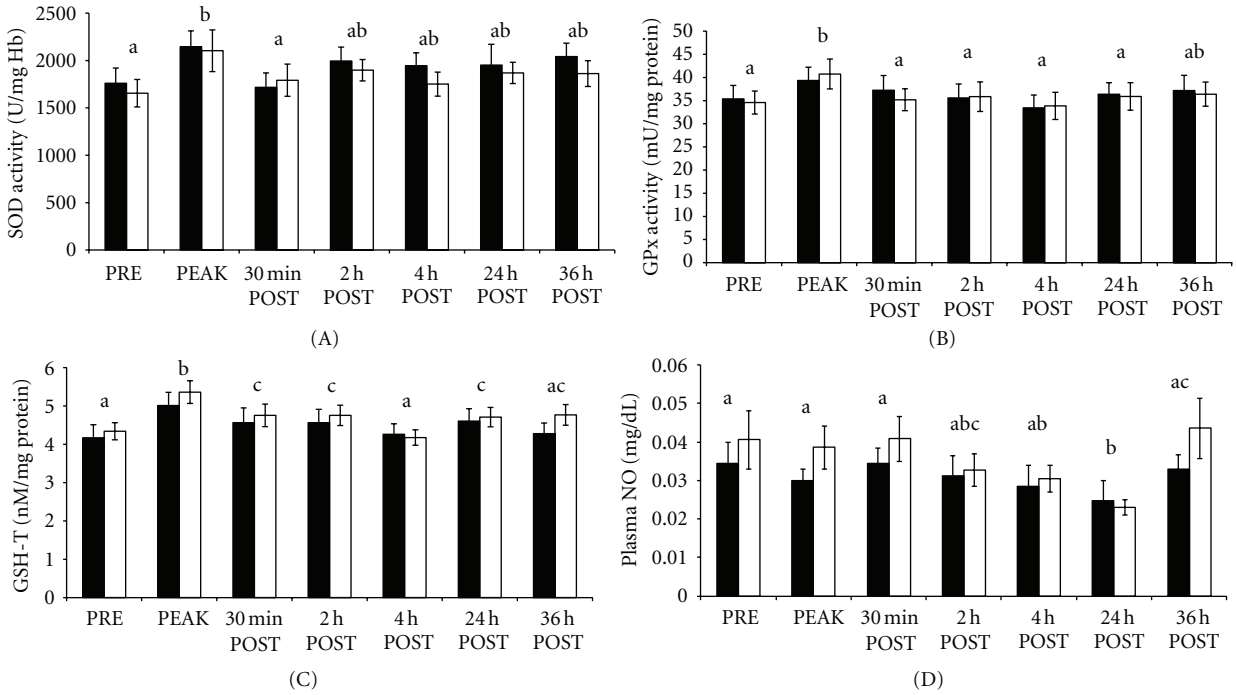

Figure 1: (A–D). exercise trial: erythrocyte superoxide dismutase activity (SOD; A), glutathione peroxidase activity (GPx; B), total glutathione (GSH-T; C), and plasma total nitrite as an indirect biomarker of nitric oxide (NO; D) before (PRE), during (PEAK), 30 min, 2 h, 4 h, 24 h, and 36 h after exercise (POST). Solid bars represent TRT and clear bars CON groups. Data are presented as mean ± SE; means without a common superscript differ ($P < 0.05$) between sample times.

Table 3: Exercise trial: interferon γ (IFNγ), interleukin-1β (IL-1β), and interleukin-10 (IL-10) relative mRNA transcript (RMT) in peripheral blood of Standardbred mares before exercise (Pre), 30 min after exercise (Post), 2 h, and 24 h Post. qRT-PCR data are presented as the mean fold change in target gene expression ± SE. Means without a common superscript differ between sample times ($^{a,b}P < 0.05$; $^{x,y}P < 0.1$). No TRT effects were detected.

	Pre		30 min Post		2 h Post		24 h Post	
	TRT	CON	TRT	CON	TRT	CON	TRT	CON
IFNγ, RMT	1.1 ± 0.2^{a}	1.2 ± 0.2^{a}	2.7 ± 1.1^{b}	3.6 ± 1.9^{b}	0.9 ± 0.1^{a}	1.1 ± 0.3^{a}	1.3 ± 0.3^{a}	1.2 ± 0.3^{a}
IL-1β, RMT	1.2 ± 0.2^{a}	2.1 ± 1.0^{a}	1.1 ± 0.2^{a}	1.2 ± 0.2^{a}	8.2 ± 3.3^{b}	3.8 ± 0.3^{b}	1.4 ± 0.2^{a}	1.8 ± 0.2^{a}
IL-10, RMT	1.6 ± 0.5^{x}	2.0 ± 0.5^{x}	3.5 ± 1.6^{y}	2.5 ± 0.7^{y}	2.3 ± 0.6^{x}	2.3 ± 0.8^{x}	1.4 ± 0.4^{x}	1.2 ± 0.3^{x}

Synovial fluid concentrations of CS were elevated ($P < 0.01$) at 30 min POST compared to PRE and 2 h POST samples, and when compared to all other sample times, synovial fluid CS was higher ($P < 0.0001$) at 24 h POST (Figure 2(a)). Hock joints were found to have higher ($P < 0.0001$) concentrations of CS when compared to carpus joints (Figure 2(a)).

Main effects for PGE_2 concentrations in synovial fluid were not detected ($P > 0.05$); however, after adjusting the data by subtracting the PRE value from each POST value, and evaluating changes relative to pre-exercise values, effects of exercise ($P = 0.03$) and joint ($P = 0.04$) were detected (Figure 2(b)). The relative increase in PGE_2 at 24 h POST was greater than that at PRE ($P = 0.03$) and demonstrated a trend for being greater ($P = 0.08$) when compared to relative changes at the 30 min POST sample time (Figure 2(b)). Similar to synovial fluid CS concentrations, there were greater relative increases ($P = 0.04$) in PGE_2 concentrations in hock joints when compared to carpus joints (Figure 2(b)).

4. Discussion

4.1. Effect of Repeated Arthrocentesis. The impact of repeated arthrocentesis on biomarkers of inflammation and joint health are conflicting in the literature. Therefore, the primary objective of the preliminary trial was to identify possible confounding factors arising from the specific repeated arthrocentesis protocol that was implemented in the subsequent exercise trial, in a sample population of healthy horses. Data from this trial indicated that repeated synovial fluid sampling did not elicit a detectable inflammatory response based upon statistically similar PGE_2 concentrations, but may have mildly impacted aggrecan turnover as evidenced by elevated CS at the last sample time. These data are in partial contrast to a previous study which demonstrated increases in synovial fluid concentrations of PGE_2 and nitric oxide following 2 consecutive joint taps with a 12-hour interval between taps, when compared to baseline values [51]. Increases in

Table 4: Exercise trial: correlations of indices of performance[a], muscle membrane integrity[b], antioxidant status[c], inflammation[d] for hematocrit (Hct), plasma total protein (TP), plasma lactate (LA), serum creatine kinase (CK), erythrocyte glutathione peroxidase (GPx), erythrocyte total glutathione (GSH), plasma total nitrite (NO), interferon gamma (IFNγ), interleukin-1β (IL-1β), interleukin-10 (IL-10), tumor necrosis factor-α (TNFα), and interleukin-6 (IL-6) transcripts. Associations were detected between each biomarker heading, and those indices within their respective columns.

Hct[a]	TP[a]	LA[a]	CK[bc]	GPx[c]	GSH-T[c]	NO[cd]	IFNγ[d]	IL-1β[d]	IL-10[d]	TNFα[d]	IL-6[d]
TP	LA	Hct	Hct	TP	Hct	TP	Hct	IL-6	Hct	Hct	IL-1β
$r = 0.40$	$r = 0.24$	$r = 0.61$	$r = 0.48$	$r = -0.38$	$r = 0.29$	$r = 0.22$	$r = 0.50$	$r = 0.25$	$r = 0.37$	$r = 0.37$	$r = 0.25$
$P < 0.0001$	$P = 0.02$	$P < 0.0001$	$P < 0.0001$	$P = 0.0002$	$P = 0.004$	$P = 0.03$	$P < 0.0001$	$P = 0.02$	$P = 0.0002$	$P = 0.0002$	$P = 0.02$
LA	GPx	TP	LA	NO	TP	GPx	TP	TNFα	LA	LA	TNFα
$r = 0.61$	$r = -0.38$	$r = 0.24$	$r = 0.39$	$r = -0.22$	$r = 0.29$	$r = -0.22$	$r = 0.23$	$r = 0.33$	$r = 0.37$	$r = 0.29$	$r = 0.59$
$P < 0.0001$	$P = 0.0002$	$P = 0.02$	$P < 0.0001$	$P = 0.04$	$P = 0.004$	$P = 0.04$	$P = 0.03$	$P = 0.001$	$P = 0.0002$	$P = 0.004$	$P < 0.0001$
CK	GSH-T	CK	GSH-T	GSH-T	CK	IFNγ	LA		CK	CK	
$r = 0.48$	$r = 0.29$	$r = 0.39$	$r = 0.28$	$r = 0.21$	$r = 0.28$	$r = 0.31$	$r = 0.50$		$r = 0.33$	$r = 0.28$	
$P < 0.0001$	$P = 0.004$	$P < 0.0001$	$P = 0.006$	$P = 0.04$	$P = 0.006$	$P = 0.003$	$P < 0.0001$		$P = 0.001$	$P = 0.005$	
GSH-T	IFNγ	IFNγ	IFNγ		GPx	TNFα	CK		IFNγ	NO	
$r = 0.29$	$r = 0.23$	$r = 0.50$	$r = 0.31$		$r = 0.21$	$r = 0.20$	$r = 0.31$		$r = 0.58$	$r = 0.20$	
$P = 0.004$	$P = 0.03$	$P < 0.001$	$P = 0.003$		$P = 0.04$	$P = 0.05$	$P = 0.003$		$P < 0.0001$	$P = 0.05$	
IFNγ		IL-10	IL-10				NO		TNFα	IFNγ	
$r = 0.50$		$r = 0.37$	$r = 0.33$				$r = 0.31$		$r = 0.57$	$r = 0.57$	
$P < 0.0001$		$P = 0.0002$	$P = 0.001$				$P = 0.003$		$P < 0.0001$	$P < 0.0001$	
IL-10		TNFα	TNFα				IL-10			IL-1β	
$r = 0.37$		$r = 0.29$	$r = 0.28$				$r = 0.58$			$r = 0.33$	
$P = 0.0002$		$P = 0.004$	$P = 0.005$				$P < 0.0001$			$P = 0.001$	
TNFα							TNFα			IL-10	
$r = 0.37$							$r = 0.57$			$r = 0.57$	
$P = 0.0002$							$P < 0.0001$			$P < 0.0001$	
										IL-6	
										$r = 0.59$	
										$P < 0.0001$	

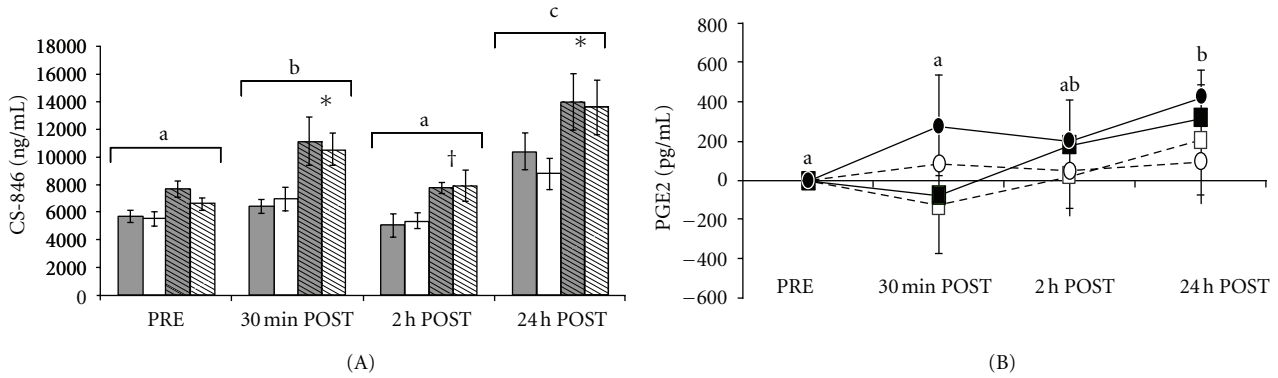

Figure 2: (A, B). Exercise trial: (A) synovial fluid concentrations of chondroitin sulfate-846 epitope (CS-846; A) before exercise (PRE), 30 min, 2 h, and 24 h following exercise (POST). Open bars represent carpus samples, hashed bars represent hock samples, grey bars represent TRT groups, and white bars represent CON groups. Data are presented as mean ± SE. (B) Relative synovial fluid concentrations of PGE_2 30 min, 2 h, and 24 h following exercise (POST) compared to samples collected 24 h prior to exercise (PRE). Squares indicate TRT group, circles indicate CON group, solid shapes indicate hock samples, and open shapes indicate carpus samples. Data were adjusted by subtracting PRE values from each sample time, and are presented as mean relative change from PRE ± SE. Different superscripts denote relative increases from PRE values. Means without a common superscript differ ($P < 0.05$) between sample times; *infers significant differences ($P < 0.05$) and †infers a trend towards significant differences ($P < 0.1$) between joint spaces within a sample time. Greater increases ($P = 0.04$) in PGE_2 in hock joints compared to carpus joints were detected.

glycosaminoglycans (GAG) were also detected in radiocarpal and tarsocrural joints after 2 consecutive taps with a 60 hour interval between taps, in mature healthy horses. Another study reported increased matrix metalloproteinase-1, a biomarker for tissue remodeling, in equine synovial fluid, following repeated arthrocentesis within a 60 h time-frame [52]. These findings are in partial contrast to additional reports in the literature. A previous study [6] did not find a repetitive arthrocentesis protocol, similar to the present trial's, to modulate synovial fluid concentrations of nitric oxide, in radiocarpal and tibiotarsal joints of mature, healthy mares following intense exhaustive treadmill exercise. Another study reported no effect of repeated synovial fluid sampling (4 taps over 10 days with intervals ranging from 12 h to 168 h between taps) on the pro-inflammatory regulator TNFα, in synovial fluid of normal equine joints [53]. Furthermore, a study where healthy horses underwent weekly synovial fluid aspiration for a total of 13 wks did not report evidence of repeated sampling effects on keratin sulfate or CS in control radiocarpal joints [54]. Authors of a similar study, during which healthy young horses underwent repeated arthrocentesis for several weeks, concluded that any effects of the repeated synovial fluid sampling were minor and most likely did not confound experimental data [22]. Relative to the preliminary trial, the specific arthrocentesis protocol that was executed did not appear to influence PGE_2 at any sample time, although aggrecan synthesis may have been impacted, indicated by mildly elevated CS concentrations at the last sampling time, and should be taken into consideration when interpreting similar data from the exercise trial.

4.2. Response to Exercise. The RSET was strenuous enough to elicit increases in muscle enzymes, pro- and anti-inflammatory cytokine gene transcripts, as well as markers of antioxidant status, discussed below. Normal increases in Hct and TP from PRE to PEAK samples were expected findings in the present study and are consistent with previous studies evaluating hematologic and plasma biochemical measurements in exercising horses [55, 56].

The LA increase during and after exercise in the present study indicates that mares were working at a velocity consistently above their anaerobic threshold and in a state where the lactate efflux mechanism was saturated. Lactate response in the present study was positively correlated with CK, pro-inflammatory cytokine IFNγ and TNFα transcripts, as well as the anti-inflammatory cytokine IL-10, which further demonstrates that the type and intensity of the RSET was a determinant of the systemic inflammatory response. Supplementation with a nutraceutical blend containing SOD has been shown to be beneficial in humans, resulting in an improvement in the velocity at which lactate threshold is reached during intense exercise [30]; however this was not seen in the present study.

Elevated PEAK CK values in the present study are comparable to those of horses having engaged in racing and training regimes intense enough to induce overtraining syndrome [57]. Exercise-induced increases in CK in the present study may indicate increased myocyte membrane permeability [58], which was not attenuated by SOD supplementation. Increases in CK have also been positively correlated with increases in ROS formation and subsequent increases in lipid peroxidation in horses [11, 59, 60]. Therefore, it is plausible that exercise-induced increases in ROS may influence myocyte membrane permeability permitting the muscle enzymes to enter circulation [61]. Creatine kinase was also positively correlated with IFNγ and IL-10 in the current study, suggesting that changes in myocyte integrity, resulting from exercise and increased ROS production, coincide with a pro-inflammatory immune response.

Systemic Inflammatory Response. Increases in pro-inflammatory cytokine transcripts at PEAK and sample times following the RSET indicate a pro-inflammatory response to exercise and a counter anti-inflammatory response. These physiological changes are most likely in response to incurred tissue microdamage, increased ROS generation, and possibly mild endotoxemia following the intense, exhaustive exercise test, although direct measurements of neutrophilia and circulating endotoxin were not quantified in the present study.

Positive correlations detected between the cytokines evaluated in the present study further illustrate the close relationships between these cytokines. For example, IFNγ is considered a pro-inflammatory cytokine that serves to activate macrophages, augment the synthesis of other pro-inflammatory cytokines, and induce nitric oxide synthase and ROS formation [49, 62]. The pro-inflammatory response also signals anti-inflammatory cytokines, such as IL-10, which is also thought to possess antioxidant properties. Interleukin-10 inhibits several cytokines including IFNγ, IL-1β, TNFα, and IL-6, mainly by initiating the degradation of mRNA for these cytokines as well as the inhibition of antigen presentation [63, 64] and release of ROS [65]. The inhibition of proinflammatory cytokines, NO production, and superoxide anion formation in endothelial tissue [66], can have an impact on neutrophil priming for oxidative burst [67]. Furthermore, antioxidants have been show to upregulate IL-10 in antigen-IgE-activated mast cells *in vitro* [68].

Changes in Antioxidant Status. In the present study, changes in SOD activity, GPx activity, GSH-T, and a reduction in plasma NO concentrations were detected, indicating that the RSET was effective in eliciting changes in antioxidant defenses and is indirectly suggestive of exercise-induced oxidative stress, although direct analysis of oxidative stress biomarkers was not performed. The authors speculate that increases in SOD, GPx, and GSH-T at PEAK were compensatory responses to exercise-induced oxidative stress in the current study. Several studies have demonstrated similar exercise-induced changes in antioxidant status or oxidative stress after a single bout of intense exercise in humans [69–71] and horses [9, 12, 56, 72–75], and following a bout of prolonged intense exercise in horses [10, 60].

Alterations in SOD, GPx, and GSH-T in response to exercise vary within the literature. With regard to the current study, GPx and GSH-T were positively correlated and increased as the mares were fatiguing at the end of the RSET. This increase in the antioxidant enzyme and its thiol substrate is in accordance with previous studies reporting similar increases in SOD, GPx, and GSH-T in horses [59, 74], GSH-T and GPx in rat muscle [76], and GSH-T in human plasma [77], either during or following strenuous exercise. Elevations in antioxidant defenses indirectly suggest that the exercise stress increased ROS production. Increased SOD activity suggests generation of superoxide anions, and the increases in GPx and GSH-T indicates increased production, most likely from the metabolism of superoxide by SOD, and subsequent elimination of hydrogen peroxide via oxygenation of reduced glutathione, in the present study. Furthermore, GSH-T and CK were positively correlated in the present study, as has been reported previously [60], suggesting that increasing ROS formation may be altering muscle membrane integrity allowing for increases in circulating muscle enzymes and antioxidant defenses.

In contrast to the present study's findings, several other studies reported no changes in erythrocyte GPx or GSH-T [12] relative to an acute bout of exercise in horses, reduced erythrocyte GPx activity, and no changes in SOD activity in trained Thoroughbred horses following intense exercise on a race track (1000 m at 15 m s^{-1}; [78]). Decreases in GPx and GSH-T in endurance horses following an 80 km race [79] and a 140 km race [10] have also been reported. It should be noted that the exercise modalities, exercise intensities, duration, and fitness status of the experimental models differ from study to study, and comparison between studies should be made with this in mind. Interestingly, in a majority of the studies that did not report changes in antioxidant defenses, the experimental subjects were exercise trained and fit for their respective exercise test, indicating that antioxidant systems most likely adapt to the specific conditions of regular training [80].

The decrease in plasma NO concentration, along with upregulation of cytokine transcripts following exercise, was comparable to responses noted in previous reports in horses following a single bout of intense exhaustive exercise, with the exception of TNFα and IL-6 transcripts remaining unchanged, in the present study [6, 81]. The decline in NO may be due to increased utilization, increased excretion, and decreased production or bioavailability of NO resulting from increased oxidative stress, in the present study. Plasma NO was positively correlated with pro-inflammatory cytokines IFNγ and TNFα and negatively correlated with GPx in the present study. The inverse relationship of NO to GPx may reflect the increase in ROS and subsequent decrease in NO bioavailability, in conjunction with increased GPx to counter oxidative stress. Exercise-induced generation of superoxide and NO lends to the formation of peroxynitrite a damaging reactive nitrogen species, therefore, reducing NO bioavailability [82]. The positive association of NO with proinflammatory cytokines most likely reflects the postexercise decline in IFNγ and NO in the present study, indicating recovery from exercise-induced inflammation and increased utilization or depletion of NO.

Implications on Joint Health. Healthy articular cartilage serves to distribute load in a pain and friction-free manner. Cartilage consists of specialized cells (chondrocytes) distributed in three distinct layers within an intracellular matrix consisting primarily of proteoglycans and type II collagen [83]. The integrity of the matrix is dependent on chondrocyte metabolism, which has been shown to be influenced by stressors, including shear and compressive strain, which occur with exercise [83]. One study, using normal human and bovine articular chondrocyte culture, demonstrated that fluid-induced shear stimulated glycosaminoglycan (GAG)

synthesis, and after 48 h of shear stress PGE_2 production was increased when compared to controls [84]. Furthermore, evidence of ROS (specifically superoxide anion) in the joint has been reported [85]. Presence of ROS in joints can lead to proteoglycan cleavage [86], compromising the integrity of the cartilage matrix. The relative increase in PGE_2 from PRE to 24 h POST in the exercise trial suggests a slightly delayed onset of inflammation following the RSET. It is plausible that the type of joint loading resulting from the RSET in the current study induced enough fluid-induced shear and change in hydrostatic pressure gradient, to induce a delayed increase in PGE_2 relative to PRE concentrations. Elevated concentrations of PGE_2 have previously been shown to occur in inflamed and osteoarthritic joint tissues of horses [22, 38, 51, 87, 88]. Furthermore, these elevated prostaglandins may compromise the cartilage matrix by decreasing proteoglycan content [89, 90] as well as initiating a localized pain response via sensitization of peripheral nociceptor terminals [20]. In a previous study, increases in circulating proinflammatory cytokine transcripts (interleukin-1 beta) were detected 2 h following intense exhaustive treadmill exercise [6]. Interleukin-1 beta is a regulator of PGE_2 release from joint tissues, which may also help explain why the increase in PGE_2 is delayed past the 2 h POST sample in the current study. It is unknown if this was a transient increase, or if PGE_2 levels remained elevated for a sustained period of time as sampling was not carried out past 24 h POST. The resting and postexercise PGE_2 concentrations were higher in the current study compared to previous reports in horses [22], which might be attributable to different analytical techniques (extraction of PGE_2 from SF versus evaluation of concentrations in diluted synovial fluid).

The increase in synovial fluid concentrations of CS at 30 min POST, which declined back to pre-exercise levels by 2 h POST in the exercise trial, suggests a transient increase in aggrecan synthesis. The magnitude of change from pre- to postexercise samples in the exercise trial was similar to that reported in chronically exercised horses [22]. Furthermore, absolute concentrations of CS in the present study were lower than those reported in the same chronically exercised healthy horses and in horses with clinical osteoarthritis [22]. When magnitude of change from T_2 to T_4 and 2 h POST to 24 h POST were compared between the preliminary and exercise trials, respectively, the increase in CS was 2.6 times greater in the exercise trial. Furthermore, absolute concentrations of CS were approximately 2057 ng mL^{-1} higher for the 2 h POST sample in the exercise trial compared to the T_2 sample in the preliminary trial. This data suggests that the RSET did elicit changes in cartilage turnover, most likely in response to cyclic high compressive loading [91–95]. Several other studies have reported exercise-induced changes in cartilage metabolism. At the end of a 6 wk training study in horses, investigators reported an increase in newly synthesized proteoglycan from an *ex vivo* cartilage culture when compared to cartilage from nonexercised controls [96]. Another study reported a decrease in aggrecan synthesis and concomitant increase in decorin synthesis in equine cartilage explants that persisted for 16 wks of rest following 17 wks of strenuous exercise [97]. Increases in CS and keratin sulfate (catabolic marker of cartilage metabolism) have been associated with cases of osteochondral fragmentation in horses [24] and repetitive exercise over time [22, 98]. Healthy horses seem to be able to return to homeostatic conditions in joint tissues fairly quickly as was the case in the present study. However, if this modality and intensity of exercise were repeated over time without sufficient recovery periods, it could result in a chronic degenerative state, ultimately compromising the stability and functionality of the articular cartilage. Although it is difficult to determine whether the increase in synovial fluid CS concentrations at 24 h POST in the exercise trial is an artifact of repeated arthrocentesis, as was identified in the preliminary study, or a response to exercise, the possibility that the delayed increase in PGE_2 following exercise may have contributed to the mild increase in aggrecan turnover, should not be completely discounted. Significant associations between serum and synovial fluid biomarkers of cartilage metabolism have been reported [22]; however, the same associations were not detected in the exercise trial.

Synovial fluid concentrations of both PGE_2 and CS were higher in hock joints when compared to carpal joints in the exercise trial. This finding is in accord with a previous study which found higher concentration of nitric oxide in hock joints compared to that of carpus samples from mature healthy Standardbred mares [6]. The differences between hock and carpus joints in the present study may be attributable to conformational and biomechanical features unique to Standardbred trotters or pacers, with a higher degree of dynamic compressive and shear stress occurring in the hock joints. Another study also demonstrated increased nitric oxide synthesis in response to shear stress in bovine articular chondrocytes in culture, which in turn mediated an increase in GAG synthesis [99]. It has previously been reported [100] that 14.3% of 753 young Standardbred trotters sampled were diagnosed with osteochondrosis in the tibiotarsal joint and 11.8% were diagnosed with bony fragments in the palmar/plantar portion of the metacarpo and metatarsophalangeal joints. These data provide further evidence that Standardbreds, compared to other breeds, experience more exercise-induced stress in the hock joints compared to joints in the forelimbs and should be considered in light of athletic conditioning and rehabilitation programs.

4.3. Effect of Nutritional Supplementation. It was hypothesized that SOD supplementation in horses would mirror the beneficial findings reported in the literature demonstrating oral bioavailability [29, 30, 101] and subsequent benefits including anti-inflammatory [102, 103], antioxidant [104, 105], and chondroprotective properties [34, 106, 107] reported in several other animal models. Data from a porcine ischemia reperfusion injury model [105] and a human hyperbaric oxygen oxidative stress model [108] suggested that supplementation of a vegetal preparation of SOD combined with a wheat-gliadin biopolymer (SOD/gliadin) is protective against oxidative DNA damage and decreases markers of lipid peroxidation in circulation. Mice supplemented with SOD/gliadin for 28 d showed a rise in

circulating antioxidant enzyme activity which was positively correlated with an increased erythrocyte resistance to oxidative induced hemolysis. This study also found increases in hepatic antioxidant defenses which were correlated with a significant decrease in hepatocyte apoptosis in the presence of Sin-1 (peroxynitrite chemical donor) when compared to controls [29]. In another study, peritoneal macrophages activated by an intraperitoneal injection of IFNγ, collected from mice after a 28 day supplementation of SOD/gliadin, and stimulated with IgG1IC *ex vivo*, exhibited a reduction in TNFα production and elevated IL-10 production as well as decreased superoxide, NO, and peroxynitrite concentrations [102].

In human studies, supplementation of SOD/gliadin in soccer athletes has been shown to reduce oxidative stress. Specifically, a decrease in the magnitude of 8-iso PGF2α response was observed in the experimental group following a graded step-wise exhaustive treadmill exercise test [30]. Similarly, college football athletes supplemented with a proprietary nutraceutical drink blend containing SOD, coenzyme Q10 and beta glucans during a 7-wk training period demonstrated anti-inflammatory (reduced IL-6), antioxidant (reduced 8-iso PGF2α), and less muscle membrane leakage of CK both after acute and chronic bouts of exercise compared to nonsupplemented controls [103]. Lastly, in patients with HIV or AIDs exhibiting compromised antioxidant status, it was determined that SOD/gliadin (1000 IU d^{-1}) supplementation for 3 wks normalized the patients' circulating SOD activity and total antioxidant status (TAS), demonstrating that oral supplementation with vegetal SOD supplement can improve systemic antioxidant defenses [104]. A previous study examining aspects of joint health demonstrated that addition of SOD to bovine synovial fluid was protective against hyaluronic acid depolymerization by superoxide derived hydroxyl radicals *in vitro*, thereby preserving the viscoelastic properties characteristic of healthy synovial fluid [106]. Palosein (generic name Orgotein), an FDA-approved, injectable form of a Cu-Zn protein with high-SOD enzyme activity derived from bovine liver [33], has also been shown to ameliorate free radical-induced (superoxide) loss in synovial fluid viscosity in horses [34]. Contrary to these reports, data from the present study suggest that supplemental SOD has no effect on biomarkers of inflammatory response, antioxidant status, or joint health in an equine exercise model.

Several factors may have contributed to the ineffectiveness of SOD supplementation in equine exercise model used in the exercise trial. The SOD may not have been effectively delivered to the target tissue, or the enzyme activity may have been compromised in the digestive and/or absorption processes. Historically, intravenous administration of SOD was the most common route of delivery, but therapeutic capabilities were limited due to its short half-life of less than 30 min [109]. Oral administration of SOD is a newer practice and the use of delivery technologies is a growing area of interest. The gliadin coating used in the present study is a hydrophobic gliadin-biopolymer demonstrated to be an effective carrier for oral delivery of active food ingredients, specifically lipophilic molecules [110]. Gliadin biopolymer has been shown to enhance oral pharmacology of SOD [111] delaying enzyme release thereby preserving the enzyme activity throughout the gastrointestinal (GI) tract as well as purportedly increasing intestinal permeability via increased zonulin release which opens the enterocyte tight junctions [112, 113]. It has been demonstrated that the SOD enzyme requires protection during GI transit [29]. Failure of SOD efficacy in the present study may be due to the gliadin biopolymer releasing the active ingredient at an inappropriate time during GI transit, rendering it incapable of being absorbed into circulation. Previous studies have investigated the biodegradation of Gliadin bioadhesion properties, pharmacokinetic properties, and active ingredient stability of various products [114, 115]. Subsequent studies should take these into consideration for the target species studied.

Failure of the oral SOD formulation to elicit increases in erythrocyte SOD activity may have been due to dosing rate. In the present study, 3000 IU d^{-1} SOD was fed at an average rate of 5.84 ± 0.18 IU kg bw^{-1} d^{-1} to the research mares. A similar SOD preparation was fed to pigs at rates of 0.316 and 1.2 IU kg bw^{-1} d^{-1} which allegedly resulted in 25% and 41% increases in plasma SOD activity, respectively [116]. In dogs, SOD has been supplemented at the rate of approximately 10 IU kg bw^{-1} d^{-1} [116], and mice have received SOD supplement at the rate of 0.003 IU kg bw^{-1} d^{-1} [29]. Although the horses in the present study were fed at a substantially higher rate than the other species, this dose could be insufficient and future research in horses should investigate different supplementation rates in excess of 3000 IU d^{-1}.

Furthermore, an unfit animal model was thought to be more susceptible to tissue microdamage, inflammation, and oxidative stress resulting from the RSET compared to that of a physically fit model conditioned to a specific modality of exercise. This unfit model theoretically would have experienced the greatest benefit from SOD supplementation when compared to a fit, conditioned individual who might be better able to cope with exercise-induced ROS formation and inflammation, as previously demonstrated in horses and humans [30, 56, 117, 118]. Despite their healthy but unfit status, endogenous antioxidant defenses of the horses used in the present study appear to have been sufficient to handle the magnitude of oxidant stress relative to the RSET, therefore, rendering any benefits of exogenous SOD supplementation undetectable.

5. Conclusion

Repeated arthrocentesis within a 48 h time frame did not seem to influence PGE_2 whereas CS concentrations did increase in joints of healthy mature horses in the present study. Therefore, impact of sampling techniques, exclusive to any treatment effects, should be evaluated and considered in the experimental design and subsequent data interpretation. Fluctuations in biomarkers of inflammation, antioxidant status, or aggrecan synthesis were not altered by SOD supplementation in Standardbred mares having

undergone intense exercise in the present study. The RSET did result in a proinflammatory response as well as up-regulated antioxidant defenses, which suggests that it is an appropriate exercise modality for investigating systemic inflammatory responses and antioxidant status and oxidative stress in horses. The use of intense treadmill exercise to induce inflammation provides a realistic yet controlled, repeatable, and relatively noninvasive model of inflammation and joint metabolism under dynamic physiologic stress. The inflammatory and metabolic changes detected in synovial fluid in the exercise trial are thought to be normal physiological responses to a single bout of intense exercise. These data suggest that joint health in mature healthy horses is not compromised following a single intense bout of exhaustive exercise. However, repetitive exercise at this intensity, without sufficient recovery periods could result in sustained localized joint inflammation resulting in a chronic degenerative state. Oral supplements may provide practical solutions to support optimal health, performance, and the prevention of chronic disease in a variety of athletic species. Despite previous evidence of SOD efficacy in other species, further investigation is needed to elucidate benefit(s) of SOD supplementation and effective supplement delivery technologies in horses.

Acknowledgments

The authors would like to acknowledge the New Jersey Agricultural Experiment Station State Equine Initiative for partial funding of this study as well as the Rutgers University Animal Science Department undergraduate student research group and Animal Care staff. Lastly, a special thanks are due to Dr. Elizabeth O'Byrne (Rutgers University), Dr. David Horohov (University of Kentucky, Gluck Equine Research Center), Dr. Amy O. Burk and Tim Shellem (University of Maryland), and Drs. Michael Orth and Cara Robison (Michigan State University) for their help with sample analysis.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2012, Article ID 973927, 10 pages
doi:10.1155/2012/973927

Research Article

Assessment of a Standardized ROS Production Profile in Humans by Electron Paramagnetic Resonance

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Received 14 March 2012; Revised 6 June 2012; Accepted 7 June 2012

Academic Editor: Steve R. McAnulty

Despite the growing interest in the role of reactive oxygen species (ROS) in health and disease, reliable quantitative noninvasive methods for the assessment of oxidative stress in humans are still lacking. EPR technique, coupled to a specific spin probe (CMH: 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine) is here presented as the method of choice to gain a direct measurement of ROS in biological fluids and tissues. The study aimed at demonstrating that, differently from currently available "a posteriori" assays of ROS-induced damage by means of biomolecules (e.g., proteins and lipids) spin-trapping EPR provides direct evidence of the "instantaneous" presence of radical species in the sample and, as signal areas are proportional to the number of excited electron spins, lead to absolute concentration levels. Using a recently developed bench top continuous wave system (e-scan EPR scanner, Bruker) dealing with very low ROS concentration levels in small (50 μL) samples, we successfully monitored rapid ROS production changes in peripheral blood of athletes after controlled exercise and sedentary subjects after antioxidant supplementation. The correlation between EPR results and data obtained by various enzymatic assays (e.g., protein carbonyls and thiobarbituric acid reactive substances) was determined too. Synthetically, our method allows reliable, quick, noninvasive quantitative determination of ROS in human peripheral blood.

1. Introduction

Reactive oxygen species (ROS) are a group of compounds endowed with high reactivity and short half-life because of their tendency to give or receive electrons to attain stability.

On the other side, cells are exposed to a large variety of ROS by means of both exogenous and endogenous sources. Nevertheless, despite the extremely strong exposure of our whole organism to ROS coming from exogenous sources, endogenous ROS play the most important and extensive role, since, in the time course of our life, each body cell is continuously exposed to them. The major responsible of ROS production are mitochondria [1]; enzymes are another endogenous source of ROS. While most enzymes can produce ROS as byproducts of their activity (xanthine oxidase is a clear example) some of them seem specifically designed to produce ROS: nitric oxide synthase yields NO radicals; NADPH oxidase complex utilizes electrons to produce superoxide radicals from oxygen molecules [2].

The continuous ROS efflux from endogenous and exogenous sources results in a uninterrupted and cumulative oxidative damage to cell components so altering a lot of cell functions.

The most vulnerable biological targets for oxidative damage are proteins, membrane lipids, and DNA. Indeed the most usually adopted techniques for oxidative stress quantification are based on the determination of specific end products of the damage resulting from the interaction of ROS just with these biological macromolecules [3, 4]. However, all these methods return an indirect ROS determination, while the only technique capable of providing a direct free-radicals detection is electron paramagnetic resonance (EPR).

As a matter of facts, also this technique is uncapable of a direct radical detection because of the short half-life of a radical with respect to the EPR time scale [5]. In order to overcome this gap, generally a trick is therefore used: a stable chemical compound traps the radical, becoming a radical in turn, but with a half-life compatible with the EPR time scale, and so, is EPR detectable. The used compounds are classified into two main groups: spin traps, not biological and to be used at high concentration, and spin probes, working in the same way but at lower concentration and higher efficiency [6]. Moreover, just by its nature, EPR is a quantitative technique, because the signals are proportional to the number of the excited electron spins. Thus, using a reference compound, absolute concentration levels can be attained.

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 [5, 7].

A new commercial EPR spectrometer (e-scan, Bruker) is able to overcome the gap, for biological and medical applications, as it operates at the common microwave frequency and deals with very low concentration levels (nanomolar) in small sample volumes (50 microliters), also responding to both easy portability and handling features. By principle it can be applied to many samples and biological environments, like cultured cells, organs or *in vivo* to animals or human blood. Up to now, blood is the most commonly used biological sample to measure antioxidant markers and oxidation products induced by ROS production in human studies, whereas muscle biopsy is generally ethically impractical due to its invasive nature. To further reduce the invasiveness of the technique, hence increasing its clinical and diagnostic potential, herein, we investigated the application of the radical-probe approach to the measurement of ROS formation in human capillary blood. To this aim, we tested our method to monitor ROS in peripheral blood of healthy human volunteers after implementing two treatments known to affect redox status.

As is well known, one way of imposing oxidative stress is by means of physical exercise [8]: a unique relationship with oxygen free-radical formation is established, so providing an excellent model for examining the dynamic balance between oxidative challenge and antioxidant defence machinery in biological systems [9]. Indeed physical exercise is known to increase the generation of ROS [10, 11] in response to increased oxygen utilization causing a disturbance in the prooxidant/antioxidant balance in favour of the former which results in oxidative stress. On the other side of the same coin, antioxidant compounds may alter redox status too, by reducing ROS production [12, 13].

Aim of this study was to evaluate the efficacy of ROS generation assessment by a new mini-invasive procedure adopting radical probe EPR technique and altering redox status by two interventions, namely, exercise and antioxidant administration. In addition a correlation of the obtained EPR data to oxidative damage production, this latter measured by enzymatic assays of the principal biomarkers, during both at rest and after controlled physical exercise was attempted.

In particular, *ex vivo* formation of ROS, reflecting metabolic activity of blood cells and production of ROS *in vivo*, was analyzed in capillary blood using bench-top electron spin resonance spectrometer e-scan. On the other side, it is worth noting that several biomarkers are available to quantify the oxidatively modified macromolecules in biological samples, although none of them can alone adequately describe oxidative damage; therefore, several assays have been proposed in order to reliably monitor oxidative stress/damages in biological specimens. As extensively reviewed [4], the assessment of thiobarbituric acid-reactive substances (TBARS) and protein carbonyl (PC) content are among the most widely employed assays used to determine lipid peroxidation and oxidative damage to proteins, respectively. For all these reasons a possible correlation between EPR results and TBARS and PC collected data was attempted.

2. Materials and Methods

2.1. Exercise Treatment. Eighteen ($n = 18$) male elite athletes (aged 19.70 ± 1.16 years; height 1.78 ± 0.04 m; body mass 77.65 ± 6.97 kg) from the Varese hockey team were recruited to participate in the study. The subjects visited the laboratory two times. On the first day, anthropometric measures were collected and an incremental test on treadmill (1% of grade) up to voluntary exhaustion to assess gas exchange threshold (GET) and peak O_2 uptake (VO_2 peak) was also performed. After six minutes of warmup exercise (according to the subject's estimated level of physical fitness), the speed was increased 1 km · h^{-1} every minute. The mean VO_2 peak measured was 51.57 ± 1.36 mL · kg^{-1} · min^{-1}. On the second visit, at least seven days after, the subjects performed a 10-minutes constant-load submaximal exercise (CLE) at heavy-intensity and speed corresponding to a VO_2 equal to ∼50% of the difference between GET and VO_2 peak.

2.2. Antioxidant Treatment. In the acute investigation, ten ($n = 10$) healthy sedentary women (aged 48.80 ± 5.32 years; height 1.64 ± 0.03 m; body mass 56.81 ± 10.35 kg) were treated with R-thioctic acid (1.6 g).

2.3. Blood Sampling. Each subject reported to the laboratory at 9:00 a.m. after an overnight fast for blood sampling. Subjects, all nonsmokers, refrained from alcohol and caffeine consumption for at least 24 h, and were asked not to perform any form of exercise for 48 h before testing. A written informed consent was signed by all participants after being informed of all risks, discomforts, and benefits involved in the study. Procedures were in accordance with the Declaration of Helsinki, and institutional review board approval was received for this study.

2.4. EPR Protocol for ROS Detection. The experimental protocol adopted for ROS detection is shown in Figure 1. For each subject, recruited for exercise procedure, capillary blood was taken from the fingertip before and after (immediately, 10, and 20 minutes) a constant-load exercise.

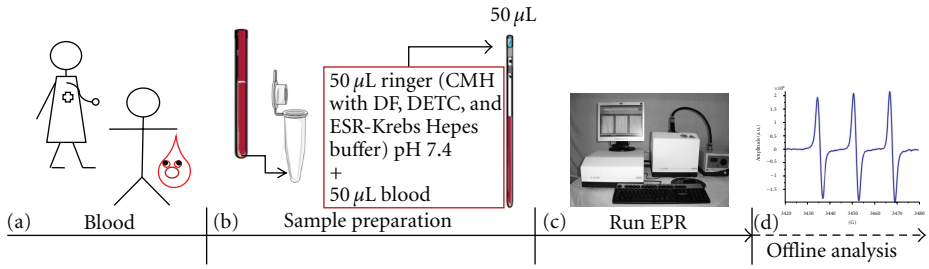

Figure 1: Step by step sketch of the EPR experimental protocol adopted to measure the ROS production by EPR.

In the antioxidant treatment, for each subject, capillary blood was drawn from the fingertip before and after R-thioctic acid administration (at 20, 40, 60, 90 minutes, 2, and 3 hours). Control sampling at rest, at the same interval time, was carried out on the same subjects two days before supplementation.

For both experimental procedures, 50 μL of blood, collected in heparinized capillary tubes (Cholestech LDX, Germany), were analyzed (Figure 1(a)). Among spin trapping (otherwise labelled probe) molecules, suitable for biological utilization, 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH, Noxygen Science Transfer & Diagnostics, Germany) was adopted. A 1 mM CMH solution was prepared in buffer (Krebs-Hepes buffer (KHB) containing 25 μM deferroxamine methane-sulfonate salt (DF) chelating agent and 5 μM sodium diethyldithio-carbamate trihydrate (DETC)) at pH 7.4. Blood was immediately treated with CMH (1 : 1). 50 μL of the obtained solution was put in the glass EPR capillary tube (Noxygen Science Transfer & Diagnostics, Germany), that was placed inside the cavity of the e-scan spectrometer (Bruker, Germany) for data acquisition (Figure 1(b)). The actual amount of solution analyzed was chosen to fill the entire sensitive area of the resonator cavity. Acquisition EPR parameters were: microwave frequency = 9.652 GHz; modulation frequency: 86 kHz; modulation amplitude: 2.28 G; center field: 3456.8 G; sweep width: 60 G; microwave power: 21.90 mW; number of scans: 10; receiver gain: $3.17 \cdot 10^1$. Sample temperature was firstly stabilized and then kept at 37°C by the temperature and Gas controller "Bio III" unit, interfaced to the spectrometer. An example of the recorded EPR signal showing the triplet coming from the interaction of the ^{14}N–OH group of CMH with the ROS oxygen unpaired electron ($NOH + O_2^{\bullet} \rightarrow NO^{\bullet} + H_2O_2$) is displayed in Figure 1(d). The radicals generated by the reaction of the probe with the blood radicals were acquired and the spectra sequentially recorded for about 5 min in order to calculate the ROS production rate. The EPR signal is proportional to the unpaired electron numbers and could, in turn, be transformed in absolute produced micromoles (μmol $\cdot$ min^{-1}): the stable CP$^{\bullet}$ (3-Carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy) radical signal was recorded in a separate session and used as reference.

The high reproducibility of the EPR measurements is shown up in the plots reported in Figure 2. The data are referred to a couple of EPR measurement data (test I (open squares), test II (closed squares)) performed on blood capillary samples taken from the same healthy subject six hours apart. The data are expressed as arbitrary units and refer to EPR signal double integrals. The regression lines obtained from the collected data show an excellent correlation coefficient ($R^2 = 0.99$) resulting in almost superimposable plots: test I (slope: 7.98, intercept: 15.49); test II (slope: 7.95, intercept: 15.95). About 0.5% discrepancy between the ROS absolute production (μmol $\cdot$ min^{-1}) in the two tests was calculated.

2.5. Limits of Detection and Quantification with the Selected EPR Method. The limits of detection (LOD) and quantification (LOQ) can be estimated using the ICH Guidelines [14] that defines these parameters as the analytic concentrations at which the signal-noise ratios (SNR) are at least 3 : 1 and 10 : 1, respectively, and in EPR they depend upon the acquisition parameters, especially on the number of scans (NS), that influence linearly the SNR and the experimental time. In the EPR spectrum of a solution of ROS at known concentration (6 μM) recorded under the same acquisition parameters adopted in the present study, the SNR of the line belonging to the ROS signal with NS = 10 was found to be 600. Therefore LOD and LOQ are immediately calculated as, respectively, $6\,\mu M \times 3/600 = 30 \cdot 10^{-3}\,\mu M$ and $6\,\mu M \times 10/600 = 100 \cdot 10^{-3}\,\mu M$.

2.6. Enzymatic Assays. Venus blood samples were taken at rest before and after (immediately, 20 minutes, 1, and 2 hour from the exercise end) constant-load submaximal exercise. Approximately 3 mL of blood were drawn from an antecubital vein, with subjects lying on a bed. The blood samples were collected in heparinized vacutainer tubes (Vacutainer, Becton Dickinson, USA), and plasma was separated by centrifuge (5702R, Eppendorf, Germany) at 1000 g for 10 min at 4°C. The samples were then stored in multiple aliquots at −80°C until assayed. Samples were thawed only once before analyses, which were performed within two weeks from collection.

2.6.1. Thiobarbituric Acid-Reactive Substances (TBARS). The measurement of TBARS is a well-established method to detect lipid peroxidation. We used TBARS assay kit (Cayman Chemical, USA) which allows a rapid photometric detection of the thiobarbituric acid malondialdehyde (TBAMDA) adduct at 532 nm. Samples were read by a microplate reader

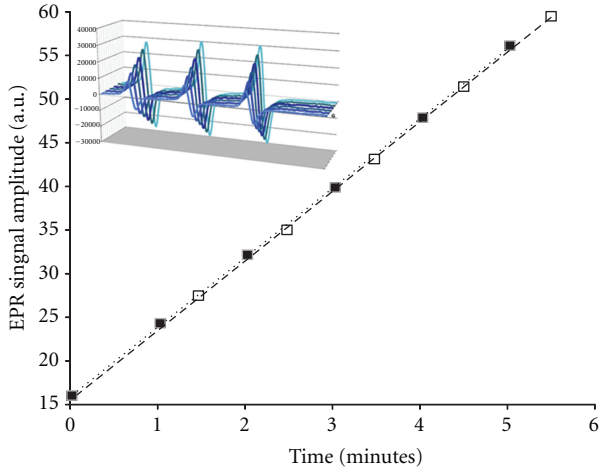

Figure 2: The high reproducibility of the EPR measurements is well demonstrated by the plots displayed in the figure showing the calculated EPR signal levels versus the elapsed time. Two tests were performed from a healthy subject taking 6 hours from each other. The best fitting straight lines ($R^2 = 0.99$) were found almost superimposable: about the 0.5% discrepancy in the ROS absolute production rate (μmol · min^{-1}) was calculated between the measurements. The stacked plot of the recorded EPR spectra during a single experiment is displayed at the upper left corner. The spectra are centered at $g = 1.997$. For each spectrum, the greatest signal amplitude difference in the triplet (arbitrary units) is returned by the acquisition routine, resulting in a point of the displayed graph. The ROS production rate (arbitrary units) is estimated by the best fitting line. It can be, in turn, converted in the absolute ROS production rate level (μmol · min^{-1}) throughout the acquisition of a stable radical compound like CP•.

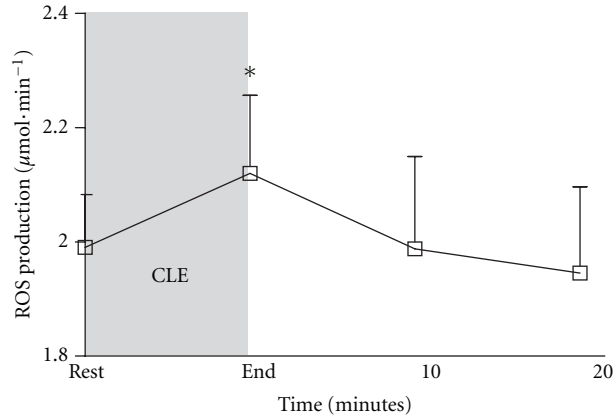

Figure 3: Time course of ROS production rate detected by EPR technique before (REST), immediately after the CLE (END) and at 10 and 20 min of recovery. Results are expressed as mean ± SD. Changes over time were significant at $P < 0.05$ immediately post CLE compared to rest (*symbol).

spectrophotometer (Infinite M200, Tecan, Austria). A linear calibration curve was computed from pure MDA-containing reactions. All samples were determined in duplicate and the interassay coefficient of variation was in the range indicated by the manufacturer (about 10%).

2.6.2. Protein Carbonyls (PC). Reactive species produced directly or indirectly through lipid peroxidation intermediates also may oxidatively modify proteins. The accumulation of oxidized proteins was measured by content of reactive carbonyls. A Protein Carbonyl assay kit (Cayman Chemical, USA) was used to evaluate colorimetrically oxidized proteins. The samples were read at 370 nm, by a microplate reader spectrophotometer (Infinite M200, Tecan, Austria), as described in detail by the manufacturer. Oxidized proteins values obtained were normalized to the total protein concentration in the final pellet (absorbance reading at 280 nm), in order to consider protein loss during the washing steps, as suggested in the kit's user manual. All samples were determined in duplicate and the interassay coefficient of variation was in the range indicated by the manufacturer.

2.7. Data Analysis. All EPR spectra were obtained by using a software standardly supplied by Bruker (version 2.11, Win EPR System). All spectra were collected by adopting the same protocol, as shown in Figure 1 and above reported.

Statistical analysis was performed using the GraphPad Prism package (GraphPad Prism 5, Software Inc. San Diego, CA). Data were analyzed using repeated Shapiro-Wilks W tests. In the Shapiro-Wilk W test, the null hypothesis is that the sample is taken from a normal distribution. This hypothesis is rejected if the critical value P for the test statistic W is less than 0.05. Experimental data were compared using one-way ANOVA with a Bonferroni post-hoc test. $P < 0.05$ statistical significance level was accepted. All values were reported as means ± standard deviation (SD).

3. Results

3.1. Exercise. Exercise-induced EPR detectable enhancement in *ex vivo* ROS formation in capillary blood. The results are summarized in Figure 3. The kinetics of ROS production estimated by the EPR signal intensity levels variation at rest, immediately after CLE and during the 20 min of recovery after is shown.

Compared with resting data, a statistically significant ($P < 0.05$) increase of ROS production immediately at the end of CLE was observed, thereafter the ROS production returned to the preexercise condition.

At the same time, as can be observed in Figure 4(a), TBARS concentration increased immediately after exercise, significantly ($P < 0.05$) peaked 20 minutes after exercise and returned toward baseline levels thereafter.

Also PC concentration increased immediately after the end of CLE, even if showing a slower rate. Its level became statistically significant ($P < 0.05$) at 20 minutes after exercise, nevertheless the highest values were reached at 1 hour after the end of the exercise and declined thereafter (see Figure 4(b)).

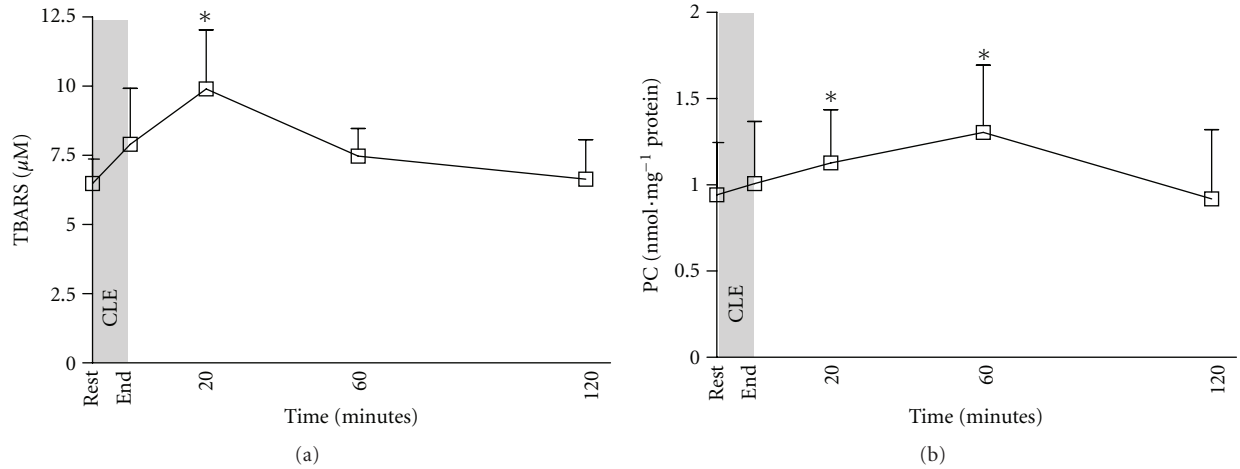

Figure 4: Time course of thiobarbituric acid reactive substances (a) and protein carbonyls (b) concentration before (REST), immediately after the CLE (END) and at 20 min, 1, and 2 hours of recovery. Results are expressed as means ± SD. Changes over time were significant at $P < 0.05$ compared to rest (*symbol).

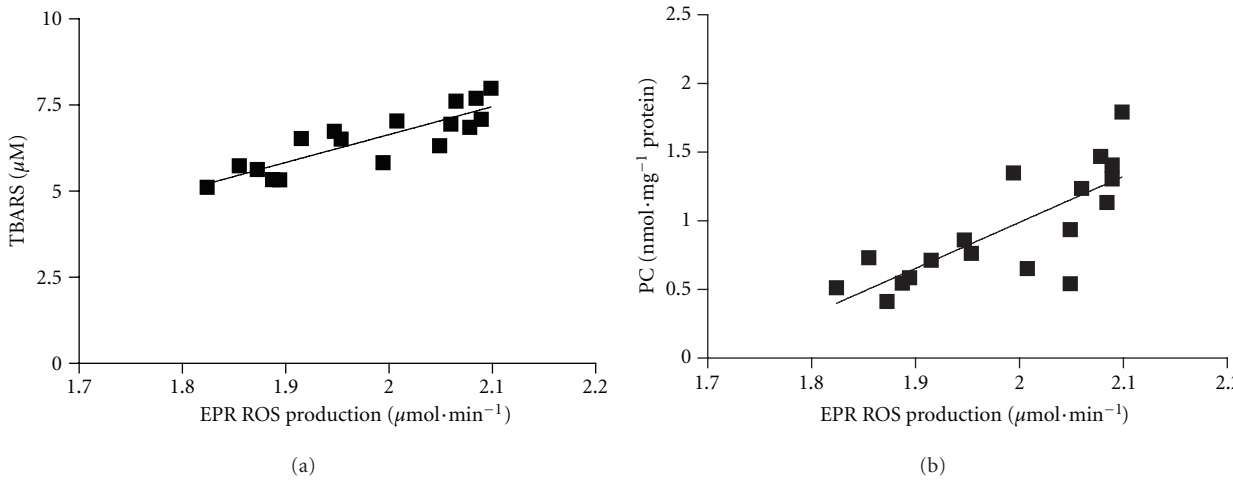

Figure 5: TBARS (a) and protein carbonyl PC (b) content as determined by enzymatic assays methods versus the ROS production rate (μmol · min^{-1}) calculated by EPR data (solid symbols). The linear regression lines (solid lines) are reported. The variance analysis (Pearson product-moment correlation) indicated a positive association for both TBARS and PC (R^2 values = 0.74 and 0.60, $P < 0.05$, resp.).

3.2. Correlation between ROS Production and Biomarkers of Oxidative Damage. Table 1 reports the mean values of plasma TBARS and PC and the ROS production values of capillary blood at rest. A positive relationship was found at rest between ROS production and plasma TBARS concentrations ($R^2 = 0.74$, $P < 0.05$) (Figure 5(a)) and with plasma PC concentrations ($R^2 = 0.60$, $P < 0.05$) (Figure 5(b)). At high-resting ROS production rate levels corresponded greater plasma TBARS and PC concentrations.

Table 1: Plasma TBARS and protein carbonyl levels and ROS production rate in capillary blood of hockey athletes at rest. Results are presented as mean ± SD.

TBARS (μM)	6.49 ± 1.01
Protein carbonyls (nmol·mg^{-1} protein)	0.94 ± 0.40
ROS (μmol·min^{-1})	1.99 ± 0.09

3.3. Antioxidant Supply. Antioxidant supplementation induced EPR detectable changes in formation of *ex vivo* ROS in capillary blood, the results are summarized in Figure 6. The kinetics of ROS production estimated by the EPR signal intensity at rest, immediately and during the 3 hours after R-thioctic acid administration is shown in the figure together with the data recorded without supplementation at the corresponding interval time. The ROS production rate level, subsequent to the supply, increased after 20 minutes, then decreased (40 minutes) and kept at a lower level with respect to the resting value, reaching its lowest significant ($P < 0.01$) level 90 minutes after supplementation, then returning to the baseline. No significant difference was observed between

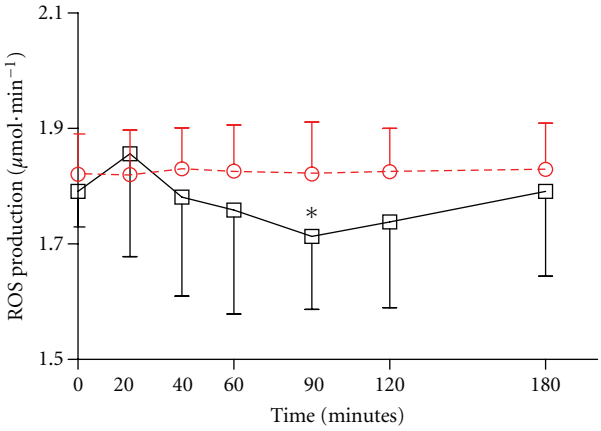

Figure 6: Time course of ROS production rate (μmol · min^{-1}) calculated by the EPR acquisition data without supplementation (red circle) and following antioxidant (R-thioctic acid) supply (black square). Results are expressed as mean ± SD. Changes over time resulted significant ($P < 0.01$) at 90 min post, compared to pre-supplementation (*symbol).

experimental baseline and control data recorded without supplementation.

4. Discussion

Electron spin resonance/electron paramagnetic resonance (ESR/EPR) spectroscopy is, without any doubt, the only direct way to detect and measure free radicals. EPR spectroscopy can be used to directly study free radicals in tissues or tissue fractions [15], while spin-trapping agents can be used to stabilize free radicals and make them easier to detect.

However, the technique suffers of serious limitations, most notably a lack of sensitivity at the concentrations of free radicals typically found in biological systems.

The relatively simple blood withdrawal procedure, compared to the more invasive tissue biopsy, seems to be the dominant factor in the overwhelming use of blood measurements in human studies.

In this case, it is also important to recognize the limitations of spin trapping, which ultimately relies on the *ex vivo* detection of relatively stable species that are formed clearly downstream of the primary reaction pathway that we assume reflecting dynamic events *in vivo*.

Nevertheless, blood interacts with all organs and tissues and, consequently, with many possible sources of reactive species. In addition, a multitude of oxidizable substrates are already present in blood and carrying a multitude of substances that are considered oxidative stress markers (e.g., TBARS, protein carbonyls). Changes in the blood concentrations of these markers reflect corresponding changes in the tissue of interest (most of the time skeletal muscle) [16].

As a matter of facts, the vast majority of the relevant human studies have measured the redox status by using plasma or serum. This choice was probably adopted after considering that plasma better reflects tissue redox status together with the ease of plasma collecting procedure. However, with this latter choice we cannot exclude potential artefacts generated by *in vitro* chemistry during the preparation and incubation phase of the spin probe or spin trap used, although this limitation is not exclusive to EPR sample preparation since it has interpretive implications for any reactive metabolite measured *ex vivo*, especially in human plasma.

Moreover, since the classic tests have mainly quantified the ROS levels in human plasma but not those associated with erythrocytes, these studies have not taken into account the role of circulating cells. Ginsburg et al. [17] raised serious doubts whether reports on antioxidant quantifications carried out exclusively in plasma can be trusted to represent true oxidant-scavenging abilities.

Indeed red blood cells may exert both antioxidant and prooxidant activity. Because of the high-iron concentration (~20 mM), the red blood cell (RBC) can be considered an "iron mine" but, paradoxically, it is also one of the major components of blood antioxidant capacity and one of the most resistant to oxidative stress cell. A very efficient intracellular reducing machinery, coupled with its high-cell density makes the erythrocyte an effective "sink" of reactive species. Probably not only the blood per se but, more important, the whole organism can benefit from RBC scavenging ability. The reverse side of RBC antioxidant power is its capability of being, in turn, a source of reactive species. The superoxide radical generated within the RBC by deoxygenated or partially oxygenated hemoglobin, usually found at low levels and likely under physiologic conditions, does not represent a big hazard for the cell. Similarly, the ability of RBC to scavenge or generate nanomolar concentrations of NO$^{\bullet}$ can be easily handled by the methemoglobin reductase/NADH/glycolysis system. Completely different may be the situation when the erythrocyte crosses a tissue where an intense production of reactive oxygen/nitrogen species is occurring. Under these conditions, the RBC may accumulate oxidative damage, in turn reflecting the oxidative stress of other tissues and organs. For this reason, oxidative status of RBCs is potential candidate for monitoring the overall oxidative stress status.

During exercise, since reactive species are generated by both blood and muscle, it is reasonable to assume that there is a bidirectional movement of reactive species from the muscle to the blood, and vice versa, until equilibrium is reached. The same may hold true for exchanges among blood constituents, namely, plasma, erythrocytes, leukocytes, and platelets [18], once that certain basic assumptions are met: reactive species with adequate half-life have the ability to cross membranes and generate reactive species at the vicinity of the compartments considered.

Based on all these considerations and aiming at reducing the invasiveness of the method and hence increasing its clinical and diagnostic potential, herein, we investigated the application of the radical-probe approach to the measurement of oxidative stress status in peripheral blood. Moreover fresh, rather than frozen, samples were used for the EPR measurements to be able to gain an estimation of the ROS production rate, instead of a single level, well assuming that this procedure allowed us to attain more precise and reliable results.

Unpaired electron(s) carrying species are, as such, EPR visible. However ROS half-life (superoxide $[O_2^{\bullet -}]$ $t_{1/2}$ (s): 10^{-4}; nitric oxide $[NO^{\bullet}]$: 0,4 at ambient temperature) is too short if compared to the EPR time scale so they are EPR-invisible. Therefore the species have to be "trapped" and transformed to a more stable radical species to become EPR detectable. Among spin trapping or probe molecules, suitable for biological utilization, CMH was adopted, since it is a molecule capable of diffusion in all cell compartments, including mitochondria [6]. Indeed, due to its peculiar physical-chemical properties, CMH probe is able to cross biological membranes, thereby detecting ROS both in plasma and intracellular compartments. In this way, EPR measurements allowed us to attain a relative quantitative determination of ROS production rate in human blood samples.

In addition, owing to its high efficiency in radical detection, CMH probe can be used at very low concentrations (0.5–1 mM) compared to spin traps (10–50 mM), which minimizes side-effects of the probes on the biological samples. Moreover CMH rapidly reacts and allows radical detection in a single chemical reaction, while other probes require at least two reactions, which may cause artefacts [19].

The high reproducibility of the measurement was demonstrated by performing the experiments two times on the same subject six hours apart. In fact the procedure itself makes impossible to repeat the same experiment several times on the same blood sample. Nevertheless, the collected data suggested that repeated experiments from a resting subject gave almost superimposable results (see Figure 2 and red circle Figure 6).

Considerable evidence has linked exhaustive exercise with extensive free radicals formation. Davies et al. [8] were the first to establish exercise-induced free radicals formation after exhaustive physical activity by demonstrating a heightened EPR signal (around $g = 2.004$) in muscle and liver homogenates. Other studies [20, 21] have also been able to demonstrate heightened EPR signals with exercise. There are numerous reports that provide reasonable support to the notion that exercise increases the production of reactive oxygen species, and that mitochondria are important sources of these oxidants [9, 22]. Other sources of oxidative stress during physical exercise are inflammatory responses mediated by neutrophils [23], the release of transition metals, such as iron, that supports Fenton chemistry with formation of hydroxyl radical, the interaction of metmyoglobin and methemoglobin with lipid peroxides [24], and the activity of xanthine oxidase [25], possibly within an ischemia-reperfusion model [26]. It may be surmised that high-intensity physical exercise disrupts the fragile balance between oxidants and antioxidant defences and that the mitochondrial respiratory chain and other pathways contribute to free radicals generation. As indicated above, blood is a tissue of paramount importance in regulating redox status changes appearing during exercise.

Using ESR spectroscopy, our study clearly demonstrated in human capillary blood that a short-term constant-load submaximal exercise (CLE) at heavy intensity, induced oxidative injuries since a significant increase in ROS production was detected (Figure 3).

As reported elsewhere [27], professional athletes showed a rapid increase in ROS on starting exercise. This was followed by a gradual decrease in the magnitude of the ROS production, reaching the initial value after 20 min. This is in agreement with the concept that adaptive responses to aerobic training programs render athletes' enzymes less responsive over time to further significant activation, and that increased ROS generation caused by physical exercise overwhelms the capacity of the body to detoxify ROS.

Parenthetically, we would like to underline that main aim of the present study was to evaluate the efficacy of ROS generation assessment by adopting a mini invasive procedure. Just for this reason we have been forced to use arterial capillary blood samples even if we are completely aware that arterial blood, because of circulatory system design, is very far from muscles that are the main ROS sources during exercise. Therefore, we expect that when using a systemic determination the ROS detected levels will be surely lower mainly because part of the produced ROS have been buffered by blood itself. Bailey et al. [28] showed, in typical EPR spectra, a positive venoarterial concentration of α-phenyl-*tert*-butylnitrone (PBN) adducts, detected in the arterial and venous circulation, at moderate intensity exercise. In the same paper the authors reported an increase in the EPR signal amplitude, according to the exercise intensity both in the arterial and venous circulation. Nevertheless the ROS detected level was always found lower in arterial with respect to venous blood samples. Indeed it is worth noting that, despite the moderate exercise levels adopted in the present study, the method was found suitable to detect a significant difference; demonstrating its reliability even under the adopted experimental conditions. On the other side, under the adopted acquisition protocol, the ROS levels measured in the present study resulted over the estimated limit of detection and quantification.

Increased production of reactive oxygen and nitrogen species after cessation of exercise may have contributed to elevated levels of lipid and protein peroxidation markers after exercise (Figure 4). The measurement of these latter only in plasma is enough to describe the changes in erythrocyte and muscle redox status because of a strong communication between the different compartments [16]. Indeed results from the present study indicate that lipid peroxidation, as measured by TBARS, was elevated immediately and until 20 min after exercise (Figure 4(a)). Many studies have provided indications for substantial increases in plasma lipid-peroxidation levels after aerobic exercise [29–31]. The delay in TBARS clearance, compared to more rapid ROS production kinetics, is in agreement with the observation of Echtay et al. [32] who proposed that lipid peroxidation products regulate mitochondrial ROS production by inducing the expression of proteins that inhibit mitochondrial $O_2^{\bullet}$ production by inducing uncoupling. Otherwise, removal of oxidized proteins from blood is, presumably, a time-consuming process, also considering that oxidative modifications in protein can occur by the effect of ROS directly or indirectly through conjugation to lipoxidation end products. For these reasons, protein carbonyls concentration remained elevated for a prolonged period (1 h) after exercise (Figure 4(b)).

Other studies generally have reported increases similar to ours immediately after exercise, whereas the increases in protein carbonyls mostly disappeared after 0.5 to 6 h of recovery [29, 33–35]. The different life response of protein carbonyls reported may be partly attributed also to the different intensity and more or less muscle-damaging exercise mode used in the studies and to differences in the physical fitness of the participants employed [36].

Secondary aim of this study was to examine whether measuring blood oxidative stress markers, a currently common practice in biomedical research, is indicative of ROS-production. Although almost all oxidative stress biomarkers have been criticized for their reliability (including those used in the present study) [37, 38], it is apparent that, at rest, all changes indicating increased oxidative stress are directly related to ROS production (Figure 5). However the time-course changes of the used markers of oxidative stress were delayed and longer (Figures 4(a) and 4(b)) than ROS-production kinetics (Figure 3), and no correlation is possible in dynamic conditions. In other words, it must be stressed that the good correlation found between EPR and enzymatic assays data do not represent a validation test for the here proposed EPR technique. By principle, the adopted enzymatic methods can quantitate the damage arising from ROS production, while EPR is the only technique capable, by using suitable probes, of quantitating the ROS production itself. These are the reasons why we could not hypothesize an "a priori" existing relationship between these methods we cannot absolutely affirm that ROS production will at the same time produce damage. On the other hand the data collected in the present study seem to suggest that the practice of the vast majority of the relevant studies to collect one blood sample immediately after or at some other early point postexercise can potentially lead to inaccurate deductions [29].

Similar consideration may be expressed for the analysis of the effects of antioxidant supplementation too. Thioctic acid (α-lipoic acid) was adopted for the study. In fact this molecule is an endogenous antioxidant which, in its reduced form, that is, dihydrolipoic acid, forms a thiol-disulphide redox system [39–41]. Moreover this moiety may also replenish intracellular GSH levels [42, 43] and is an essential cofactor for the multienzyme complexes and pyruvate dehydrogenase.

Indeed, in our study, the time corresponding to the peak activity of R-thioctic acid is delayed well after supplementation (Figure 6), as reported by other authors too [44]. Antioxidants may be defined as molecules that prevent cell damage against free radicals and are critical for maintaining optimum health in both animals and humans. In all living systems, cells require adequate levels of antioxidant defences in order to avoid the harmful effect of an excessive production of ROS, and so preventing the associated damage. Indeed excess of ROS production might play a role in pathophysiology of many disease conditions, including cancer, Alzheimer's disease, and atherosclerosis. Many basic research studies and observational epidemiologic studies in humans suggest that antioxidants can prevent oxidative damage. However, this is still a controversial issue because the results of clinical trials have been inconsistent [45]. Indeed there is a need for large multicentre prospective randomized control trials to assess the effects of different types and doses of antioxidant supplementation in selected groups of subjects. The methodological approach adopted in this study might be an efficient and practical tool to solve the question both in acute or chronic conditions.

5. Conclusion

Although the healthcare field is increasingly aware of the importance of free radicals and oxidative stress, screening and monitoring has not yet become a routine test. The method herein presented allows reliable, rapid, and noninvasive measurement of the instantaneous concentration of ROS directly in human peripheral blood. Due to its simplicity coupled with the high sensitivity and specificity of EPR spectroscopy, it compares favourably with the few currently available methods that have been successfully applied to measure radical species in human blood. After accurately testing and defining the most appropriate assay procedure, this kind of EPR determination can become an even turnkey, automated technique for a lot of routine and medical diagnostic applications on the time course of oxidative stress too. Moreover these findings seem to be valid both at rest and after interventions able to alter the redox status of a living system. In conclusion, ROS production assessed by the here proposed EPR measurement procedure in capillary blood seems to be well suitable to provide a reliable indication about the free radical-mediated changes that appear in skeletal muscle, heart, and liver.

Acknowledgments

The authors wish to thank the Scientific Commission of Italian Federation of Sport Medicine for the financial support. They are grateful to all the athletes and trainers of hockey Varese team and to all subjects that participated in the experimentation. The authors also thank Dr. Manuela Liberi and Dr. Roberto Melzi of Italian Bruker Biospin for their kind technical support.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2012, Article ID 935483, 11 pages
doi:10.1155/2012/935483

Research Article

Interaction between Overtraining and the Interindividual Variability May (Not) Trigger Muscle Oxidative Stress and Cardiomyocyte Apoptosis in Rats

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Received 9 April 2012; Accepted 16 April 2012

Academic Editor: Michalis G. Nikolaidis

Severe endurance training (overtraining) may cause underperformance related to muscle oxidative stress and cardiomyocyte alterations. Currently, such relationship has not been empirically established. In this study, Wistar rats ($n = 19$) underwent eight weeks of daily exercise sessions followed by three overtraining weeks in which the daily frequency of exercise sessions increased. After the 11th training week, eight rats exhibited a reduction of 38% in performance (nonfunctional overreaching group (NFOR)), whereas eleven rats exhibited an increase of 18% in performance (functional overreaching group (FOR)). The red gastrocnemius of NFOR presented significantly lower citrate synthase activity compared to FOR, but similar to that of the control. The activity of mitochondrial complex IV in NFOR was lower than that of the control and FOR. This impaired mitochondrial adaptation in NFOR was associated with increased antioxidant enzyme activities and increased lipid peroxidation (in muscle and plasma) relative to FOR and control. Cardiomyocyte apoptosis was higher in NFOR. Plasma creatine kinase levels were unchanged. We observed that some rats that presented evidence of muscle oxidative stress are also subject to cardiomyocyte apoptosis under endurance overtraining. Blood lipid peroxides may be a suitable biomarker for muscle oxidative stress that is unrelated to severe muscle damage.

1. Introduction

The upper limit of human sports performance has yet to be identified. To maximize physical performance, athletes and coaches manipulate training load through adjustments in duration, frequency, and intensity or through a reduction of the regenerative period. However, a cumulative imbalance between exercise load and recovery can lead to persistent, undesirable underperformance associated with training and/or nontraining stress, which is generally referred to as overtraining (OT).

Sports science researchers have used a variety of terminologies to describe both the means and the consequences of OT [1]. In 2006, to encourage a consensus among researchers in the field, the European College of Sport Science defined OT as a continuous process of intense training that can generate different performance states [2]. The functional overreaching (FOR) state is characterized by performance maintenance or by an increase in performance after a brief recovery period of days to weeks. The nonfunctional overreaching (NFOR) state is characterized by a prolonged decay of performance that is reversed only by a long regenerative period of weeks to months. Finally, overtraining syndrome (OTS) is the most extreme state of OT; performance recovery in OTS may take years to occur or may never occur.

Lehmann et al. [3] reported that individual variability in recovery potential, exercise capacity, stress tolerance, and training tolerance explains the diverse vulnerabilities of athletes to OT under identical training stimuli. However, the metabolic and physiological factors responsible for the varying individual responses to OT and the causes of persistent underperformance are not well understood. Therefore, we have investigated the potential effects of endurance OT in Wistar rats using a standardized, 11-week treadmill endurance OT regimen [4]. Using this OT protocol, we observed lower citrate synthase (CS) activity in the red gastrocnemius (RG) muscle in rats that displayed a long-term reduction of performance in their training regimen [4]. In contrast, an increase in CS activity is an expected positive adaptation to endurance training as a known marker of mitochondrial oxidative capacity [5].

It has been proposed that 0.1% of the O_2 consumed by the mitochondria is converted to superoxide anions ($O_2^{\bullet -}$) [6]. Therefore, exercise may increase reactive oxygen species (ROS) levels because it increases whole-body and tissue rates of oxygen consumption [7]. The ROS generated during exercise can reduce the ATP yield in mitochondria by potentially damaging protein complexes in the electron transport chain, mitochondrial enzymes, membrane lipids or mitochondrial DNA [8–11] Nevertheless, a series of enzymatic and nonenzymatic antioxidants limit the biological activity of ROS inside the mitochondria and in the cytosol [12], and, accordingly, it was shown [13] that contractile activity in isolated muscle is related to an immediate enzymatic antioxidant response.

Chronic endurance training, however, does not result in a predictable adaptation of antioxidant enzyme activity in parallel to oxidative capacity in rat skeletal muscle [14–17]. Therefore, persistently high concentrations of ROS during severe endurance training can overwhelm cellular defense mechanisms and generate oxidative stress [18]. In this sense, the relationship between oxidative stress and underperformance in OT was previously proposed [19].

Severe endurance exercise can also increase the production of ROS in cardiac muscle [20]. Studies of ultraendurance activities have highlighted cardiac risks, such as the transient loss of ventricular function, increased heart tissue damage and the subsequent appearance of myocardial injury biomarkers in the blood [21, 22]. In rats, treadmill running to exhaustion was associated with increased deletion of mitochondrial DNA ($mtDNA^{4834}$) and increased apoptosis in the left ventricle (LV) [23]. It was thus shown that H_2O_2 and $O_2^{\bullet -}$-induce apoptosis [24] by opening the permeability transition pore and triggering the release of proapoptotic proteins into the cytosol [25]. For obvious reasons, we have not found studies reporting possible morphological modifications or an altered redox status in the cardiac tissue of athletes subjected to OT that might correlate changes to the myocardial tissue structure with underperformance. Unlike in skeletal muscle, the impairment of cardiac function by severe alterations of cardiac tissue structure can not only decrease performance but also can be fatal. Therefore, it is essential to establish specific blood biomarkers for severe endurance training to indicate individual intolerance to training regimens and to provide empirical evidence for the risks assumed [26].

The aim of this study was to provide support for the hypothesis that muscular oxidative stress and lowered mitochondrial capacity are metabolic features that underlie persistent underperformance due to endurance OT. Furthermore, we investigated the potentially dangerous effect of endurance OT on heart tissue, targeting the relationship between individual vulnerability to muscle oxidative stress, cardiac apoptosis, and histological alterations.

2. Materials and Methods

The rationale for all methodological procedures concerning the development and the characterization of the OT animal model used in this study is published elsewhere [4]. We present here sufficient description for an independent reading.

2.1. Animals. Fifty-six 21-day-old male albino Wistar rats were acquired from the Multidisciplinary Center for Biological Investigation on Laboratory Animal Science (CEMIB), UNICAMP. The animals were housed in a climate-controlled environment at 25 ± 1°C with an inverted 12 h light-dark cycle and were fed *ad libitum*. The experimental protocols were approved by the Animal Experimentation Ethics Committee of the Institute of Biology (IB)-UNICAMP.

When they were 60 days old, the rats were adapted to the treadmill for two weeks prior to beginning the training protocol. During the adaptation phase, the rats were placed on a treadmill 5 days/week for 10 minutes at a speed of 12 m/min. This initial training distinguished the animals that ran voluntarily ($n = 50$) from the rats that refused to run ($n = 6$) to ensure that only animals that ran were included in the study.

2.2. Training Protocol. The training protocol was designed to induce a training-to-OT continuum as evaluated by six performance tests (T1 to T6) (Table 1). A motorized treadmill with twelve individual lanes and no inclination was used. A shock grid at the back of the treadmill provided a mild shock (1.5 mA) if the pace of the rats fell below the treadmill speed. The training week consisted of five consecutive days of training sessions followed by two days of rest. The amount of training in minutes of each training session was individually quantified. The animals were allowed to recover for 24 hours between training sessions during the adaptative training phases (AT1 and AT2). The recovery period between sessions was gradually decreased during T2x, T3x, and T4x (Table 1).

2.3. Performance Test. All tests were performed 60 hours after the last training session of the week (between 1 : 00 pm and 5 : 00 pm). The tests began with the animals running on the treadmill at an initial speed of 12 m/min. Every 2 minutes, the speed was increased by 1 m/min until 20 m/min was reached. After that, the speed was increased by 2 m/min every 3 minutes until exhaustion, which was defined as the time at which the animals touched the shock grid five times in one

Table 1: Training Protocol.

Experimental weeks	Training phases	Test number	Training speed (m/min)	Training time (min)	Number of daily sessions	Recovery between training sessions (h)
	Acclimatization	T1	(—)	(—)	(—)	(—)
1st	AT1	no tests	15	20	1	24
2sd	AT1	no tests	20	30	1	24
3th	AT1	no tests	22,5	45	1	24
4th	AT1	T2	25	60	1	24
5th to 7th	AT2	no tests	25	60	1	24
8th	AT2	T3	25	60	1	24
9th	T2x	T4	25	60	2	4
10th	T3x	T5	25	60	3	3
11th	T4x	T6	25	60	4	2

AT1: adaptative training 1. AT2: adaptative training 2. T2x, T3x, T4x: training week w/2, 3, and 4 daily sessions.

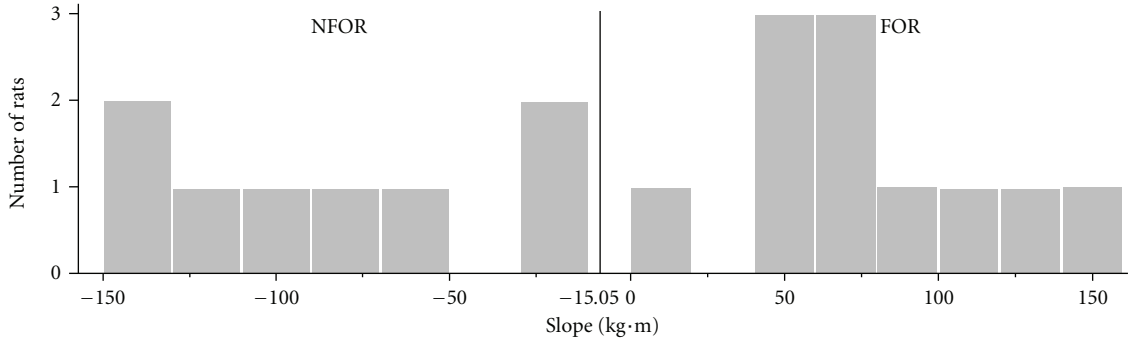

Figure 1: Histogram of the Pr slopes of the NFOR ($n = 8$) and FOR ($n = 11$) groups.

minute. The body weight of the animals was measured before each performance test.

2.4. Performance Quantification. To better evaluate the effect of training over time, we quantified animal performance using a mass-dependent model. This calculation allows the performance of each rat to be measured by a quantity that is proportional to the mechanical work performed by the rat as it runs on the treadmill, specifically, power times distance, as shown in (1):

$$\mathrm{Pr} = \Sigma \mathrm{Pr}_i = \Sigma m V_i \; T_i = \Sigma m D_i = mD, \tag{1}$$

where (Pr) represents the rat's performance, (Pr_i) is the performance of the rat at each stage (i), (m) is the body mass, (V_i) is the stage velocity, (T_i) is the stage running time, (D_i) is the stage distance; and (D) is the total distance covered by the rat during the test. In this paper, Pr will be expressed in kilograms multiplied by meters (kg·m).

2.5. Animal Groups. Only those rats with Pr values that ranged between 70 and 230 kg·m (28 out of 50 running rats) in test 1 (T1; Table 1) were selected for the study. The rejected rats were transferred to other studies. A control group (CO, $n = 9$) was randomly selected from among the 28 rats chosen for this study. During the 11 weeks of training, the animals of the CO group were subjected to 10 min of running at 12 m/min twice a week to become accustomed to the treadmill and handling. The other 19 rats were subjected to the training protocol and were finally divided in two groups: nonfunctional overreaching (NFOR) and functional overreaching (FOR).

2.6. Establishment of the Functional Overreaching and Nonfunctional Overreaching Groups. The rats were empirically assigned to the NFOR and FOR groups based on their individual performances in T4, T5, and T6. The test results were quantified by measuring the slope (α) of a least-squares fit line through the Pr for T4, T5, and T6. The critical slope value for group separation was obtained from the CO group from our previous study [4], which had a slope of -3.26 ± 11.79 kg·m (means ± SD). The critical value for separating the NFOR and FOR groups was the mean minus one standard deviation, that is, $\alpha_{\text{critical}} = -15.05$ kg·m. Figure 1 shows the histogram of the observed slopes. Rats with $\alpha < -15.05$ kg·m were designated as NFOR ($n = 8$), and those with $\alpha \geq -15.05$ kg·m were designated as FOR ($n = 11$).

2.7. Tissue and Blood Sample Collection. The animals were euthanized 48 h after T6 (Table 1) using a combination

of the anesthetic Zoletil (50 mg/kg body weight) and the muscle relaxant xylazine (10 units/kg body weight), which was administered by intramuscular injection in the right quadriceps. Samples of blood, heart (left ventricle, LV), and red gastrocnemius (RG) muscle were collected in that order from the CO, NFOR, and FOR groups.

2.8. Activity of Muscle Citrate Synthase and Mitochondrial Complexes I/V and IV/V. Red gastrocnemius samples (30 mg) were prepared as described by Zerbetto et al. [27] with the modifications described by Molnar et al. [28]. The samples were homogenized (Polytron PT-MR 2100, Kinematica, Switzerland) in 2 mL of ice cold buffer (20 mM 3-(N-Morpholino) propanesulfonic acid (MOPS), pH 7.2, 440 mM sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), and 5 mM phenylmethylsulfonyl fluoride (PMSF). The samples were centrifuged at 20,000 ×g for 20 minutes. The supernatant was used to spectrophotometrically assay CS (EC 4.1.3.7) activity by determining the rate of the increase in absorbance at 412 nm due to the formation of thionitrobenzoic acid ($å = 13.6\,mL \cdot \mu mol^{-1} \cdot cm^{-1}$) as described by Srere [29]. The pellet was resuspended in 80 μL of buffer (1 M 6-aminohexanoic acid, pH 7.0, 50 mM BIS-TRIS, and 5 mM PMSF) with 30 μL of 10% n-dodecylmaltoside. This solution was centrifuged for 35 minutes at 100,000 ×g. The supernatant was collected, and the procedure was repeated to increase the sample yield. Both aliquots were mixed (20 μL) and stored at −80°C.

The blue native polyacrylamide gel electrophoresis (BN-PAGE) was performed as described by Schagger and von Jagow [30] using a 1 × 70 × 82 mm minigel (Mini-Protean, Bio-Rad). Following electrophoresis, the gels were cut into three pieces, each containing samples from one animal from each of the three experimental groups. The colorimetric analysis of the catalysis reactions (histochemistry) of complexes I and IV and the determination of the abundance of complex V (mitochondrial protein marker) were performed according to Molnar et al. [28].

The wet gels were digitally scanned using LABSCAN 5.0 software, and the histochemical staining intensity was quantified using the IMAGE MASTER 2D PLATINUM 6.0 software. The staining of complexes I and IV was measured as arbitrary units relative to the staining of complex V to allow the results to be analyzed both qualitatively and quantitatively as the specific activity.

2.9. Muscle and Heart Antioxidant Enzymes and TBARS Assays. The LV and RG sections (60 mg each) were frozen at −80°C and later homogenized (Polytron PT-MR 2100, Kinematica, Switzerland) in an ice bath at maximum speed for 10 s. The tissue was solubilized in a 1 : 20 (w/v) ratio in 50 mM phosphate buffer, pH 7.0, to analyze catalase (EC 1.11.1.6), GR (EC 1.8.1.7) and total SOD (EC 1.15.1.1). An additional 90 mg section was solubilized in a 1.15% KCl solution at a 1 : 10 (w/v) ratio for the thiobarbituric acid-reactive substance (TBARS) analysis.

The catalase activity was determined according to Aebi [31] by the decrease in absorbance due to H_2O_2 consumption ($å = 0.04\,mL \cdot \mu mol^{-1} \cdot cm^{-1}$) measured at 240 nm. The glutathione reductase activity was quantified as described by Smith et al. [32] by the rate of the increase in absorbance at 412 nm due to the formation of thionitrobenzoic acid. One unit (U) of catalase, GR, and CS represents the activity in μmol/min.

The total SOD quantification was conducted with an R&D Systems kit (MN, USA) according to the protocol of the manufacturer. One unit of SOD was defined as the activity that inhibits the rate of NBT-diformazan formation by 50%.

TBARS was quantified similarly to the methods described by Uchiyama and Mihara [33] and Ohkawa et al. [34] using 1,1,3,3-tetramethoxypropane (TMP) as the standard. All analyses were performed in triplicate using a Beckman DU-640 spectrophotometer. The means of the coefficients of variation (CV) from the triplicate LV homogenate samples were 3.2%, 5.4%, 1.3% and 8.8% for catalase, GR, SOD, and TBARS, respectively. For RG muscle, we obtained CV values of 3%, 5.8%, 1.3%, and 9.1% for catalase, GR, SOD, and TBARS, respectively.

2.10. Heart Histology. The heart was removed in diastole (filled with 1.15% KCl), cleaned with 0.9% saline solution at 4°C to remove excess blood and weighed immediately following the cleaning. The left ventricle (LV) was fixed in 4% paraformaldehyde for 24 h and subsequently embedded in paraffin. The paraffin blocks were sectioned into 5 μm slices using a microtome and were immediately mounted onto glass slides. The sections were stained with hematoxylin and eosin (HE) or picrosirius (sirius red 3BA in saturated picric acid solution), which was visualized without polarization. The cellular area and the LV collagen fibers were measured on the HE and Sirius Red slides, respectively. We quantified 30 cells on the HE slides and counted 30 fields from three animals of each experimental group on the Sirius Red slides. The cell area was determined in μm^2, and the collagen area was measured as the percentage of total fields observed. The slide visualization and image recording were performed using an Olympus (USA) optic microscope, and the quantitative analysis was performed using Image Pro-Plus 6.0 (Amersham Biosciences).

2.11. Heart Apoptotic Index. A longitudinal section of the anterior portion of the LV was immersed in a 30% sucrose solution at 4°C for 30 min for cryopreservation, embedded in Tissue-Tek, and stored at −15°C. Twelve consecutive 5 μm slices were cut in a cryostat (Leica, model CM1850, Germany) and placed on slides that had been previously coated with silane (methacryloxypropylmethoxysilane, Sigma, USA). The apoptotic index was obtained using the *In Situ* Cell Death Detection kit (Roche Diagnostics, Mannheim, Germany) and was calculated as the ratio of marked nuclei (apoptotic, brown) to nonmarked nuclei (nonapoptotic, purple). We used five optical fields from five animals in each experimental group for a total of 25 fields per group. The nuclei were counted using an Olympus (USA) microscope by an experienced researcher who was blinded to the identity of the samples.

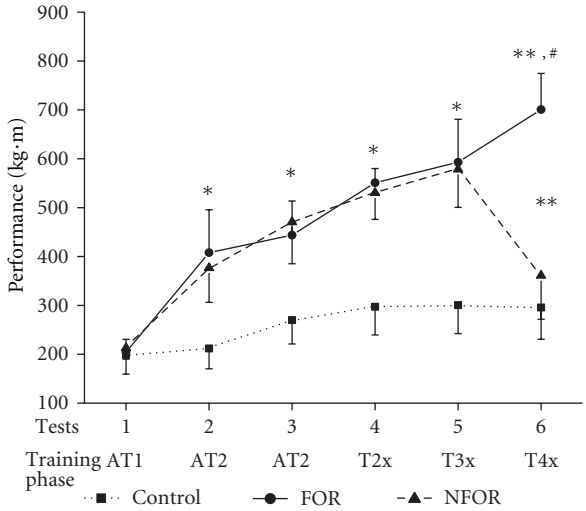

Figure 2: Performances of the CO ($n = 9$), FOR ($n = 11$) and NFOR ($n = 8$) groups in the six performance tests during the 11-week running training protocol (means ± SD). *Significant difference of the FOR and NFOR groups relative to the CO group ($P < 0.001$). **Significant difference between test 6 and test 5 in the paired analysis of FOR and NFOR groups ($P < 0.01$ for FOR and $P < 0.001$ for NFOR). #Significant difference of the FOR group relative to the NFOR and CO groups ($P < 0.001$).

2.12. Plasma Creatine Kinase and Lipid Peroxides. The blood was collected in heparin-coated tubes and was immediately centrifuged at 1,232×g for 15 minutes at 4°C to separate the plasma. The plasma CK was measured by the CK-NAC kit (Wiener Lab, Rosário, Argentina) using an Autolab Analyzer (Boehringer Mannheim, Germany), and the lipid peroxides were measured with a lipid peroxidation assay kit (Colorimetric Calbiochem kit, Frankfurt, Germany). This colorimetric assay is designed to measure malondialdehyde (MDA) in combination with 4-hydroxyalkenals. Malondialdehyde was used as the standard.

2.13. Statistical Analysis. The results are presented as the means ± standard deviation (SD). We used the t-test to compare the differences between the means of two groups. To compare the means of more than two groups, we used unpaired ANOVA followed by Tukey's post hoc test. Comparisons with P-values less than 0.05 were considered significant.

3. Results

3.1. Performance. Both trained groups exhibited a significant increase in performance relative to the CO group throughout the protocol until the 10th week of training (Figure 2). When the training load was increased to four sessions a day (T4x), the performances of the trained rats differed. Some rats showed a significant performance increase in T6 relative to T5 (FOR group), whereas others showed a significant performance drop in T6 relative to T5 (NFOR group). There were no significant differences in the training volumes of the NFOR and FOR groups until the 10th week (T3x). The total training volume of the NFOR group was significantly lower than that of the FOR group after the 11th week (T4x) (4348 ± 110 min and 4552 ± 70 min, resp., $P < 0.01$).

3.2. Antioxidant Activities of SOD, Catalase, and GR and Concentration of TBARS in Muscle and Heart. The activities of SOD, catalase, and GR and the concentration of TBARS were significantly higher in the muscle of the NFOR group than in those of the CO and FOR groups (Figure 3). In the heart, the SOD activity of the NFOR group was significantly higher than that of the CO and FOR groups. The catalase activity of the NFOR group was significantly higher than that of the FOR group only. The GR activity of both trained groups, FOR and NFOR, was significantly higher than that of the CO group. Finally, there were no significant differences in the concentration of TBARS among the three groups.

Comparing heart with muscle within the CO group, we found that the activities of catalase and GR and the concentration of TBARS were significantly higher in the heart than in the muscle.

3.3. Activities of Muscle Citrate Synthase and Mitochondrial Complexes I/V and IV/V. The activity of CS was significantly higher in the FOR group than in the CO and NFOR groups (Table 2). The histochemical staining of complex I activity normalized to the complex V abundance (Complex I/V) was not significantly different between the groups. The equivalent analysis for complex IV/V staining produced a significantly lower value for the NFOR group than for the CO and FOR groups. The staining of complex IV was noticeably weaker for the individual samples from the NFOR group compared to the samples from the FOR and CO groups within the same gel.

3.4. Cardiac Morphometric Analysis. There was no significant difference in the heart mass between the different groups (Table 3). The ratio between the heart mass and the body mass was significantly greater for the FOR and NFOR groups than for the CO group. The cellular areas of tissue for the FOR and NFOR groups were significantly greater than that of the CO group.

3.5. Histology of the Left Ventricle

3.5.1. Hematoxylin and Eosin (HE) and Picrosirius (Sirius Red). Figure 4 shows representative HE (Figure 4(a)) and sirius red (Figure 4(b)) images of left ventricle tissue from an individual rat from each of the experimental groups. The HE staining did not reveal any observable differences in the LV cell structure among the CO, FOR, and NFOR groups. We did not discern any alterations related to the presence of cellular infiltration, as samples from all of the groups showed similar levels of this phenomenon in the peripheral region and around blood vessels. We did not observe cardiac steatosis. The sirius red staining did not reveal any quantitative differences in collagen between the different groups (Table 3).

Table 2: Citrate synthase activity and specific activity (as arbitrary units) of complex I/V and IV/V in BN-PAGE gels.

	CO	FOR	NFOR
Citrate synthase (U/g wet)	18 ± 2	35 ± 3**	21 ± 7
Complex I/V	1.04 ± 0.36	1.06 ± 0.40	0.88 ± 0.23
Complex IV/V	1.04 ± 0.22	1.06 ± 0.22	0.73 ± 0.26*

CO ($n = 8$). FOR ($n = 8$). NFOR ($n = 8$). *Significantly different from CO and NFOR ($P < 0.05$). **Significantly different from CO and NFOR ($P < 0.001$). Bands representing the histochemical staining of complexes I and IV and the protein content of complex V (Coomassie Blue G-250). Means ± SD.

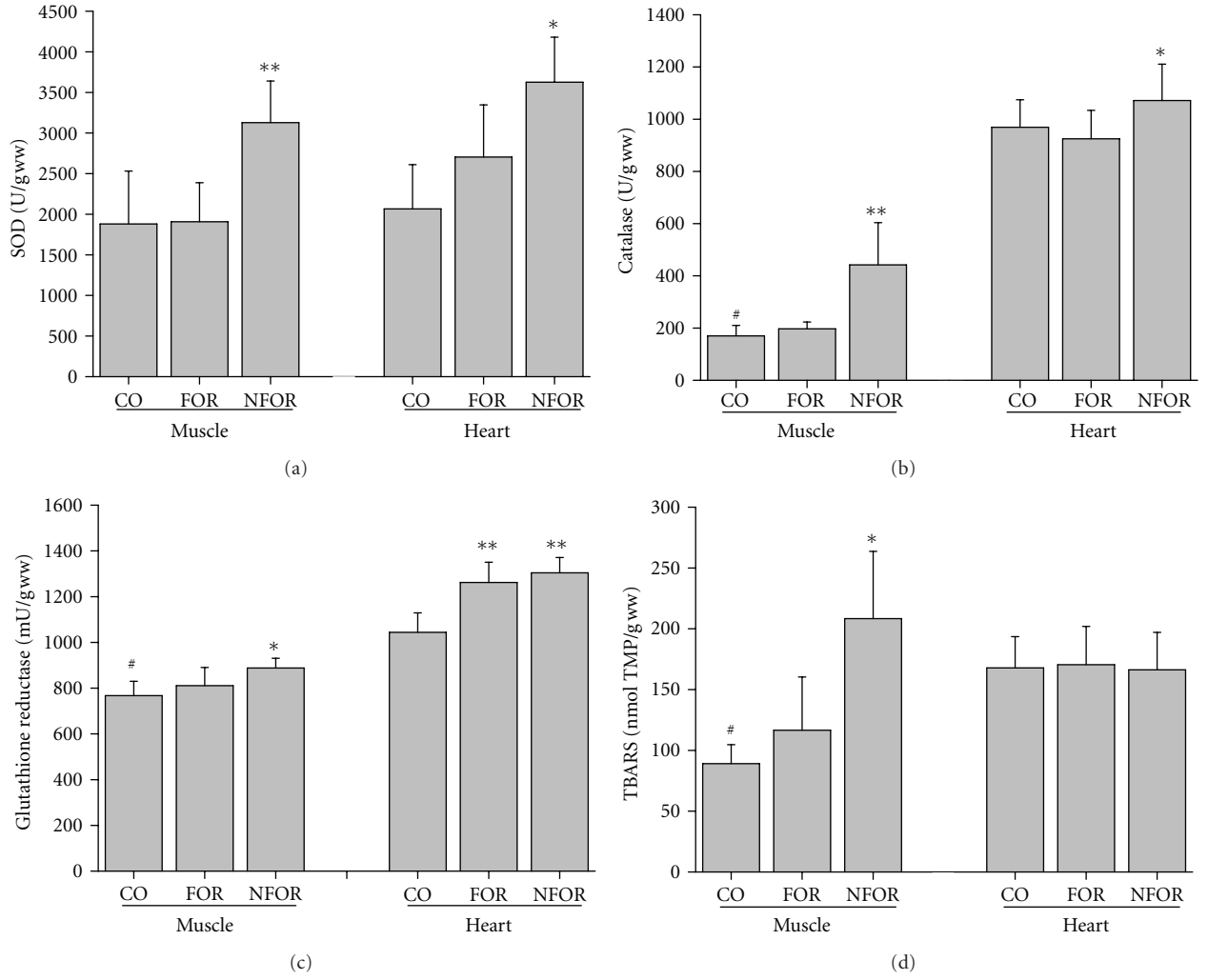

Figure 3: Activity of antioxidant enzymes and concentration of TBARS in heart and skeletal muscle tissue (means ± SD). Comparisons were made between groups within the same tissue (muscle or heart) and between muscle and heart of the CO group. CO ($n = 9$), FOR ($n = 11$), and NFOR ($n = 8$). (a) SOD: *difference with FOR ($P < 0.05$) and CO ($P < 0.001$); **difference with FOR and CO ($P < 0.001$). (b) Catalase: *difference with FOR ($P < 0.05$); **difference with FOR and CO ($P < 0.001$); #difference with CO heart ($P < 0.001$). (c) GR: *difference with FOR ($P < 0.05$) and CO ($P < 0.01$); **difference with CO ($P < 0.001$); #difference with CO heart ($P < 0.001$). (d) TBARS: *difference with FOR and CO ($P < 0.001$); #difference with CO heart ($P < 0.01$).

Table 3: Heart morphometric analysis.

		CO	FOR	NFOR
Cellular area	(μm^2)	184.30 ± 70.72	220.85 ± 59.39*	204.04 ± 55.64*
Collagen	(%)	1.43 ± 1.16	1.56 ± 1.42	1.38 ± 1.49
Heart mass	(mg)	1196.70 ± 140.57	1198.43 ± 105.89	1203.18 ± 111.60
Body mass	(g)	416.20 ± 41.14	366.89 ± 24.12*	370.29 ± 33.03*
Heart/body mass	(mg/g)	2.88 ± 0.22	3.27 ± 0.27*	3.25 ± 0.17*

Cellular area and collagen from the left ventricle: CO, FOR and NFOR ($n = 3$). A total of 30 cells (cellular area) and 30 fields (collagen) per animal were quantified, for a total of 90 cells and 90 fields per group. Heart mass and body mass: CO ($n = 9$), FOR ($n = 11$), NFOR ($n = 8$). *Significantly different from CO ($P < 0.05$). Mean ± SD.

3.6. Apoptotic Index of the Left Ventricle. Figure 5 shows the apoptotic index of the left ventricle tissue. The ratio of the number of fields that showed apoptotic nuclei to the total number of fields quantified is displayed above the SD marker for each group. This ratio is an additional index of the proportional incidence of cardiomyocyte apoptosis in each group.

The NFOR group exhibited a significantly higher apoptotic index than the CO group. The number of positive stained fields (9 out of 25) highlights the increased incidence of apoptosis in the NFOR group. Additionally, apoptosis was observed in samples from four animals out of five in the NFOR group, whereas apoptosis was only observed in two FOR animals and just one CO animal.

3.7. Blood Biomarkers: CK and Lipid Peroxides. The plasma CK concentration was not changed in the FOR (253 ± 121 U/L) or NFOR (271 ± 25 U/L) groups compared with the CO (355 ± 82 U/L) group.

The plasma lipid peroxides were significantly higher ($P < 0.01$) in the NFOR group (1766 ± 538 nmol/L) compared to the CO (925 ± 190 nmol/L) and FOR (1018 ± 249 nmol/L).

4. Discussion

In the OT animal model used here, all of the animals in the FOR and NFOR groups showed the same progressive increase in performance relative to the same starting performance level (Figure 2) until the 10th training week, suggesting that both groups adapted similarly to the training protocol until that time (T3x; Table 1). The critical distinction between the results in the FOR and NFOR groups appeared with an increase in the daily training frequency (T4x; Table 1) at the 11th week. Therefore, our data showed that OT is necessary to maximize the increase in endurance performance for some individuals, as shown in the FOR group; however, it can be detrimental to the adaptive training process for other individuals, as shown in the NFOR group, due to the intrinsic characteristics of each animal that cannot be predicted by their initial performance level.

4.1. Muscle Analyses. Treadmill running had no effect on muscle catalase activity in the FOR group (Figure 3(b)). This result is consistent with previous studies on rats that provide little evidence for the ability of endurance exercise to increase catalase activity in skeletal muscle (10, 29). Other investigators, however, have reported reduced catalase activity resulting from exercise training [14, 35]. Similarly, the unchanged total SOD activity in the red gastrocnemius muscle of rats in the FOR group (Figure 3(a)) is consistent with a number of studies [16, 17, 36, 37], but other studies have shown increased SOD activity after exercise training [38, 39]. These discrepancies could be explained by differences in training protocols, muscle fiber type composition [17, 40] or muscle recruitment pattern in different muscles. Hollander et al. [15] showed that the training induced upregulation of inner mitochondrial Mn-SOD, but not cytosolic Cu/Zn-SOD, and catalase occurs primarily in type IIa fibers in the deep vastus lateralis after endurance training in rats. In contrast, Hollander et al. [15] also showed that in type I fibers in the soleus and in mixed fibers in the plantaris, there was no significant upregulation in antioxidant enzymes activities. The red gastrocnemius, plantaris and soleus are collectively referred to as the triceps surae muscle, and the plantaris muscle acts with the gastrocnemius [41]. The red gastrocnemius of Wistar rats is also a mixed fiber muscle [41]. Our data confirm that the mixed fiber type red gastrocnemius is less likely to show an increase in SOD and catalase after endurance training; these results are similar to those observed by others [17].

In contrast, we observed a significant increase in the activities of skeletal muscle SOD and catalase in the NFOR group when compared to the FOR and CO groups. Reactive oxygen species are involved in both transcriptional regulation and the increase expression of SOD and catalase [42, 43]. Therefore, our data suggest that the increase in antioxidant enzymes activities in the NFOR group may reflect a specific adaptation to increased ROS production during the later stages of this OT protocol.

To date, no study has specifically shown the relationship between ROS, gene transcription and GR activity. Furthermore, there are fewer studies on GR adaptation to endurance training than those on SOD and catalase adaptation. In this study, the GR activity of the FOR group remained unchanged relative to CO (Figure 3(c)). In contrast, GR activity was increased in the NFOR group. Other studies [40, 44] showed that GR activity in rat skeletal muscle decreases after endurance training. The GR response presented here supports the argument that after severe endurance training, a more challenging prooxidant milieu was generated in the muscles of rats in the NFOR group, thus causing a higher

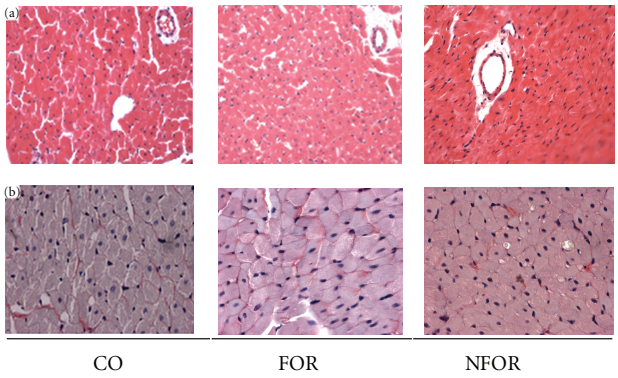

Figure 4: Left ventricle histology of representative animals from each of the three experimental groups. (a) Hematoxylin and eosin (40x). (b) Sirius red (40x). There was no observable difference in the cell structure

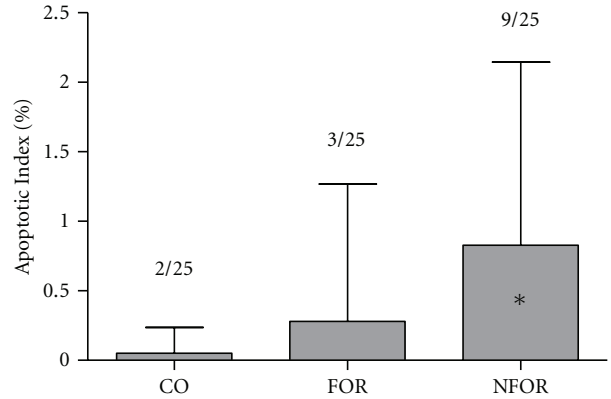

Figure 5: The apoptotic index of left ventricle from the CO ($n = 5$), FOR ($n = 5$), and NFOR ($n = 5$) groups (means ± SD). The ratio of the number of fields that showed apoptotic nuclei to the total number of fields quantified is displayed above the SD marker for each group. *Significant difference relative to CO ($P < 0.05$).

antioxidant response adaptation. In addition, we observed a significant increase in muscle TBARS in the NFOR group compared to the CO and FOR groups (Figure 3(d)), suggesting increased lipid peroxides levels. The increase in muscle TBARS has not been frequently observed after endurance training; other studies have shown decreased or unchanged levels of TBARS in rat muscle [45–47], as we observed in the FOR group.

Complex IV of the electron transport chain, cytochrome c oxidase, is particularly vulnerable to ROS attack. Some authors have reported that an increase in markers of oxidative stress damage is associated with lower complex IV activity [48, 49]. In this study, the activity of complex IV was lower in NFOR rats than in FOR rats (Table 2). Furthermore, when compared to CO rats, the specific activity staining of complex IV/V in the NFOR group showed a decreased activity after endurance OT. The oxidation of thiol groups (–SH) in mitochondrial proteins by ROS may explain this lowered complex IV activity [50]. Analogously, oxidizing thiol groups within CS can also compromise its activity (Table 2), as shown experimentally by Ji et al. [51] after exhaustive exercise in rats. Complex I, however, may not be affected by ROS to the same extent that complex IV is. It has been suggested that several of the constituent subunits of complex I neutralize oxidative damage, thereby protecting the redox groups and stabilizing the core catalytic subunits [52, 53]. Because of the differences in the structures of these two electron transport chain complexes and the sensitivity of these structures to ROS exposure, the BN-PAGE technique allowed individual identification of the abnormal activity of complex IV, as shown by others [54].

Taken together, lowered mitochondrial oxidative capacity after endurance training, increased activity of antioxidant enzymes in the RG muscle and increased lipid peroxide levels provide complementary evidence for a prooxidant alteration in muscle redox homeostasis associated with underperformance, particularly in the NFOR group. Our data for the NFOR group also suggest that the prooxidant cellular milieu in the RG muscle cannot be entirely avoided by the increase in activity of the antioxidant defense system during endurance OT. Similarly, Franco et al. [55] have shown that rat muscle cells exposed to certain concentrations of ROS exhibit increased levels of not only the gene expression and activity of SOD and catalase but also of oxidized proteins. Therefore, a critical level of ROS production that can overcome the response of antioxidant enzymes to exercise may characterize muscle oxidative stress in subjects intolerant to severe endurance training.

4.2. Heart Analyses. Our results demonstrated that 11 weeks of endurance training had no effect on heart catalase activity in the FOR group (Figure 3(b)) and that the FOR group showed a trend of SOD activity upregulation compared to CO ($P = 0.0523$, Figure 3(a)). Accordingly, Powers et al. [56] showed that the general adaptation to several endurance-training protocols is characterized by SOD upregulation and unchanged catalase activity in the left ventricle. In agreement with muscle data, however, only the NFOR group presented a significant increase in the activities of both SOD and catalase in the heart.

Observing the CO group, the higher basal concentration of TBARS in the heart compared to muscle (Figure 3(d)) may illustrate a natural, elevated production of ROS by cardiac tissue [57]. However, when the NFOR group was subjected to endurance OT, the naturally higher GR and catalase activities of the heart, as shown by the CO group (Figures 3(b)-3(c)), and the further increase of SOD, catalase, and GR activities seem to be sufficient antioxidant adaptations to maintain the heart's redox homeostasis. The greater adaptation of antioxidant defense activity in the heart compared to muscle may have maintained the levels of TBARS observed in both the FOR and NFOR groups (Figure 3(d)). This finding is similar to the effect of moderate exercise on levels of TBARS previously reported by others [58–60].

The cardiac morphometric analysis showed no differences in heart mass among the CO, FOR and NFOR groups (Table 3). However, we noted a significant increase in the heart mass/body mass ratio in the FOR, and NFOR groups

compared to the CO group. This increase resulted from a loss of bodyweight in the animals of both training groups during the protocol, as shown elsewhere [61]. Additionally, structural analysis of the left ventricle not only showed normal tissue architecture in the FOR and NFOR groups (Figure 4) but also showed remodeling due to increased cell area (i.e., cardiomyocyte hypertrophy) in trained animals compared to the CO group (Table 3). According to Fenning et al. [62], these adaptations improve contractile capacity in rats and lead to an optimization of cardiac function with a concomitant increase in cardiac output.

Despite the expected positive adaptation of cardiac function of trained rats, the increase in the apoptotic index for the NFOR group was significant compared to the CO group (Figure 5). Our data are comparable to the observations of Huang et al. [23], who have shown a preservation of the myocardial histological structure and an increase in apoptosis in the left ventricle of rats after an endurance-training protocol. In contrast to the results of Huang et al. [23], however, we only observed significant cardiomyocyte apoptosis in the NFOR group which was vulnerable to muscle oxidative stress during severe endurance exercise.

4.3. Plasma CK and Lipid Peroxides. Plasma CK concentrations did not change in the FOR and NFOR groups compared to CO. This finding suggests that increased ROS production during exercise is not always related to severe muscle cell damage. Studies that induced electric contractile stimulation have shown that muscle cells rapidly generate ROS during exercise associated with little or no cell damage [13, 63]. However, increased plasma CK is mainly related to the eccentric component in rat treadmill running [64, 65], the sloping run, which is not a relevant component of our training model. These observations also exclude subclinical chronic muscular damage as the cause for the chronic performance drop, which is a necessary condition to define the NFOR state [2].

The significant increase in plasma lipid peroxides in the NFOR group is in agreement with increased muscle TBARS. Many ROS (such as $NO^{\bullet}$, $O_2^{\bullet -}$ and H_2O_2) and reactive aldehydic products are membrane permeable and, as a result, are potentially able to diffuse into the surrounding medium [18, 66]. However, it is not yet possible to exclude other sources of ROS in the NFOR group because other organs or blood cells, such as leukocytes and erythrocytes, may be additional sources of ROS during the exercise regimen [67]. In the context of the training protocol used here, lipid peroxide levels were more useful than CK as a blood biomarker to detect underperformance related to a prooxidant status.

5. Conclusion

To our knowledge, this is the first study showing that severe endurance exercise impairs mitochondrial adaptation related to oxidative stress in selective subjects. The persistent underperformance and the higher incidence of cardiomyocyte apoptosis in the NFOR group indicate that increasing training sessions and reducing recovery in the pursuit of high performance levels must be undertaken with caution. Our study also showed that blood biomarkers that are directly related to oxidative cellular damage can be used to identify individual vulnerability to oxidative stress during endurance OT, whereas CK, a frequently used marker of muscle tissue damage, may not be as effective in this case. Finally, the intrinsic characteristics that underlie the different adaptations to intense endurance training between the FOR and NFOR groups are currently only speculative. Our results support further studies with isolated mitochondria of NFOR rats to elucidate the intrinsic causes of subject-selected oxidative stress during severe endurance training.

Authors' Contribution

R. Ferraresso and R. de Oliveira contributed equally to this work.

Acknowledgments

The authors thank A. M. M. Porto for technical support. R. Ferraresso (07/57512-2) and R. de Oliveira (07/57511-6) received scholarships from FAPESP. R. Hohl received a postdoctoral fellowship grant from FAPESP (09/51125-2). This study was also supported by FUNCAMP (927.7-0100).

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2012, Article ID 628352, 9 pages
doi:10.1155/2012/628352

Research Article

Low-Frequency Fatigue as an Indicator of Eccentric Exercise-Induced Muscle Injury: The Role of Vitamin E

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Received 7 April 2012; Accepted 14 June 2012

Academic Editor: Chad M. Kerksick

This study investigates whether vitamin E can attenuate eccentric exercise-induced soleus muscle injury as indicated by the amelioration of *in situ* isometric force decline following a low-frequency fatigue protocol (stimulation at 4 Hz for 5 min) and the ability of the muscle to recover 3 min after the termination of the fatigue protocol. Adult male Wistar rats were divided into vitamin E-supplemented or placebo-supplemented groups studied at rest, immediately post-exercise or 48 h post-exercise. Daily DL-α-tocopheryl acetate intraperitoneal injections of 100 mg/kg body mass for 5 consecutive days prior to exercise doubled its plasma levels. Fatigue index and recovery index expressed as a percentage of the initial tension. FI at 0 h post- and 48 h post-exercise respectively was 88% ± 4.2% and 89% ± 6.8% in the vitamin E groups versus 76% ± 3% and 80% ± 11% in the placebo groups. RI was 99% ± 3.4% and 100% ± 6% in the vitamin E groups versus 82% ± 3.1% and 84% ± 5.9% in the placebo groups. Complementally to the traditionally recorded maximal force, low-frequency fatigue measures may be beneficial for assessing injury-induced decrease in muscle functionality.

1. Introduction

Physical activities involving eccentrically biased muscle contractions have been associated with skeletal muscle injury [1–4] resulting in skeletal muscle functional alterations and performance impairment [5–7]. It has been suggested that a highly reliable and widely used functional index for evaluating eccentric contraction-induced muscle injury in animals is the measurement of the electrically elicited maximal isometric tetanic force (P_o), whereas in humans is the measurement of maximal voluntary contraction torque [8].

Interestingly though, several lines of evidence from human and animal studies have recognized that after eccentric contractions the reduction in muscle force is more profound at submaximal tension elicited by low frequencies of stimulation, rather than maximal tetanic tension elicited by high frequencies of stimulation. Although Westerblad and Allen [9] have proposed the terminology delayed low-frequency recovery to describe the prolonged fatigue-induced weakness at low stimulation frequencies, yet the term widely used to describe this physiological phenomenon is low-frequency fatigue.

In humans, the loss of muscle force was more profound at low-frequency stimulation when muscle was exercised isometrically at a long length [10] or after exercise in which muscle was actively stretched [11]. In animal studies, the force depression at low frequencies of stimulation following

eccentric contractions has been recorded in single mouse fibers in vitro [5], in rat muscle bundles preparation in vitro [12], as well as in entire rat muscle in vitro [13]. Low-frequency fatigue is caused by a reduction in intracellular calcium (Ca^{2+}) concentration, probably as a consequence of a reduced Ca^{2+} release from the sarcoplasmic reticulum in the injured muscle fibers during the excitation-contraction (E-C) coupling [14–19].

Vitamin E is a nutritional antioxidant widely used to attenuate oxidative stress and muscle injury associated with eccentric exercise [20–22]. The potential protective effect of vitamin E against exercise-induced muscle injury and disturbance of redox homeostasis is attributed to its properties to act as a potent chain breaking antioxidant [23] and stabilizer of membrane structure [24–26]. Vitamin E, as an integral part of the membrane bilayer, may prevent oxidation of membrane lipids by scavenging free radicals [23] and contribute to maintaining sarcolemma integrity [24, 26].

The relatively little data available from human [27–31] and animal [32–34] studies have failed to show consistently any protective effect of vitamin E on muscle force deficit caused by eccentric contraction-biased exercise. Only in the study of Shafat et al. [35], in which though a combination of vitamins E and C was administered, attenuation of the force decline following eccentric exercise in the supplemented group was reported. It is worth mentioning that in all these studies the potential efficacy of vitamin E treatment against muscle injury was mainly based upon maintaining maximal muscle force, as assessed by maximal voluntary contraction and/or electrically evoked maximal tetanic tension; rarely was a measure of low-frequency stimulation used.

Undoubtedly, the use of maximal force as a functional measure of exercise-induced muscle injury is of great importance, because it reflects the maximal strength that the entire contractile machinery can produce under maximal stimulation. However, maximal tetanic force, which is characterized by prolonged and maximal calcium ion release from the sarcoplasmic reticulum, may not serve as a sensitive indicator of muscle microtrauma as it would be a low-frequency stimulation protocol characterized by transient and limited Ca^{2+} release. In this regard, stimulation at low frequencies may better reflect Ca^{2+} kinetics, thus revealing any potential E-C coupling failure induced by eccentric exercise. Moreover, any potential protective effect of vitamin E or other nutritional intervention on muscle injury should be strong enough for this effect to be statistically shown, whereas weaker, yet true, effects might not be detected.

Indeed, it has been recently demonstrated in vitamin-E-supplemented rats that, even though maximal isometric tetanic force (P_o) was not clearly spared following eccentric contraction-biased exercise, single twitch force (P_t), which reflects Ca^{2+} kinetics in the muscle [36], declined significantly less compared to the placebo conditions [32]. Based on this evidence, we hypothesized that any potential effect of vitamin E treatment to attenuate muscle injury-induced force decline could be better tested by utilizing a low-frequency stimulation protocol. The implementation of a low-frequency fatigue protocol may be a sensitive screening method for detecting alterations in muscle function associated with microdamage that, otherwise, could not be detected under conditions of maximal stimulation.

If this is the case, then, along with the regularly used P_o measure, the incorporation of a low-frequency fatigue test as a standard procedure for assessing muscle functionality following eccentric contraction-biased exercise would provide a more comprehensive approach to muscle injury and the effectiveness of the interventions implemented to attenuate the injury. For, unlike maximal force which is rarely used in everyday activities, low-frequency stimulation is commonly used in everyday tasks [37]. In this regard, the force deficit at low frequencies of stimulation may be of even greater physiological and clinical importance than the deficit in maximal force, because the former may compromise functionality in daily activities.

Therefore, the aim of the present study was to explore the possibility that a low-frequency fatigue protocol can clearly detect small alterations in muscle force induced by muscle-damaging exercise. To accomplish this objective, vitamin E, a nutritional compound know to have some prophylactic effects on injury-induced force deficit [32, 35], was employed. Vitamin-E-treated or placebo-treated rats ran downhill on a treadmill and studied at rest, immediately postexercise or 48 h postexercise. Muscle injury was assessed by implementing a very-low-frequency fatigue protocol (stimulation at 4 Hz for 5 min) in the soleus muscle *in situ*. The ability of the muscle for immediate recovery was also estimated by recording isometric single twitch tension 3 min after the completion of the fatigue protocol.

2. Materials and Methods

2.1. Animals. The project was approved by the institutional review board and the appropriate state authority. All procedures were in accordance with the European Union guidelines for the care and use of laboratory animals, as well as the "Principles of laboratory animal care" (NIH publication No. 86-23, revised 1985). Sixty adult (8 to 10 week-old) male Wistar rats, weighing 220–270 g, were used in the study. The animals were housed under a 12 h light : 12 h dark cycle, controlled temperature (18–21°C), and controlled humidity (50–70%). Commercial rat chow (ELVIZ, Plati, Greece) and tap water were provided *ad libitum*.

Rats were randomly assigned into six groups as follows: placebo-treated sedentary control (Pb-Sed), placebo-treated and studied immediately postexercise (Pb-Ex0), placebo-treated and studied 48 h postexercise (Pb-Ex48), vitamin-E-treated sedentary control (VE-Sed), vitamin-E-treated and studied immediately postexercise (VE–Ex0), and vitamin E-treated and studied 48 h postexercise (VE-Ex48). Experimental design is shown in Figure 1.

We examined muscle injury at 0 and 48 h postexercise because these time points highlight the two main stages (i.e., initial/autogenetic and phagocytic) of the four-stage muscle injury process, as proposed by Armstrong [38]. The

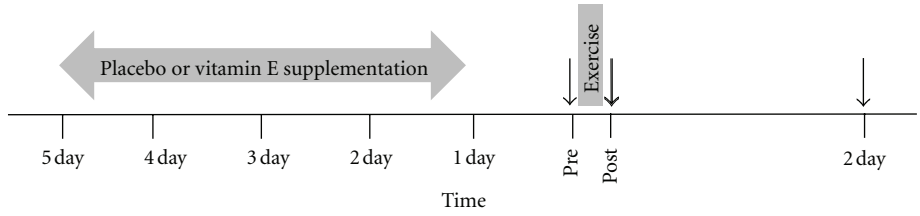

Figure 1: Experimental design. Rats were injected intraperitoneally with either placebo (grain oil) or vitamin E (100 mg/kg body mass of DL-α-tocopheryl acetate per day) for 5 consecutive days prior to examination (placebo-treated and vitamin-E-treated sedentary control groups) or exercise (placebo-treated and vitamin-E-treated exercised groups studied immediately post and 2 days post exercise), respectively. Downward arrows indicate the time of assessment.

initial/autogenetic stage reflects primarily the initiation of degrading cellular structures via Ca^{2+}-activated proteolytic and lipolytic events initially induced by the mechanical component of eccentric exercise. During the phagocytic stage, which is marked by a typical inflammatory response in the tissue, the injury is mainly attributed to the metabolic events associated with the production of reactive oxygen species, the breakdown of the injured myofibrils, and the removal of fiber debris [38].

2.2. Vitamin E Supplementation. The animals of the vitamin-E-treated sedentary control group and those of the vitamin-E-treated exercised groups were injected intraperitoneally (i.p.) with 100 mg/kg body mass of DL-α-tocopheryl acetate per day for 5 consecutive days prior to examination or exercise, respectively [39, 40]. Vitamin E was diluted in grain oil at a concentration of 4% (w/v). Accordingly, the placebo-treated sedentary and exercised groups were injected with vehicle. Previous administration of similar vitamin E doses have increased vitamin E concentration in muscle [33] and lung [40] and inhibited lipid peroxidation in the lungs [40] and the liver [39]. Based on the vitamin E content of the rat diet (100 mg/kg chow), the estimated daily food consumption of an adult laboratory rat in the size range of 220–270 (10–20 g a day), and the injected daily dose (100 mg/kg body mass), it is estimated that for rats in this size range the daily vitamin E intake was 22–27 mg for vitamin-E-treated rats and 1-2 mg for placebo-treated rats. This is approximately 15–20 times the vitamin E intake through the normal diet.

2.3. Exercise Protocol. Exercise was performed after an overnight fast and 16–18 h after the last injection. The exercise protocol consisted of 90 min of intermittent downhill running on a motor-driven treadmill. After a few minutes of familiarization on the treadmill, the rats performed 18 bouts of 5-minute running at a speed of 16 m/min down a 16° inclination, with a 2 minute rest between bouts. After the completion of exercise protocol, the placebo-treated and vitamin-E-treated rats to be studied 48 h postexercise were placed back in their cages with free access to food and water until they were examined. Previous studies have shown that this exercise protocol induced injury in the soleus and other rat hindlimb muscles [1, 4, 41, 42].

2.4. Plasma Vitamin E Concentration. Blood samples were collected either at rest (16–18 h after the last injection) or immediately after exercise or 48 h following exercise as per group as follows. Under deep anaesthesia with chloral hydrate (4.5%, 1 mL/100 g body mass i.p.), about 1 mL of blood was drawn from the right jugular vein using a heparin-treated syringe. Blood was then centrifuged at $3000 \times g$ for 10 min and the resulting plasma was collected in eppendorf vials, snap frozen in liquid nitrogen and stored at $-80°C$ until analysis. In 3 animals from each group, plasma vitamin E was determined using high-performance liquid chromatography (Jasco HPLC System, Great Dunmow, UK) according to Nierenberg and Nann [43]. Each assay was run in duplicate and the interassay coefficient of variation (CV) was less than 10%.

2.5. In Situ Isometric Tension Recording. After blood sampling, *in situ* isometric tension of the soleus muscle was recorded following a procedure previously described [32]. Under the same anaesthesia as above (chloral hydrate 4.5%, 1 mL/100 g body mass i.p.), the hair of the lower limbs was shaved up to the knee joint and the area was cleaned lightly with 70% ethanol. The skin was then sectioned and a longitudinal incision was made on the lateral surface of the right hindlimb over the area covered by gluteus superficialis. Adjacent muscles were gently reflected using blunt-tip forceps to expose the sciatic nerve. Likewise, a small incision was made on the back of the ankle, thus uncovering the distal (Achilles) tendon. The gastrocnemius muscle was retracted carefully to avoid rupturing the blood vessels, and the intact soleus muscle was exposed. The tendon was then tied with 3/0 silk thread and cut distally.

The rat was then placed prone on a stable rodent surgery table and was prepared for tension recording. Steel pins were inserted through the knee and ankle joints to stabilize the hindlimb, and cloth tape was used to secure the foot perpendicularly to the lower leg. Pins were supported with magnetic stand holders throughout the experiment. The tendon of the soleus muscle was attached to a strain-gauge transducer (UFI, Morro Bay, CA) by short silk suture, and bipolar silver electrodes were placed under the sciatic nerve, which was held in a relaxed position. Isometric contractions were evoked by stimulating the sciatic nerve (Digitimer DS9A stimulator, Hertfordshire, UK) using supramaximal (3–8 V) square pulses of 0.5 ms. Tetanic twitch stimulation

was set at 350 ms. The signal from the transducer was amplified by a DC amplifier (Neurolog NL 107, Digitimer, Hertfordshire, UK), displayed on an oscilloscope screen (Fluke PM 3380A, Everett, WA), stored in a computer, and processed using data acquisition software (FlukeView combiscope software, Everett, WA). A pulse programmer (Digitimer D4030, Hertfordshire, UK) controlled all devices during tension recording.

The muscle was adjusted to the optimal length (L_o) using a micromanipulator (Prior Scientific, Rockland, MA) allowing motion in all three directions. L_o was defined as the muscle length at which maximal twitch tension was obtained. It took 5-6 single twitch trials to set L_o. Once this was set, a 1-minute resting period was allowed before the actual recordings. Throughout the recordings, the longitudinal axis of the muscle remained aligned to the longitudinal axis of the transducer, and both remained parallel to the tibia.

A very low-frequency fatigue protocol (stimulation at 4 Hz for 5 min) was implemented to assess soleus muscle fatigability as described below. Throughout tension recording, the rat was kept warm with a heating pad. A diffuse heat source was also placed in close proximity to the surgery table. The depth of anaesthesia was assured by the constriction of the pupils as well as simple sensory tests, such as the absence of eye blinking when the eyelid was touched and the absence of foot withdrawal when the foot was pinched. When necessary, anaesthesia was maintained by administering approximately 10% of the initial dose. The sciatic nerve and soleus muscle were kept moist by periodically sprinkling Krebs solution at 37°C. Immediately after tension recording, the soleus muscle was excised and weighed on an electronic scale with an accuracy of 0.001 g. Animals were then euthanized by an overdose of chloral hydrate injected intravenously. Normalization of the data from muscles of different sizes was achieved by expressing generated force per muscle mass.

2.6. Fatigue Protocol. A muscle fatigue protocol of very low-frequency (stimulation at 4 Hz for 5 min) was applied to test for fatigability. The decrease in tension after the 5 min stimulated contraction period was expressed as a percentage of the initial tension, denoting the fatigue index (FI). To estimate the ability for immediate muscle recovery, isometric single twitch tension (P_t) was recorded 3 minute after the completion of the fatigue protocol. The tension recorded after the 3 min recovery period was expressed as a percentage of the initial tension, denoting the recovery index (RI). For both FI and RI, an initial tension was considered the greatest force of the first three twitches recorded in the beginning of the 5 min fatigue protocol. To ensure that the muscle retained its L_o after repeated contractions during the 5 min fatigue protocol, an average of 2-3 single-twitch trials were applied immediately after the 3 minute recovery period. Once L_o was confirmed, the actual single twitch was recorded after 1 minute.

2.7. Statistical Analysis. Data were analyzed with the SPSS, version 12, software (SPSS, Chicago, IL) and presented as mean ± SD. The distribution of all dependent variables was examined by the Shapiro-Wilk test and was found not to differ significantly from normal. To evaluate the potential protective effects of vitamin E supplementation against exercise-induced muscle injury as reflected by force decline during the fatigue test and the immediate recovery rate, we performed 2×3 (supplementation by time) ANOVA followed by simple main effect analysis. The statistical significance level was set at $\alpha = 0.05$. To determine the meaningfulness of the effects of exercise and vitamin E supplementation on muscle injury markers, effect sizes (ESs) were calculated for FI and RI as the difference between post- and preexercise mean values divided by the standard deviation of the preexercise value. According to the original Cohen's scale, effect size is 0.2 for small, 0.5 for moderate and 0.8 for large effects [44] and according to the modified Cohen's scale, effect size is 0.2 for small, 0.6 for moderate, 1.2 for large, 2.0 for very large, and 4.0 for nearly perfect effects (http://sportsci.org/resource/stats/). Due to the pre-post characteristic of our intervention and an improved response in fact being a reduction in the post value, the ES inherently will be negative. To avoid confusion in the interpretation of the calculated ES, we decided to present ES as absolute values.

3. Results

3.1. Plasma Vitamin E Concentration. The data referring to vitamin E concentration in plasma have been initially presented in a recently published paper of a relative work from our group [32]. Because of the small sample size used for the particular analysis ($n = 3$ per group), only descriptive statistics were applied on these data. No further inferential statistical comparison was performed on these values. Vitamin E administration doubled the concentration of vitamin E in plasma in the sedentary control group (VE-Sed group: 18.5 mg/L ± 3.3 mg/L, range 15.9–22.2 mg/L versus Pb-Sed: 9.3 mg/L ± 0.4 mg/L, range 8.9–9.7 mg/L). Furthermore, the vitamin-E-treated exercised rats had consistently higher vitamin E plasma levels compared to that of the placebo-treated exercised counterparts, both immediately postexercise (VE-Ex0: 12.3 ± 0.7 mg/L, range 11.8–13.1 mg/L versus Pb-Ex0: 7.4 ± 2.3 mg/L, range 5.9–10.1 mg/L) and, at 48 h postexercise (VE-Ex48: 13.8 ± 1.1, range 13.0–15.0 mg/L versus Pb-Ex48 : 10.2 ± 1.3 mg/L, range 8.8–11.3 mg/L); yet these vitamin-E concentrations were lower compared to that measured in the vitamin-E-treated sedentary control group.

3.2. Fatigue Index and Recovery Index. Data on soleus muscle fatigability (as reflected by the FI) and ability for immediate recovery (as reflected by the RI) between the placebo-treated exercised groups (i.e., Pb-Ex0 and Pb-Ex48), subject to muscle damaging downhill running (injured muscle), and the placebo-treated sedentary control (Pb-Sed) group (uninjured muscle) have been initially presented elsewhere [45] and now compared with the vitamin E-supplemented condition. Briefly, these data indicated that

eccentric exercise significantly increased soleus muscle fatigability and decreased the ability for immediate recovery, suggesting that the exercise protocol used in this study induced injury in the soleus muscle.

To examine whether or not vitamin E treatment had any protective effect on muscle injury induced by downhill running, FI and RI were recorded in VE-Ex0 and VE-Ex48 vitamin-E-treated exercised groups and compared with the values obtained in the Pb-Ex0 and Pb-Ex48 placebo-treated exercised groups. A marginally nonsignificant supplementation-by-time interaction ($P = 0.056$) and significant main effects of supplementation ($P < 0.001$) and time ($P = 0.034$) in soleus muscle FI were found (Figure 2). In the within groups pairwise comparisons, FI was significantly lower in Pb-Ex0 ($P = 0.002$) and Pb-Ex48 ($P = 0.045$) rats compared to Pb-Sed rats. In the vitamin E condition, no significant decrease in FI between the exercise groups (VE-Ex0 and VE-Ex48) and the vitamin-E-treated sedentary control group (VE-Sed) was found. In the between groups pairwise comparisons, FI was significantly decreased in the placebo-treated exercised condition compared with the vitamin-E-treated exercised condition, both immediately postexercise ($P < 0.001$) and at 48 h-postexercise ($P = 0.009$). No significant difference ($P > 0.05$) in FI was detected between the Pb-Sed and VE-Sed control groups.

A significant supplementation-by-time interaction ($P < 0.001$), as well as significant main effects of supplementation ($P < 0.001$) and time ($P < 0.001$) in soleus muscle RI were found (Figure 3). In the within groups pairwise comparisons, RI was significantly lower in Pb-Ex0 ($P < 0.001$) and Pb-Ex48 ($P < 0.001$) rats compared to Pb-Sed rats. This was also the case in the vitamin E condition, as a significant decrease in RI between the exercise groups (VE-Ex0 and VE-Ex48) and the vitamin-E-treated sedentary control group (VE-Sed) was found ($P < 0.001$). In the between groups pairwise comparisons, RI was significantly decreased in the placebo-treated exercised condition compared with the vitamin E-treated exercised condition, both immediately postexercise ($P < 0.001$) and at 48 h-postexercise ($P = 0.009$). No significant difference ($P > 0.05$) in RI was detected between the Pb-Sed and VE-Sed control groups.

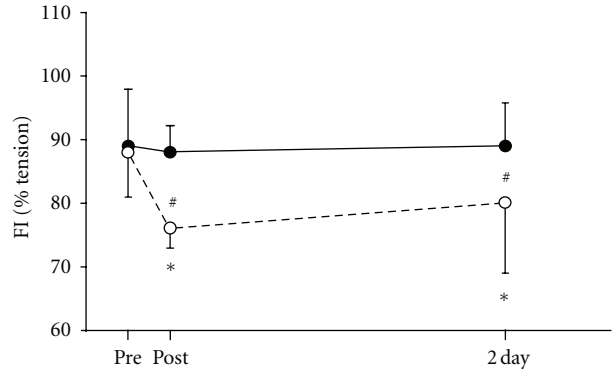

Figure 2: Fatigue index (expressed as a percentage of the initial tension) after the implementation of a low-frequency fatigue protocol (stimulation at 4 Hz for 5 min) of the soleus muscle *in situ* in the placebo-treated (dashed line and ◯) and vitamin E-treated (solid line and •) rats (mean and SD, $n = 10$ per group). *Significantly different from pre exercise values within the same group ($P < 0.05$). #Significantly different between placebo and vitamin E at the same time point ($P < 0.01$).

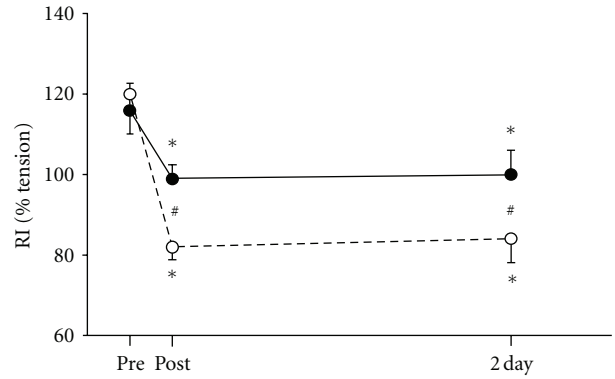

Figure 3: Recovery index (expressed as a percentage of the initial tension) 3 min following the termination of low-frequency fatigue protocol of the soleus muscle *in situ* in the placebo-treated (dashed line and ◯) and vitamin-E-treated (solid line and •) rats (mean and SD, $n = 10$ per group). *Significantly different from pre exercise values within the same group ($P < 0.001$). #Significantly different between placebo and vitamin E at the same time point ($P < 0.001$).

4. Discussion

This study is part of a larger project aimed at investigating the effects of vitamin E supplementation on muscle injury following downhill running. The objective of the present study, in particular, was to investigate whether a low-frequency fatigue protocol can clearly detect small alterations in muscle force induced by muscle-damaging exercise. The prophylactic properties of vitamin E against exercise-induced muscle injury, as a potent antioxidant and intramembrane stabilizer alike, was employed for delineating "finest" alterations in muscle force associated with microdamage. Vitamin-E-treated and placebo-treated rats studied at rest, immediately post- or 48 h postdownhill running. Alternatively, the objective of this study could be formulated as to whether vitamin E can attenuate eccentric exercise-induced muscle injury as assessed by the amelioration of force decline following the implementation of a low-frequency fatigue protocol and the ability of the muscle for immediate recovery 3 min after the completion of the fatigue protocol.

Our data demonstrate that downhill running significantly increased soleus muscle fatigability and decreased the ability for immediate recovery. The fatigability after the low-frequency fatigue protocol was more pronounced in the exercised rats immediately postexercise (Pb-Ex0: 76% ± 3%) and at 48 h-postexercise (Pb-Ex48: 80% ± 11%) compared to the sedentary rats (Pb-Sed: 88% ± 7%). The effect sizes for FI in the placebo groups at 0 h and 48 h were 1.71 and 1.14, respectively (ES are presented as absolute values). Likewise, the ability of the soleus muscle to recover was diminished in the exercised rats immediately postexercise

(Pb-Ex0: 82% ± 3.1% and at 48 h-postexercise (Pb-Ex48: 84% ± 5.9%), whereas the RI in the sedentary control group was surprisingly increased by 20% of the initial single twitch value (Pb-Sed: 120% ± 9.9%). The effect sizes for RI in the placebo groups at 0 h and 48 h were 3.83 and 3.63, respectively. This increase in the RI of the sedentary rats beyond the initial single twitch value was attributed to a staircase effect (treppe phenomenon) that could reflect an increased Ca^{2+} availability in the uninjured soleus muscle. It is also a criterion of the viability of the preparation.

It appears that the low-frequency protocol-induced minimal fatigue in the nonexercised (uninjured) group, which was fully restored 3 min after fatigue protocol, suggesting that the force decline in the exercised (injured) groups did not have a metabolic etiology but rather it can be attributed to muscle damage alone. These findings indicate that the exercise protocol used in this study induced injury in the soleus muscle, as evidenced by the significant reduction in the ability of the placebo-treated eccentrically exercised animals (injured muscles) to maintain tension at a low-frequency fatigue protocol as well as the reduced ability to recover 3 min after the termination of the fatigue protocol, compared with the sedentary control animals (uninjured muscle).

Vitamin E treatment decreased soleus muscle fatigability and preserved the ability for immediate recovery following downhill running. In the vitamin-E-treated rats, postexercise soleus muscle fatigability (VE-Ex0: 88% ± 4.2% and VE-Ex48: 89% ± 6.8%) was not different compared to the sedentary conditions (VE-Sed: 89% ± 9.9%). The effect sizes for FI in the vitamin E groups at 0 h and 48 h were 0.11 and 0, respectively. In addition, as was the case in the placebo-treated sedentary group, the RI in the vitamin-E-treated sedentary group was also increased by 16% of the initial single twitch value (VE-Sed: 116% ± 6.7%). Nevertheless, the ability for immediate recovery was essentially preserved in the vitamin-E-treated exercised rats (VE-Ex0: 99% ± 3.4% and VE-Ex48: 100% ± 6.0%). The effect sizes for RI in the vitamin E groups at 0 h and 48 h were 2.54 and 2.39, respectively.

When the placebo and vitamin conditions were compared at the same time point, the FI was less pronounced in the VE-Ex0 and VE-Ex48 (88% ± 4.2% and 89% ± 6.8%) groups as compared to that observed in the Pb-Ex0 and Pb-Ex48 (76% ± 3% and 80% ± 11%) groups. In addition, the RI was improved in the VE-Ex0 and VE-Ex48 (99% ± 3.4% and 100% ± 6%) vitamin-E-treated animals as compared to that observed in the Pb-Ex0 and Pb-Ex48 (82% ± 3.1% and 84% ± 5.9%) placebo-treated counterparts. In fact, the ability of the soleus muscle to produce tension was fully restored 3 min after the termination of the low-frequency fatigue protocol. Our findings clearly indicate that the vitamin E-treatment used in this study protected soleus muscle from the injury normally induced by downhill running. As compared to the placebo-treated groups, the vitamin-E-treated rats had significantly increased ability to maintain tension at low-frequency fatigue as well as to recover 3 minute after the termination of the fatigue protocol.

A functional measure that has been widely used to assess exercise-induced muscle injury is the maximal tetanic tension, which is associated with maximal intracellular Ca^{2+} concentration. P_o, although of great importance, may not serve as a sensitive indicator for detecting smaller functional alterations caused by muscle microtrauma or uncovering potential weaker protective effects of nutritional interventions against muscle damage. We suggest that a low-frequency stimulation protocol, characterized by low intracellular Ca^{2+} concentration, may also be used as a muscle function index complementing P_o, because the former may be a more suitable measure to distinguish muscle microdamage induced by exercise.

A considerable number of studies have substantiated that, following eccentric muscle contractions, force deficit is more pronounced at low frequency (submaximal) stimulation rather than high-frequency (maximal) stimulation. The relative isometric force reduction of the rat soleus muscle bundles in vitro at 10 min post injury induced by eccentric contractions was significantly greater at the stimulation frequencies of 10 and 20 Hz compared to higher stimulation frequencies up to 100 Hz [12]. This is in agreement with previous data showing a greater relative isometric force reduction at low frequencies postinjury in single fibers of the mouse flexor brevis muscle in vitro. More specifically, the force decline at 10 min postlengthening contractions was greater at the stimulation frequencies of 30 and 50 Hz compared to higher stimulation frequencies up to 100 Hz [5]. In addition, McCully and Faulkner [13] have demonstrated that the isometric force decline of the rat extensor digitorum longus muscle in vitro after lengthening contraction-induced muscle injury was greater at the stimulation frequencies of 100 and 200 Hz compared to maximal isometric force occurred at 300 Hz. In support to these findings, Jones et al. [10] have also shown that after eccentric contractions the decline in muscle force of the quadriceps and forearm flexors was greater at the electrically evoked low-frequency fatigue (20 : 100 Hz ratio) compared to maximal voluntary isometric superimposed contraction. In view of this, and the very slow recovery of low-frequency fatigue, the authors suggested that the force decline may be attributed to some form of damage caused by the generation of high forces during lengthening contractions [10]. In all aforementioned studies the force deficit was directly or indirectly attributed to the E-C coupling impairment.

With respect to the effects of vitamin E on muscle force preservation at low frequencies of stimulation following muscle damaging exercise, it has been shown that the subjects treated with a mixture of vitamins E and C had smaller decline in electrically evoked force at frequencies of 20 and 50 Hz immediately post- and at days 1 and 2 post-maximal eccentric contractions of the knee extensor muscles, compared to the placebo controls [35]. In addition, we have recently demonstrated that soleus muscle single twitch force declined significantly less in vitamin-E-treated rats compared to the placebo controls immediately post-downhill running, even though P_o was not clearly protected in the vitamin-E-supplemented rats [32]. These findings are in line with previous data on muscle force following downhill

running in ovariectomized rats treated with estrogen, a compound known to have antioxidant properties similar to vitamin E. More specifically, compared to the placebo-treated, the estradiol-treated ovariectomized rats had smaller decline in soleus muscle force at low stimulation frequencies of 10–40 Hz and less fatigability was recorded immediately post and 3 days post exercise, despite that P_o was equally decreased in both treatment conditions [41].

There is overwhelming evidence to support that the main mechanism involved in force decline following eccentric contraction is the E-C coupling failure affecting Ca^{2+} kinetics [5, 17, 46–48]. The mechanism of Ca^{2+} kinetics impairment supports our case that low-frequency stimulation might be used as an alternative—or rather complemental to P_o—functional index of muscle injury induced by eccentric exercise. There is a greater chance that a weaker protective effect of vitamin E against muscle injury is revealed at low stimulation frequencies, whereas this effect may be masked at maximal stimulation. Unlike P_o, which results from the application of high-frequency stimuli causing prolonged and maximal Ca^{2+} release from the SR, unfused tetanic force is characterized by a train of low-frequency stimuli challenging the Ca^{2+} release and Ca^{2+} reuptake process by the SR. The lower the stimulation frequency, the more transient and limited the Ca^{2+} release by the SR. In P_t, for example, the SR Ca^{2+} channels open abruptly with depolarization and then close rapidly once the membrane is repolarized, thus allowing a very short time for Ca^{2+} release [36]. In this context, the maximal stimulation of the E-C coupling system may compensate for any potential impairment in SR Ca^{2+} kinetics, and therefore, may mask any functional/force decrements that would normally be manifested under submaximal E-C coupling stimulation. Hence, we proposed that any impairment of Ca^{2+} kinetics, which was thought to reflect microinjury, is more easily detected via the recordings of single twitch tension and unfused tetanic force at very low stimulation frequencies (e.g., 5 to 30 Hz). These measures can be either assessed alone or in a train of stimuli incorporated in a low-frequency fatigue protocol. For example, a low-frequency fatigue protocol of 10–20 Hz and duration of 5–10 min might be a very suitable functional tool to detect the microtrauma induced by eccentric-biased exercise.

A limitation of the present study was that plasma vitamin E concentration was determined only in a small number of samples, thus not allowing further inferential statistical comparisons rather than descriptive statistics on these data. Another limitation of the present study was that we did not measure redox homeostasis and oxidative stress biomarkers which could have provided an estimate of the potential contribution of reactive species to low-frequency fatigue as well as whether vitamin E maintained redox homeostasis and alleviated oxidative stress. It has been proposed that the long lasting effects of low-frequency fatigue can be in part attributed to increased generation of reactive species causing structural alterations of the proteins involved in the E-C coupling process, thus affecting Ca^{2+} kinetics and myofibrillar Ca^{2+} sensitivity [49–51]. However, data from skinned and intact fibers suggest that the increased sensitivity of the SR Ca^{2+} release channels to reactive species is not necessarily followed by a respective physiological activation generated by an action potential [50, 52, 53]. Nevertheless, there is evidence from animal and human studies that support the role of vitamin E in reducing oxidative stress induced by eccentric exercise. Vitamin E supplementation decreased the susceptibility of the rat soleus muscle homogenates to oxidative stress [34], attenuated the increased protein carbonyls in rat soleus and vastus intermedius muscles [54], and reduced human muscle conjugated denies and urinary thiobarbituric acid reactive substances [55] following downhill running. An interesting approach to look into the role of reactive species in muscle force generation and the potential effectiveness of antioxidants against muscle injury-induced force decline would be to examine the issue within a hormetic framework [56].

In conclusion, our findings suggest that vitamin E supplementation at the doses and duration administered resulted in a two-fold increase of its concentration in plasma. This dose was effective to protect soleus muscle from injury induced by eccentric contraction-biased exercise as indicated by the decreased fatigability at a low-frequency of stimulation and the almost complete recovery of single-twitch force immediately after fatigue in the vitamin E condition compared to the placebo condition. This protective effect of vitamin E at low-frequency fatigue is considered critical and physiologically significant given that low-frequency firing of the neuromuscular system is common in daily activities and, hence, force deficit at these frequencies may possibly compromise everyday functionality. This is not contradictory to the statement we have made elsewhere [32] reporting no physiologically significant protective effects of vitamin E against eccentric exercise-induced muscle injury. In that study, the physiological significance with respect to muscle injury attenuation was defined merely on the basis of the P_o. The criterion was the difference in P_o between the vitamin E and the placebo conditions, only to show the inadequacy to comprehensively evaluate eccentric exercise-induced force deficit when P_o alone is used. For a comprehensive assessment of injury-induced decrease in muscle functionality, it may be beneficial that low-frequency fatigue measures, complementally to the traditionally recorded P_o, are also determined.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

This work was partially funded by a grant award to A. Kyparos by the Research Committee (Research Dissemination Center) of the Aristotle University of Thesssaloniki, Greece. No other funding has been received. Vitamin E was a generous gift by G. A. Pharmaceuticals S. A. Professor Themistoklis Christides is acknowledged for the valuable methodological and technical advice on vitamin E administration. The authors are grateful to Professor Nikolaos

Kokolis, Department of Physiology at the Veterinary School of the Aristotle University of Thessaloniki, for kindly opening his laboratory to the authors to perform a portion of the experiments.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2012, Article ID 349710, 13 pages
doi:10.1155/2012/349710

Review Article

Exercise in the Metabolic Syndrome

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Received 27 March 2012; Accepted 13 May 2012

Academic Editor: Steve R. McAnulty

The metabolic syndrome is a clustering of obesity, diabetes, hyperlipidemia, and hypertension that is occurring in increasing frequency across the global population. Although there is some controversy about its diagnostic criteria, oxidative stress, which is defined as imbalance between the production and inactivation of reactive oxygen species, has a major pathophysiological role in all the components of this disease. Oxidative stress and consequent inflammation induce insulin resistance, which likely links the various components of this disease. We briefly review the role of oxidative stress as a major component of the metabolic syndrome and then discuss the impact of exercise on these pathophysiological pathways. Included in this paper is the effect of exercise in reducing fat-induced inflammation, blood pressure, and improving muscular metabolism.

1. Introduction

The metabolic syndrome (MS) describes a constellation of hypertension, diabetes, and dyslipidemia that is caused by abdominal obesity [1, 2] and has also been variously termed X syndrome, insulin resistance syndrome, and the deadly quartet [3]. The diagnostic criteria for MS have been set out by different organizations with slight variations in these criteria as shown in Table 1. The global increase in prevalence of the MS that is rampant in both industrialized and developing countries is associated with an increase in obesity. For example, in a study of 12363 US men and women using the National Cholesterol Education Program's Adult Treatment Panel III guidelines, the MS was diagnosed in 22.8% and 22.6% of the men and women, respectively [4]. This syndrome was present in 4.6%, 22.4%, and 59% of normal weight, overweight, and obese men, respectively, and a similar distribution was observed in women. Higher body mass index (BMI), current smoking, low household income, high carbohydrate intake, and physical inactivity were associated with increased odds.

The MS can be present in different forms, according to the combination of the different components of the syndrome, and it is well established that it increases the risk for the development of cardiovascular disease, type II diabetes, and cancer [5–7]. It is not yet known how the MS is triggered or how the different components are causally linked, but insulin resistance is strongly suspected as a common pathophysiologic link [8, 9], since it is clear that there is a positive correlation between body weight and insulin resistance and the risk of developing all the metabolic abnormalities associated with insulin resistance [9]. However, recent data suggests that MS and obesity do not always occur in concordance as there is some evidence for conditions of benign obesity [10–14]. For example, some studies suggest that frank obesity does not necessarily translate into insulin resistance and increased risk for metabolic comorbidities. In a cross sectional study of 5440 participants of the National Health and Nutrition Examination Surveys 1999–2004, 31.7% of obese adults (BMI $\geq$ 30) were metabolically healthy [12]. In general, healthy obesity describes the lack of any metabolic disorder including type II diabetes, dyslipidemia, and hypertension in an obese individual. To date, there is no prospective study of the healthy obese phenotype and there are a myriad of questions that can be addressed by studying this subtype of obesity. Amongst these are the following questions : do healthy obese subjects represent a delayed onset of obesity related insulin resistance, or is it a permanent condition? What are the causal factor(s) that lead the transition between healthy and unhealthy obese phenotypes? What

Table 1: Comparison of definitions of the metabolic syndrome.

WHO	EGIR	NCEP	IDF
Presence of one of the following:	Insulin resistance AND two or more of the following:	Presence of three of the following (2001):	Central obesity[13] AND any two of the following:
(i) DM	(i) Central obesity[4]	(i) Central obesity[6]	(i) Raised TG[14]
(ii) IGT	(ii) Dyslipidemia[5]	(ii) Dislipidemia[7]	(ii) ↓ HDL[15]
(iii) IFG	(iii) BP ≥ 140/90	(iii) BP ≥ 130/85	(iii) ↑ BP[16]
(iv) Insulin resistance	(iv) FBG ≥ 6.1 mmol/L (110 mg/dL)	(iv) FPG ≥ 6.1 mmol/L (110 mg/dL)	(iv) ↑ FBG[17]
AND two of the following:		Update (2004):	
(i) BP ≥ 140/90		(i) Elevated waist circumferences[8]	
(ii) Dyslipidemia[1]		(ii) Elevated TG[9]	
(iii) Central obesity[2]		(iii) Reduced HDL[10]	
(iv) Microalbuminuria[3]		(iv) Elevated BP[11]	
		(v) Elevated fasting glucose[12]	

BP: blood pressure, DM: diabetes mellitus, EGIR: European Group for the Study of Insulin Resistance, FBG: fasting blood glucose, HDL: high density lipoproteins, IDF: International Diabetes Federation, IFG: impaired fasting glucose, IGT: impaired glucose tolerance, NCEP: US National Cholesterol Education Program, TG: triglycerides, WHO: World Health Organization.
[1] TG ≥ 1.695 mmol/L and HDL ≤ 0.9 mmol/L (male), ≤1.0 mmol/L (female).
[2] Waist/hip ratio > 0.90 (male) >0.85 (female), or body mass index > 30 kg/m^2.
[3] Urinary albumin excretion ratio ≥ 20 μg/min or albumin/creatinine ratio ≥ 30 mg/g.
[4] Waist circumference ≥ 94 cm (male), ≥80 cm (female).
[5] TG ≥ 2.0 mmol/L and/or HDL < 1.0 mmol/L or treated for dyslipidemia.
[6] Waist circumference ≥ 102 cm or 40 inches (male), ≥88 cm or 36 inches (female).
[7] TG ≥ 1.7 mmol/L (150 mg/dL) and HDL < 40 mg/dL (male), <50 mg/dL (female).
[8] Men, greater than 40 inches (102 cm) and women, greater than 35 inches (88 cm).
[9] Equal to or greater than 150 mg/dL (1.7 mmol/L).
[10] Men, Less than 40 mg/dL (1.03 mmol/L) and women, Less than 50 mg/dL (1.29 mmol/L).
[11] Equal to or greater than 130/85 mm Hg or use of medication for hypertension.
[12] Equal to or greater than 100 mg/dL (5.6 mmol/L) or use of medication for hyperglycemia.
[13] Defined as waist circumference with ethnicity specific values (If BMI is >30 kg/m^2, central obesity can be assumed and waist circumference does not need to be measured).
[14] TG > 150 mg/dL (1.7 mmol/L), or specific treatment for this lipid abnormality.
[15] HDL < 40 mg/dL (1.03 mmol/L) in males, <50 mg/dL (1.29 mmol/L) in females, or specific treatment for this lipid abnormality.
[16] systolic BP > 130 or diastolic BP > 85 mm Hg, or treatment of previously diagnosed hypertension.
[17] FPG > 100 mg/dL (5.6 mmol/L), or previously diagnosed type 2 diabetes.

is known with some clarity is that android fat distribution, visceral and ectopic fat accumulation, and insulin resistance are critical factors and potential causative parameters for the development of unhealthy obesity [15–17].

The association between the MS and inflammation is well documented [18]. In an attempt to clarify the relationship between adiposity and inflammation, Welsh et al. [19] used a bidirectional Mendelian randomization approach and deduced that adiposity leads to higher C-reactive protein (CRP) levels, with no evidence for any reversal of this pathway. Accumulating evidence demonstrates a close link among the metabolic syndrome, a state of chronic inflammation, and oxidative stress [20]. In fact, the oxidative stress-inflammation pathway has important roles in all the individual components of MS including vascular alterations [20–24].

2. Oxidative Stress and Ectopic Fat

Ectopic fat refers to the accumulation of triglycerides within cells of nonadipose tissue; these tissues normally contain only small amounts of fat. Visceral areas, liver, heart, and/or muscle are common sites for deposition of ectopic fat [25]. The amount of ectopic fat is directly related to insulin resistance [26], triglyceride level, blood pressure [26, 27], and in general with the metabolic syndrome [27]. The role of adipose tissue in secreting metabolically active substances has been known for some time and it is now believed that a balance between antiatherosclerotic adipokines (such as leptin and adiponectin) and proatherosclerotic cytokines (such as interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and monocyte chemotactic protein-1 (MCP-1)) adjusts metabolic and cardiovascular homeostasis at both local and remote sites. Mazurek et al. showed inflammatory properties of cardiac fat, as an example of ectopic fat, by a paired sampling of epicardial and subcutaneous adipose tissues before the initiation of cardiopulmonary surgery [28]. Higher levels of IL-1β, IL-6, MCP-1, and TNF-α mRNA and protein occurred in epicardial adipose stores irrespective of clinical variables such as diabetes, BMI, and drug use. Expression levels of the TNF gene were higher in abdominal adipose tissue compared to subcutaneous fat, and importantly, greater TNF gene expression occurs in the adipose tissues of obese animals [29] and humans [30]. At the cellular level,

TNF-dependent activation of stress-related kinases inhibits insulin signaling, causing cellular insulin resistance. Some of these stress-related kinases also promote further production of TNF, perpetuating a positive feedback mechanism for sustained TNF activity and chronic insulin resistance [31]. Targeted disruptions of the genes that encode TNF [32] or TNF receptors [33] markedly improve insulin sensitivity in obese mice. On the other hand, visceral fat obesity is associated with decreased concentrations of insulin-sensitizing and anti-inflammatory adipokines [25]. During lipolytic activity, more fatty acids are released from visceral adipose tissue compared to subcutaneous adipose tissue [34, 35]. Increased TNF levels induce hepatic uptake of these fatty acids that is accompanied by reduced fatty acid oxidation and triglyceride export. These events cause accumulation of fat within hepatocytes (hepatic steatosis). Indeed, nonalcoholic fatty liver disease emerges as a companion of the metabolic syndrome. It is generally believed that the chain of reactions leading to hepatocyte fatty degeneration begins with increased levels of TNF and insulin resistance, which precede fat accumulation [36]. During hepatic insulin resistance, hepatic glucose production is no longer down regulated by insulin, resulting in increased hepatic glucose production and stimulation of increased insulin secretion. Chronic hyperinsulinemia desensitizes peripheral tissues to insulin and causes systemic insulin resistance. Insulin resistance increases adipocyte lipolysis, which results in the release of large amounts of fatty acids into the blood and exacerbation of hepatic steatosis and insulin resistance [37].

Lipid accumulation and insulin resistance activate a variety of hepatic reactive oxygen species (ROS) producing pathways such as (a) cytochrome P450 2E1 and 4A, which produce ROS during the metabolism of endogenous ketones [38], (b) mitochondrial NADPHs (nicotinamide adenine dinucleotide phosphate), which generate ROS continuously, and (c) peroxisomes, which produce hydrogen peroxide and are activated when mitochondrial β-oxidation is saturated or impaired [39]. TNF is a potent inducer of mitochondrial ROS [40] and increases ROS production in fatty hepatocytes. In order to mitigate or reverse this chronic oxidative stress, adaptive mechanisms such as uncoupling proteins are activated or upregulated. Mitochondrial respiration can be uncoupled by the controlled transfer of protons across the inner mitochondrial membrane, thereby dissipating the proton gradient and reduce the harmful effects of ROS. The family of inner mitochondrial membrane uncoupling proteins plays important roles in the thermogenesis of brown adipose tissue and in regulating the disposal of mitochondrial ROS in other tissues [41]. Decreases in the mitochondrial membrane potential reduce ATP synthesis and make cells susceptible to necrotic cell death [42]. These events lead to local inflammatory reactions by attracting inflammatory cells, leading to the histopathology of nonalcoholic steatohepatitis [43].

Confrontation with various stressors (oxidative stress, inflammatory cytokines, and elevated concentrations of fatty acids) activates stress kinases, including mitogen-activated protein kinases (MAPKs), c-Jun N-terminal kinase (JNK), extracellular signal regulated kinase, inhibitor of nuclear factor kappa B (NFκB)-kinase (IKK), and conventional and atypical protein kinases C (PKC) [44]. The action of these kinases induces insulin resistance through phosphorylation of insulin receptor substrate (IRS). IRS-1 serine phosphorylation disrupts insulin receptor signaling through several distinct mechanisms and blocks insulin action. These kinases also exert powerful effects on gene expression, including promoting further inflammatory gene expression through activation of activator protein-1 (AP-1) complexes and NFκB [45]. NFκB, in turn, interacts with other transcription factors such as peroxisome proliferator activated receptor (PPAR)γ, which is necessary for adipocyte differentiation. Reduction of PPARγ activity prevents normal induction of some adipocyte genes such as TNF antagonist and adiponectin, which have direct effects on intermediary metabolism [46]. Adiponectin helps in the removal of free fatty acids from the circulation and deposition in fat depots [47]. In hepatocytes, it reduces hepatic glucose production and fatty acid uptake. Adiponectin also increases fatty acid oxidation in liver and skeletal muscle, resulting in a global increase in insulin sensitivity [48–50].

Decreased antioxidant capacity accompanied with increased lipid peroxidation has been reported in patients with fatty liver, visceral obesity, and metabolic syndrome. There is a correlation between the amount of visceral fat and systemic oxidative markers, indicating that visceral fat is an independent regulator of oxidative changes [51]. In nondiabetic human subjects, lipid peroxidation (represented by plasma thiobarbituric acid reactive substance and urinary 8-epi-prostaglandin-$F_2\alpha$) was positively correlated with body mass index and waist circumference [52]. The role of the liver both as an affected organ and a contributory source for impaired redox balance in patients with the MS and visceral adiposity has been strongly implicated [53]. The increased prevalence of fatty liver with hypertension and metabolic syndrome in nonobese patients provides additional support for role of liver in this condition [54].

3. Oxidative Stress and Hyperglycemia

Hyperglycemia can induce oxidative stress by several different mechanisms including nonenzymatic, enzymatic, and mitochondrial pathways, and so accelerate the four important molecular mechanisms involved in hyperglycemia-induced oxidative tissue damage [55, 56]. Nonenzymatic sources of oxidative stress originate from the oxidative biochemistry of glucose. Hyperglycemia directly increases ROS generation since glucose undergoes autoxidation to generate $^{\bullet}$OH radicals [57]. In addition, glucose reacts with proteins in a nonenzymatic manner leading to the development of Amadori products followed by formation of advanced glycation end products (AGEs). Enzymatic sources of augmented generation of ROS in diabetes include nitric oxide synthase (NOS), NAD(P)H oxidase, and xanthine oxidase [58–60]. All isoforms of NOS require five cofactors/prosthetic groups such as flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), heme, tetrahydrobiopterin (BH4), and Ca^{2+}-calmodulin. NOS becomes "uncoupled" when the

enzyme lacks its substrate L-arginine or one of its cofactors, and when uncoupled, NOS produces $O_2^{\bullet-}$ instead of $^{\bullet}NO$ [58–61]. A major source of $O_2^{\bullet-}$ production is NAD(P)H oxidase, a membrane associated enzyme that consists of five subunits [58, 59, 62, 63]. Guzik et al. investigated $O_2^{\bullet-}$ levels in vascular specimens from diabetic patients and probed sources of $O_2^{\bullet-}$ using inhibitors of NOS, NAD(P)H oxidase, xanthine oxidase, and the mitochondrial electron transport chain and reported that the enhanced production of $O_2^{\bullet-}$ in diabetic patients is predominantly formed by NAD(P)H oxidase [59].

The mitochondrial respiratory chain is a nonenzymatic source of reactive species. During oxidative phosphorylation, electrons are transferred from the electron carriers NADH and FADH2, through four complexes in the inner mitochondrial membrane, to oxygen, and generating ATP in the process [64]. Under normal conditions, $O_2^{\bullet-}$ is immediately eliminated by natural defense mechanisms. Hyperglycemia-induced generation of $O_2^{\bullet-}$ at the mitochondrial level is thought to be the major driver of the vicious cycle of oxidative stress in diabetes [65, 66]. There is an increased generation of ROS (especially $O_2^{\bullet-}$) when endothelial cells are exposed to clinically relevant hyperglycemic conditions. The augmented generation of pyruvate via accelerated glycolysis under hyperglycemic conditions is thought to flood the mitochondria and thus generates $O_2^{\bullet-}$ formation at the level of complex II in the respiratory chain [65].

Superoxide anions can activate several pathways in diabetes including accelerated formation of AGE's, polyol pathway, hexosamine pathway, and protein kinase C (PKC), all of which have been proven to be involved in micro- and macrovascular diabetic complications. Both $O_2^{\bullet-}$ and H_2O_2 stimulate stress-related signaling mechanisms such as NF-κB, p38-MAPK, and signal transducers and activators of transcription-Janus kinases (STAT-JAK), resulting in vascular smooth muscle cell migration and proliferation. In endothelial cells, H_2O_2 mediates apoptosis and pathological angiogenesis [67]. Furthermore, $O_2^{\bullet-}$ immediately reacts with $^{\bullet}NO$ to generate cytotoxic peroxynitrite ($ONOO^-$) and this reaction itself has several consequences. First, $ONOO^-$ alters the function of biomolecules by protein nitration as well as by causing lipid peroxidation [57]. For example, potassium channels, which regulate vasorelaxation, are inhibited by nitration [68, 69]. As reviewed by Turko et al., increased levels of nitrotyrosine are associated with apoptosis of myocytes, endothelial cells, and fibroblasts in diabetes [57]. Second, $ONOO^-$ causes single-strand DNA breakage, which in turn activates nuclear enzyme poly(ADP-ribose) polymerase (PARP) (a nuclear DNA-repair enzyme that is able to cause a depletion of NAD^+) [70]. Third, it decreases $^{\bullet}NO$ bioavailability causing impaired relaxation and inhibition of the antiproliferative effects of $^{\bullet}NO$ [64]. Furthermore, $ONOO^-$ oxidizes BH_4, an important cofactor for NOS, and causes uncoupling of NOS to produce $O_2^{\bullet-}$ instead of $^{\bullet}NO$ [61]. ROS-induced peroxidation of membrane lipids alters the structure and the fluidity of biological membranes, which will have global effects that alter vascular function [61, 67–71].

4. Oxidative Stress and Hypertension

There is a growing body of evidence supporting an important central role of the renin angiotensin aldosterone system (RAAS) in the coexistence of obesity, insulin resistance, dyslipidemia, and hypertension [72–74]. Indeed, aldosterone has a firm role in the pathogenesis and progression of the MS. Adipose tissue produces a lipid soluble factor that stimulates aldosterone secretion [75], which is then (along with other glucocorticoids) able to enhance adipogenesis and increase macrophage infiltration into fat depots [75, 76]. Elevated plasma aldosterone level induces insulin resistance in fat, skeletal muscle, liver, and cardiovascular tissue, independent of other RAAS components such as angiotensin II [74, 77]. Aldosterone induces proinflammatory adipokine expression and oxidative stress, resulting in diminished insulin receptor expression and impaired insulin induced glucose uptake [78]. Studies in animal models show that mineralocorticoid receptor blockade reduces expression of proinflammatory and prothrombotic factors in adipose tissue and increases the expression of adiponectin in heart and adipose tissue [79]. In humans, there is evidence to suggest that oxidative stress drives the production of aldosterone-stimulating oxidized fatty acids. Compounds such as 12,13-epoxy-9-keto-10(trans)-octadecenoic acid, derived from linoleic acid, can affect adrenal steroid production and mediate some of the harmful effects of obesity and oxidative stress [80]. Reduction of blood pressure, plasma renin activity, and aldosterone levels in both obese hypertensive and normotensive subjects who underwent weight reduction provides further evidence for the association of excess aldosterone and fat tissue [81, 82]. The deleterious effects of aldosterone on blood vessels and skeletal muscle tissue are partly mediated by stimulation of NAD(P)H oxidase, which induces excessive amount of ROS and oxidative stress. Exogenous aldosterone induces aortic expression of NAD(P)H oxidase (NOX2) (through mineralocorticoid receptor-dependent mechanisms) and of p47 phox (subunit of NADPH oxidase) mRNA (through both angiotensin receptor (AT1) and mineralocorticoid receptor-dependent mechanisms) [83]. Mineralocorticoid receptor activation contributes to angiotensin II mediated activation of NAD(P)H oxidase in the heart and aorta [84–86]. This in turn leads to induction of redox sensitive stress kinases (PKC, MAPK, JNK, etc.), phosphorylation of IRS-1 docking protein, and finally impaired glucose utilization [87, 88]. Aldosterone induces activation of NF-κB in the heart, an effect that is prevented in NOX-2 deficient mice [85]. Activation of NF-κB by aldosterone induces further production of adhesion molecules, chemokines such as monocyte chemoattractant protein (MCP-1), and inflammatory cytokines. In a rat model of aldosterone/salt hypertension, aldosterone induced severe hypertension, increased the expression of proinflammatory molecules in the heart, and cause inflammatory arterial lesions with infiltration of perivascular macrophages [89]. Several studies show that mineralocorticoid receptor blockade improves systemic insulin sensitivity and skeletal muscle glucose uptake that is associated with reduced NADPH oxidase activity and the attenuation of ROS [72, 74]. Mineralocorticoid receptor

antagonism in hypertensive rats decreases aortic inflammation, fibrosis, and hypertrophy [90–92] while it also decreases oxidative stress and inflammation in apolipoprotein E-deficient mice fed a high-cholesterol diet, a model of atherosclerosis [93]. Other proposed mechanisms for aldosterone induced metabolic effects include the effects of hypokalemia on pancreatic β-cell function, induction of hepatic gluconeogenesis, interfering with sodium-glucose transport, and fibrosis-induced malfunction in insulin secreting or insulin sensitive tissues [87, 94, 95]. Mosso et al. investigated insulin sensitivity and insulin secretion in patients with idiopathic primary aldosteronism and showed an association between aldosterone and lower pancreatic β-cell mass [94]. There is a negative correlation between C-peptide and serum aldosterone levels that are independent of serum potassium. Other data suggests that the harmful effects of aldosterone on β-cell function are mediated through induction of islet cell inflammation and oxidative stress [95].

5. Exercise and Metabolic Syndrome

Reduced daily physical activity in healthy young adults is associated with negative metabolic consequences such as decreased insulin sensitivity and increased abdominal fat [96, 97]. Therefore, increased physical activity is likely to be the evolutionary favored pathway to prevent the development of insulin resistance during metabolic derangements. According to Nunn et al. [98], chronic subclinical inflammation associated with the metabolic syndrome could be one reason for the continued physical inactivity and the induction of a vicious cycle. In the presence of inflammation, physical activity becomes less desirable, both physically and psychologically. The "inflammatory-induced sickness behavior" in animal studies is in support of this theory [99, 100]. Injection of lipopolysaccharide (which induces cytokine release) or direct injection of cytokines results in fatigue reduced movement and depressive symptoms. In contrast, hormetic stimuli, including exercise, calorie restriction, or polyphenols, can induce anti-inflammatory effects and enhance exercise capability, leading to better biological fitness. Low/moderate amounts of ROS produced during regular skeletal muscle work, are part of hormesis, which describes the generally favorable biological responses to low exposures to toxins and other stressors. A pollutant or toxin showing hormesis has opposite effects in small versus large doses. Hormesis is characterized by stimulation at low doses and inhibition at higher doses, resulting in an inverted U-shaped dose-response effect [101]. For example, exercise-induced increased production of ROS can be beneficial by evoking specific adaptations, such as increased antioxidant/oxidative damage repairing enzyme activity, increased resistance to oxidative stress, and lower levels of oxidative damage. On the other hand, excessive production of ROS is usually associated with detrimental effects.

6. Exercise and Adipose Tissue

Several studies show a strong association between obesity and physical inactivity [102, 103]. There is an inverse relationship between physical activity, body mass index (BMI), hip-waist ratio, and waist circumference [102–104]. These studies demonstrate that maintaining an active lifestyle can prevent the development of the MS. Weight reduction, via exercise, results in less loss of muscle (compared to fat) than weight loss through diet [105]. Maintaining lean body mass is essential for better glucose transport and fat metabolism. Reduction in fat mass is helpful in increasing adiponectin levels and improving cytokine profiles; changes in adipokines and cytokines are associated with the MS [106]. Controlling the release and activity of at least two cytokines, TNF-α and IL-6, could contribute to the natural protective effects of physical activity. Interleukine-6 (IL-6) is the first cytokine to be released into the circulation during exercise, and its levels increase in an exponential fashion in response to exercise [107]. IL-6 mRNA is upregulated in contracting skeletal muscle [108] and the transcriptional rate of the IL-6 gene is also markedly enhanced by exercise [109]. IL-6 acts as both a proinflammatory and anti-inflammatory cytokine. When secreted by T cells and macrophages, IL-6 stimulates the immune response and boosts inflammatory reactions, while muscle-produced IL-6 exerts anti-inflammatory effects through its inhibitory effects on TNF-α, IL-1β, and activation of interleukin-1 receptor antagonist (IL-1ra) and IL-10 [110]. Exercise-induced increases in plasma IL-6 correlate with the muscle mass involved in exercise activity and also with the mode, duration, and, especially, the intensity of exercise [111]. Exercise also confers protection against TNF-induced insulin resistance [112]. In addition, Starkie et al. reported that infusion of recombinant human IL-6 (rhIL-6) into human subjects simulated the exercise induced IL-6 response in the prevention of endotoxin-induced increase in plasma TNF-α [113]. Exercise can also suppress TNF-α production by an IL-6-independent pathway, as demonstrated by Keller et al. who reported only modest decreases in plasma TNF-α after exercise in IL-6 knockout mice [114]. Exercise induced increases in epinephrine levels can also blunt the TNF-α response [115]. In addition, Petersen et al. showed that IL-6 enhances lipid turnover and stimulates lipolysis as well as fat oxidation via activation of AMP-activated protein kinase [116]. Consistent with this, Wallenius et al. demonstrated that IL-6 deficient mice (IL6$-/-$) develop mature onset obesity and have disturbed carbohydrate and lipid metabolism that is partly reversed by IL-6 replacement. Other data indicate that centrally acting IL-6 exerts an antiobesity effect in rodents [117]. The lipolytic effect of IL-6 on fat metabolism was confirmed in two clinical studies of healthy and diabetic subjects [116, 118]. Visceral fat is potentially a cause of low-grade systemic inflammation, which in turn leads to insulin resistance, type II diabetes, and atherosclerosis [119]. During exercise, IL-6 also increases hepatic glucose production. Glucose ingestion during exercise reduces IL-6 production by muscles, suggesting that IL-6 is released due to the reduction in glycogen levels during endurance exercise and the consequent adrenergic

stimulation of IL-6 gene transcription via protein kinase A activation [120].

7. Exercise and Glucose Metabolism in Skeletal Muscles

At least two distinct pathways are involved in glucose transport; one is stimulated by insulin or insulin mimetics and the other is activated by contraction or hypoxia [121–123]. Phosphatidylinositol 3 kinase (PI3-kinase) is involved in insulin activated (but not contraction-activated) pathway [124], while 5′AMP-activated protein kinase participates in contraction-activated reactions [125]. Insulin-stimulated tyrosine phosphorylation of IRS-1 and activity of PI3 kinase [126], and insulin-stimulated Akt kinase activity are both diminished in skeletal muscle of obese and diabetic patients. Therefore, exercise can provide an alternative way to bypass the impaired insulin signal transduction in muscles of diabetic patients [127]. Regular physical activity mends insulin function and glucose tolerance in healthy individuals [128], patients with obesity [129], insulin resistance [130], and diabetics [131, 132]. Molecular mechanisms for improved glucose clearance and insulin sensitivity following exercise are related to the increased expression and activity of signaling proteins and enzymes which are involved in skeletal glucose and fat metabolism [133, 134]. Glucose transporter isoform 4 (GLUT4) is a key enzyme in this chain of reactions and its mitochondrial biogenesis is increased due to exercise training [135, 136]. It has been reported that peroxisome proliferator-activated receptor γ coactivator-1 (PGC-1) stimulates GLUT4 expression [137]. PGC-1 is a member of a group of transcription coactivators which play a key role in the regulation of cellular energy metabolism. It increases mitochondrial biogenesis and participates in the regulation of both carbohydrate and lipid metabolism. PGC-1 promotes the remodeling of muscle tissue to a fiber type composition that has greater oxidative capacity and less glycolytic metabolism in nature [138]. A single bout of exercise can increase muscular PCG1-1 content [139]. It should be noted that exercise-induced improvement in insulin signaling is not exclusively restricted to increased GLUT4 protein expression, as its concentration is similar in sedentary diabetics and insulin sensitive control subjects [140, 141]. While exercise increases GLUT4 protein and mRNA in diabetic patients [142], increased post receptor insulin signaling, especially at the distal step of the insulin PI3-kinase cascade (which results in GLUT4 translocation and glucose uptake), is the main mechanism [134, 143, 144]. Atypical protein kinase C (aPKC) and Akt substrate of 160 kDa (AS160) are among newly characterized insulin signaling molecules [145, 146]. AS160 in the basal nonphosphorylated state acts as an inhibitor for GLUT4 translocation. Insulin stimulates AS160 phosphorylation by Akt on five of six phosphor-Akt substrate motifs, leading to increased GLUT4 membrane trafficking events [147]. The exact mechanisms of aPKC in controlling GLUT4 translocation is still not clear, however, reports suggest that parallel to Akt, activation of aPKC is essential in both the process of translocation and docking/fusion of GLUT4 to the plasma membrane [148].

8. Exercise and Lipid Metabolism in Skeletal Muscles

As stated earlier, in addition to hyperglycemia and/or hyperinsulinemia, patients with the MS show a serious dysregulation in lipid metabolism as manifested by increased levels of circulating free fatty acids (FFAs) and triglycerides, accompanied by lipid accumulation in skeletal muscles [149]. Increased intramyocellular lipids will increase cellular oxidative stress with subsequent generation of ROS, stimulating lipid membrane peroxidative injury of mitochondrial membranes. One of the basic effects of exercise training is augmenting oxidative capacity of skeletal muscles, which results in an improvement in the rate of whole body fat oxidation [150]. This increase in fat oxidation capacity is partly due to an increase in fatty acid transport proteins, which leads to increased removal of plasma FFAs [151]. $FABP_{PM}$ and CD36 are among several key proteins that have been identified as fatty acid transporter proteins in human and animal muscles [152]. The effects of exercise training on the mRNA and protein expression of CD36 and $FABP_{PM}$ in muscles have shown different results [153, 154]. It may well be that increases in those proteins is totally protocol dependent, in terms of exercise duration and intensity. Exercise also activates AMP kinase, which stimulates fatty acid oxidation, glucose uptake, and mitochondrial biogenesis.

The AMPK complex is evolutionally a well-conserved serine/threonine kinase that functions as a fuel sensor in the cell and is activated when cellular energy is depleted and the AMP/ADP ratio rises [155]. The result of AMPK activation is the inhibition of energy-consuming biosynthetic pathways and the activation of ATP producing catabolic pathways. AMPK can also affect transcription of specific genes involved in energy metabolism, thereby exerting long-term metabolic control [156]. Cellular stresses that increase the AMP/ATP ratio such as hypoxia, oxidative stress, hypoglycemia, exercise, or nutrient deprivation can affect cellular metabolic conditions partially through this pathway [155]. In vivo and in vitro studies have shown that activation of AMPK leads to reduced glucose output form the liver [157]. Insulin sensitivity is also improved through reduced triglyceride accumulation by skeletal muscles [158]. This occurs as a result of AMPK phosphorylation, and thus inactivation, of acetyl-CoA carboxylase (ACC), resulting in decreases in malonyl-coenzyme A [159, 160]. ACC is an important rate-limiting enzyme for the synthesis of malonyl-CoA, which in turn is a critical precursor of fatty acids biosynthesis and a potent inhibitor of mitochondrial fatty acid oxidation. Decreases in malonyl-CoA content result in the reduction of fatty acid synthesis and increases in fatty acid oxidation. Amplified AMP kinase activity is also associated with increased cytochrome-c content, mitochondrial density, and DNA binding activity of nuclear respiratory factor-1, a transcription factor that acts on a nuclear set of genes required for transcription of respiratory chain proteins

in addition to mitochondrial transcription and replication [161].

9. Exercise and Increased Blood Pressure

Lifestyle modifications are recommended as the initial treatment strategy for reduction of high blood pressure [162]. Regular exercise training induces a moderate antihypertensive effect, with females and relatively lean participants earning greater benefits [163]. Aerobic exercise also lowers blood pressure and improves blood pressure control among overweight adult subjects [164]. In this regard, a modest weight loss of 3–9% is associated with a significant reduction in systolic and diastolic blood pressure of roughly 3 mm Hg in overweight people [165].

Potential mechanisms for exercise training and weight reduction effects on blood pressure include functional and structural changes in the vasculature, modulation of the renin-angiotensin system, reduction of sympathetic nervous system stimulation, and increased insulin sensitivity. It is been suggested that leptin is the main link between obesity, increased sympathetic nervous system activity, and hypertension [166]. Obesity is associated with resistance to the appetite and weight reduction actions of leptin, although the renal sympathetic activation effects remain intact [167]. Human studies show an interaction between high leptin levels and increased renal sympathetic tone in obese subjects [168]. Chronic hyperleptinemia also has a pressor effect which is mediated by increased sympathetic nervous system activity. Leptin infusion in animal models increases blood pressure, heart rate, and sympathetic nervous in different tissues [169, 170]. Leptin-induced increases in ROS and ET-1 can also contribute to hypertension [171, 172].

Physical activity increases vascular expression of eNOS both in animals and human beings [173–176]. The importance of this phenomenon has been confirmed in patients with stable coronary artery disease and chronic heart failure [177, 178]. There are several reports suggesting that exercise-induced upregulation of vascular eNOS expression is closely related to the changes of frequency and the intensity of physical forces within the vasculature, especially shear stress. Exercise-induced increases in heart rate will augment cardiac output and vascular shear stress, leading to increased expression of eNOS [173]. Increased NO synthesis secondary to amplified shear stress induces extracellular superoxide dismutase (SOD) expression in a positive feedback manner so as to inhibit the degradation of NO by ROS [179]. Another parallel mechanism that participates in this harmony is upregulation of eNOS through exercise-induced ROS production, since exercise-induced increases in shear stress-stimulates vascular production of ROS by an endothelium dependent pathway [180]. Endothelial NAD(P)H oxidase has a critical role in this process [181]. Superoxides are rapidly converted to H_2O_2 by SOD; hydrogen peroxide then diffuses through the vascular wall and increases the expression and activity of eNOS [182, 183]. Thus, increased expression of SOD1 and SOD3 (which facilitate the generation of hydrogen peroxide from superoxide) augments the effect of hydrogen peroxide on exercise-induced eNOS expression. On the other hand, eNOS expression is not increased in catalase overexpressing transgenic mice [174, 184]. Another putative mechanism is exercise-induced increases in arterial compliance which is mediated by reduction of plasma ET-1 concentration as well as the elimination of ET-1-mediated vascular tone. Twelve weeks of aerobic exercise training results in increased arterial compliance, which was accompanied by decreased plasma ET-1 levels. Moreover, the increase in central arterial compliance observed with ET-receptor blockade before the exercise intervention was eliminated after the exercise-training intervention [185]. These results indicate that endogenous ET-1 participates in the mechanisms underlying the beneficial influence of regular aerobic exercise on central arterial compliance.

Exercise training has a significant impact on the morphology of various blood vessels. These structural changes are followed by functional changes and lead to improved blood flow. Exercise induces angiogenesis, which is an expansion of the capillary network by the formation of new blood vessels at the level of capillaries resistance arterioles, and arteriogenesis, which is an enlargement of existing vessels [186].

10. Summary

The metabolic syndrome is an emerging epidemic that affects roughly 20% of the population in Western industrialized countries. It has become evident that inflammation and oxidative stress, which are associated with obesity and overweight, play crucial roles in the pathophysiology of this syndrome. They also greatly impact related pathological outcomes. It seems likely that insulin resistance is at the center of several vicious cycles that exacerbate the disturbances, leading to intensification of oxidative stress. Physical inactivity, a frequent finding in obese patients, escalates these processes. Chronic subclinical inflammation associated with the metabolic syndrome could be one reason for the continued physical inactivity and perpetuation of a vicious cycle. However hormetic stimuli, such as those that result from exercise, can boost antioxidant capacity, induce anti-inflammatory effects, and improve exercise ability. Exercise regulates fat and glucose metabolism and results in an increased action of insulin, while it also lowers blood pressure and improves blood pressure control in overweight adult subjects. In spite of these benefits, the precise duration and intensity of exercise for individual patients remain to be determined.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2012, Article ID 572090, 8 pages
doi:10.1155/2012/572090

Research Article

Antioxidant Status in Elite Three-Day Event Horses during Competition

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Received 14 February 2012; Accepted 22 April 2012

Academic Editor: Manfred Lamprecht

The objective of this study was to determine if competition intensity would have an effect on antioxidant status in horses before and during a three-day event. Body weight, body condition score, and blood was sampled from CCI2* ($n = 19$) and CCI3* ($n = 23$) horses before the start of dressage, 20 to 30 min following cross-country, and 18–24 h after cross-county. Data were analyzed using a PROC MIXED in SAS. There were no differences between CCI2* and CCI3* horses during competition for plasma cortisol, lactate, α-tocopherol, retinol, or erythrocyte glutathione peroxidase. After cross-country, CCI3* horses had higher serum creatine kinase ($P = 0.003$) and aspartate aminotransferase ($P < 0.0001$) than the CCI2* horses. Plasma β-carotene was higher in the CCI2* horses compared to the CCI3* horses ($P = 0.0001$). Total erythrocyte glutathione was also higher in the CCI2* horses versus CCI3* horses ($P < 0.0001$). These results are the first report of antioxidant status of horses competing in this level of a three-day event. The changes in antioxidant and muscle enzymes observed between divisions are likely due to the increased anaerobic and musculoskeletal demand on the upper level horses and the fitness required to compete at that level.

1. Introduction

Equine athletes competing at the top levels of their disciplines experience physiological stress that may compromise health and performance. Oxidative stress results in oxidative damage to all cell components. Oxidative stress is caused by an imbalance between the reactive oxygen species (ROS) produced during cellular respiration and the body's antioxidant system used to scavenge these ROS [1]. Oxidative stress has been observed in horses exposed to intense bouts of exercise [2, 3] and endurance exercise [4, 5]. Antioxidants are vitamins, minerals, and enzymes that must be synthesized in the body or obtained from the diet. Therefore, exercise level and diet are both factors that play a role in influencing the oxidative stress and antioxidant status of the equine athlete.

Eventing is a rigorous exercise challenge, similar to the human triathlon that tests the physical ability and skill of both horse and rider during three separate phases. In dressage, a standard set of movements is performed and awarded a subjective score. Cross-country jumping requires horses to jump over 35 to 40 obstacles covering 5 to 7 km of terrain within a limited time. For stadium, jumping horses are jumped over 10 to 12 obstacles arranged in a course set in an enclosed arena. The International Federation for Equestrian Sports (FEI) governs the sport and has divided it into four levels of difficulty. The highest or Olympic level is denoted as Concours Complet Internationale Four-Star Event (CCI4*) followed by the CCI3*, CCI2*, and CCI1* divisions.

Many studies previously have researched electrolyte loss and fluid shifts, lactate response, and other biochemical measures of exercise intensity of horses performing in 3-day event competitions. However, none have measured the antioxidant status along with their dietary profile. Therefore, the main objective of this study was to determine the effects of a rigorous, high-level three-day event on the antioxidant status of horses. Recognizing that diet can influence antioxidant status, a second objective was to characterize the diet and feeding management practices of three-day event horses prior to and during the exercise challenge. It is hypothesized

Table 1: Specifications of the cross-country jumping phase in the CCI2* and CCI3* divisions of the 2006 Jersey fresh three-day event.

	CCI2*	CCI3*
Distance	4.4–5 km	5.7–6.8 km
Speed	9.2 m/s	9.5 m/s
No. of jumps	36	40
Max. height	1.15 m	1.2 m
Max. drop	1.8 m	2 m

that the oxidative stress markers would be higher after cross-country jumping in both divisions, but the CCI3* would be higher than the CCI2* due to the higher level of exercise intensity.

2. Materials and Methods

2.1. Competition. Horses and riders participating in this study were competing in either the CCI2* or CCI3* division of the Jersey fresh three-day event in Allentown, NJ from May 31 to June 4, 2006. The event consisted of a postarrival veterinary horse inspection on day 1, dressage on d 2 or 3, cross-country jumping followed by the second veterinary horse inspection on d 4, and then a third veterinary horse inspection followed by stadium jumping on d 5. Competitors rode the 2005 FEI CCI2* or CCI3* event dressage test B for their respective divisions. The distance, optimum speed and jumping efforts for the cross-country jumping phase are presented in Table 1. Mean ambient temperature during the five days of competition was 22.9°C (range: 16.1 to 33.3°C) with 5.4, 3.3, and 0.36 cm of precipitation occurring on days 3, 4, and 5 (Weather Data Services, Inc, Wichita, KS).

2.2. Subjects. Prior to the first veterinary horse inspection, horse owners or riders were asked to complete a release statement that voluntarily enrolled their horses as participants in the study. Nineteen of 33 (58%) CCI2* horses and 23 out of 41 (56%) CCI3* horses were enrolled. Horses in the CCI2* division completed dressage from 0900 to 1200 on days 2 and 3 of the competition and cross-country jumping between 0900 and 1100 on days 4. Horses in the CCI3* division completed dressage from 1300 to 1800 on d 2 and 3 and cross-country jumping between 1300 and 1600 on days 4. All stadium jumping started at 1200 on days 5 with CCI2* horses competing first, immediately followed by CCI3* horses.

2.3. Sample Collection and Processing. Heart rate, rectal temperature, and respiratory rate were recorded by a licensed veterinarian immediately following completion of the cross-country jumping phase. Whole blood was collected on d 1 immediately after the first veterinary horse inspection (PRE), on d 4 20–30 min following the cross-country jumping phase as part of the second veterinary horse inspection (XC), and on d 5 immediately after the third veterinary horse inspection but prior to the commencement of stadium jumping (POST). The POST sample was therefore taken 18 to 24 h after XC. Body weight (BW) was determined using an electronic scale, and body condition score (BCS) [6] was analyzed at the time of blood collection. Whole blood (40 mL) was collected by venipuncture into sodium heparin, EDTA, and serum separator Vacutainer tubes (Becton Dickinson and Company, Franklin Lakes, NJ), immediately placed on ice, and then processed into red blood cell, plasma, or serum aliquots at an on-site laboratory within 30 min of collection using previously described methods [7]. All processed samples were stored on dry ice until transferred to −80°C, where they remained until analysis (within 24 h of sample collection).

2.4. Sample Analysis. Whole blood was analyzed for hematocrit (Hct) using a microhaematocrit technique (Spiracrit, Oxford Labware-Division of Sherwood Medical, St. Louis, MO). Plasma was analyzed for total protein (TP) using refractometry. Plasma cortisol (CORT) was analyzed by radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA). Plasma lactate (LAC) was analyzed using YSI model no. 1500 lactate analyzer (YSI Inc., Yellowsprings, OH). Serum was analyzed for creatine kinase (CK) and aspartate aminotransferase (AST) using a VetTest 8008 analyzer (Model VT8008, IDEXX Laboratories Inc., Westbrook, ME).

The antioxidants α-tocopherol (TOC), retinol (RET), and β-carotene (BC) were analyzed by HPLC (Michigan State Diagnostic Laboratories, East Lansing, MI) by first running plasma samples through a precolumn followed by a reverse phase C-18 HPLC column eluted isocratically at 1.2 mL/min with an injection volume of 50 uL. Absorbance was measured for TOC (292 nM), RET (325 nM), and BC (450 nM). Red blood cells were analyzed for total glutathione (GSH-T) and glutathione peroxidase (GPx). Methods for analysis of GSH-T (Biotech GSH-420, kit #21023; Oxis Health Products Inc., Portland, OR; interassay CV 7.0%, intra-assay CV 5.6%) and cellular GPx (Biotech GPx-340, kit #21017; Oxis Health Products Inc., Portland, OR; interassay CV 4.2%, intra-assay CV 5.0%) were previously described [7].

2.5. Statistical Analysis. Data are summarized as mean ± SE unless otherwise noted. Effects of division, sample time, and their interaction were evaluated on antioxidant and oxidative stress markers, along with performance parameters, using a repeated model ANOVA with sample as our repeated effect (PROC MIXED, SAS Inst Inc., version 9.1, Cary, NC). To further test for differences between competition divisions and sample times, Tukey-Kramer adjustment was used with significance determined using $P < 0.05$. Associations between exercise and antioxidant variables were tested using Pearson's product-moment correlation. Horse within division was included in the model to test for significance; if insignificant, then it was removed from the model. Significance was determined using $P < 0.05$.

3. Results

3.1. Horse Data. Demographic information, initial BW, and initial BCS of horses participating in the study are shown

Table 2: Subject information for horses and riders competing in the CCI2* and CCI3* divisions of the 2006 Jersey fresh three-day event.

Item	Divisions	
	CCI2*	CCI3*
Subject participation[1], %	57.5	56.1
Age, yr	11.3 ± 0.7	11.3 ± 0.5
Sex of horse, %		
Male	78.9	91.3
Female	21.1	8.7
Breed of horse, %		
Thoroughbred	78.9	59.1
Thoroughbred cross	10.5	22.7
Warmblood	5.3	9.1
Other	5.3	9.1
Initial BW, kg	529.2 ± 7.7	529.7 ± 7.1
Initial BCS	5.2 ± 0.1	5.1 ± 0.1

[1] CCI2*, $n = 19$ out of 33 entered; CCI3*, $n = 23$ out 41 entered.

in Table 2. There were no differences in age, initial BW, and initial BCS between CCI2* and CCI3* horses. For both divisions, the majority of horses were of the male gender and Thoroughbred breed. Mean age was 11.3 ± 0.7 and 11.3 ± 0.5 yrs for CCI2* and CCI3* horses, respectively. Horses competing in the CCI2* had been competing at that level for 0 to 1 yr (50.0%), 1 to 2 yr (30.0%), or 3 to 5 yr (20.0%), whereas horses in the CCI3* had been competing at that level for 0 to 1 yr (52.2%), 1 to 2 yr (26.1%), or 3 to 5 yr (21.7%).

Twelve subjects completed the CCI2* (63.2%) with seven placing in the top 10 (1st, 2nd, 3rd, 6th, 8th, 9th, and 10th). Of the nonfinishers, three withdrew before cross-country jumping, three withdrew or were eliminated during cross-country jumping, and one did not pass the final veterinary horse inspection. In the CCI3*, 13 subjects completed the event (56.5%) with four placing in the top 10 (3rd, 6th, 9th, and 10th). Nonfinishers included one that was withdrawn before cross-county jumping, six that were withdrawn or were eliminated during cross-county jumping, and three that were either withdrawn after cross-country jumping for unknown reasons or did not pass the final veterinary horse inspection.

Mean heart rate, rectal temperature, and respiratory rate immediately following the completion of cross-country jumping were not different between divisions (data not shown). For all horses completing the cross-country jumping phase, mean heart rate was 102 ± 2.8 beats per min, rectal temperature was 40.4 ± 0.09°C, and respiratory rate was 100 ± 3.2 breaths per min. There were no correlations observed between these variables and blood variables assessed at XC (data not shown).

3.2. Diet Data. Diets of horses could not be controlled in this study, and as a result, horses were fed according to the rider or owner's preference. A nutritional management survey was conducted prior to and during the competition to gather information on each horse's feeding times; type of pasture and hours spent turned out on pasture prior to competition; type and amount of hay, concentrate, and supplements offered at competition; feeding associated with transport to the event and cross-country jumping. All feeding management data collected during the study have been previously reported [8].

3.3. Blood Data

3.3.1. Effect of Division. There was no main effect of the competition division on plasma TP, CORT, LAC, AST, TOC, RET, BC, and erythrocyte GPx; therefore, data were averaged across division (Table 3). Figure 1 shows blood variables that differed between horses competing in the CCI2* and CCI3* divisions. There was a main effect of division on Hct ($P = 0.002$), and erythrocyte GSH-T ($P < 0.0001$) with the CCI2* horses having higher blood concentrations than the CCI3* horses. For serum CK ($P = 0.04$), however, serum concentrations were lower in the CCI2* horses compared to the CCI3* horses. For Hct, CCI2* horses were higher at XC than CCI3* horses ($P = 0.001$; 55.1 ± 1.7 versus 50.1 ± 1.0%, resp.; Figure 1(a)). Serum CK was lower in CCI2* horses at XC than CCI3* horses ($P = 0.003$; 235 ± 16 versus 576 ± 123 mmol/L; Figure 1(b)). Red blood cell GSH-T was higher in CCI2* horses at XC ($P < 0.0001$; 237 ± 12 versus 163 ± 12 uM, resp.) compared to CCI3* horses (Figure 1(c)), and a time by division interaction was observed ($P = 0.006$).

3.3.2. Effect of Time. The Hct was highest at the XC sample for all horses ($P < 0.0001$, Figure 1(a)), but was not different between PRE and POST. Plasma TP was different for all three samples ($P < 0.0003$, Table 3) with the highest occurring at XC. Plasma CORT was highest after XC and remained higher than PRE levels at POST ($P < 0.005$, Table 3). Serum CK, AST, and plasma LAC were all lowest at PRE ($P < 0.01$, Figure 1(b), and Table 3, resp.). Plasma TOC did not change in horses throughout the competition (Table 3). Plasma BC was highest in horses at PRE ($P = 0.003$; Table 3) and decreased to XC and POST. Plasma RET increased in all horses from PRE to XC, but it then decreased to its lowest value at POST ($P < 0.03$, Table 3). Red blood cell GSH-T was similar from PRE to XC, but it then decreased below baseline at POST ($P < 0.0001$, Figure 1(c)). Red blood cell GPx peaked at XC compared to PRE and POST ($P = 0.004$, Table 3).

3.3.3. Correlations. Significant correlations between the exercise and antioxidant variables are shown in Table 4. Some of these include a positive correlation between LAC and GSH-T, GPx, CORT and RET ($P < 0.05$). Plasma CORT, was also positively correlated with CK ($P = 0.007$), RET ($P = 0.003$), and GSH-T ($P = 0.04$) and negatively correlated with TOC ($P = 0.04$). Plasma AST, however, was not correlated with CORT as CK was, but was positively correlated with TOC ($P = 0.002$) and negatively correlated with GPx ($P = 0.04$).

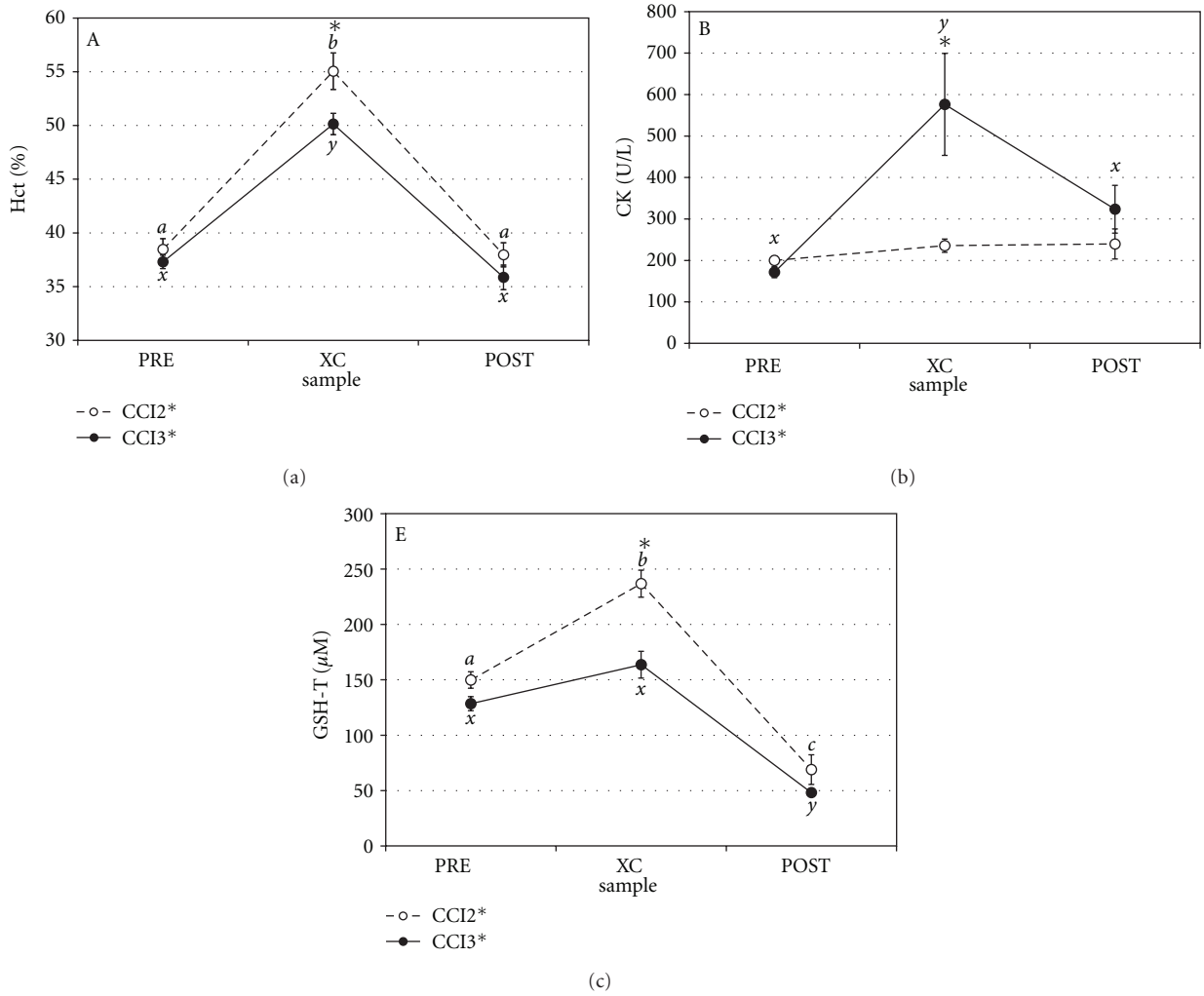

Figure 1: Hematocrit (Hct; (a)), serum creatine kinase (CK; (b)), and red blood cell total glutathione (GSH-T; (c)) in CCI2* and CCI3* horses before competition (PRE), 20–30 min after cross-country (XC), and 18–24 hr after XC but before stadium jumping (POST). *denotes difference between CCI2* and CCI3* horses within sampling time ($P < 0.001$). Letters a, b, and c denote differences across sampling time for CCI2* horses ($P < 0.05$). Letters *x*, *y*, and *z* denote differences across sampling time for CCI3* horses ($P < 0.05$).

Table 3: Blood variables assessed before the competition (PRE), 20–30 min after cross-country jumping (XC), and 18–24 h after cross-country jumping (POST) for horses competing in the CCI2* and CCI3* divisions of the 2006 Jersey fresh three-day event[a].

Variable[b]	PRE	XC	POST
Plasma TP, g/dL	7.01 ± 0.06^{x}	7.31 ± 0.07^{y}	6.76 ± 0.07^{z}
Plasma CORT, ug/dL	5.47 ± 0.43^{x}	11.1 ± 0.70^{y}	7.55 ± 0.36^{z}
Plasma AST, U/L	353.2 ± 33.8^{x}	413.4 ± 34.0^{y}	424.1 ± 36.2^{y}
Plasma LAC, mmol/L	0.82 ± 0.04^{x}	11.2 ± 1.60^{y}	0.62 ± 0.03^{x}
Plasma TOC, ug/mL	5.50 ± 0.28	5.50 ± 0.26	5.75 ± 0.33
Plasma RET, ng/mL	163.4 ± 4.6^{x}	177.0 ± 5.7^{y}	152.9 ± 4.4^{z}
Plasma BC, ng/mL	0.61 ± 0.07^{x}	0.47 ± 0.07^{y}	0.47 ± 0.07^{y}
RBC GPx, mU	260.6 ± 7.7^{x}	308.9 ± 12.8^{y}	254.5 ± 16.2^{x}

[a] For each variable, no difference between divisions was observed; therefore, data was averaged across divisions.
[b] TP: plasma total protein; LAC: plasma lactate; CORT: cortisol; AST: plasma aspartate aminotransferase; TOC: plasma alpha-tocopherol; RET: plasma retinol; BC: plasma beta-carotene; GPx: erythrocyte glutathione peroxidase.
[x,y,z] Within row, means with unlike superscripts differ ($P < 0.05$).

Table 4: Significant correlations between performance and antioxidant blood measures[a,b].

Y	X	R	P	Y	X	R	P	Y	X	R	P
BW	CORT	−0.21	0.02	TP	GPx	0.23	0.02	CORT	GSH-T	0.19	0.04
BW	TP	−0.22	0.03	TP	CORT	0.25	0.002	CORT	CK	0.25	0.007
Hct	RET	0.22	0.03	LAC	GSH-T	0.52	<0.0001	RET	GSH-T	0.40	0.01
Hct	GSH-T	0.66	<0.0001	LAC	GPx	0.19	0.04	TOC	BC	0.40	<0.0001
Hct	GPx	0.27	0.007	LAC	CORT	0.28	0.002	TOC	AST	0.30	0.002
Hct	CORT	0.44	<0.0001	LAC	RET	0.27	0.004	GSH-T	GPx	0.28	0.003
TP	CK	0.25	0.01	CORT	RET	0.28	0.003	GPx	AST	−0.19	0.04
TP	GSH-T	0.33	0.0007	CORT	TOC	−0.19	0.04	CK	AST	0.50	<0.0001

[a] Horse was included in the model to test for significance, if insignificant, then it was removed from the model.
[b] Initial BW (n = 112), hematocrit (Hct; n = 104 total points), plasma total protein (TP; n = 104), lactate (LAC; n = 114), cortisol (CORT; n = 114), alpha-tocopherol (TOC; n = 114), beta-carotene (BC; n = 114), retinol (RET; n = 114), serum creatine kinase (CK; n = 113), asparate aminotransferase (AST; n = 113), erythrocyte total glutathione (GSH-T; n= 114), and glutathione peroxidase (GPx; n = 111).

4. Discussion

The main objective of this study was to determine the effects of a rigorous exercise activity, like a three-day event, on the antioxidant status of horses. To our knowledge, this is the first paper to reveal the antioxidant levels of horses competing in upper level divisions of a three-day event. A companion study studying inflammatory parameters and diet on a similar population of competitive horses was recently published [9]. Overall, our findings suggest that the cross-country jumping phase of a three-day event was a rigorous acute exercise bout that challenged the antioxidant systems of the horse, with more of a challenge being placed on the CCI3* horses. Collectively, the competing horses had higher blood levels of RET, GPx, and GSH-T, but lower BC at XC compared to precompetition (PRE) indicating these antioxidants were mobilized to be used in a response to a challenge to the antioxidant system as a result of the cross-country jumping phase. Specifically within divisions, the CCI3* horses had 2.5 times greater serum concentrations of CK, 1.5 times greater serum concentrations of AST, approximately 50% lower plasma BC, and 70% lower erythrocyte GSH-T, as compared to the CCI2* horses at XC. This might appear to contradict the above statement; however, it might also lead us to believe that the higher level CCI3* horses were more able to handle that level of competition and therefore did not need as much GSH mobilized.

4.1. Time Effects. We found that GSH-T increased at XC in both divisions but decreased below baseline after 18 to 24 hr of recovery. One recent study did look at antioxidant status in horses during a moderate level jumping competition [10]. They found that nonprotein sulfhydryl groups (this group included glutathione) did not increase until 24 h after competition, but did theorize that the response was due to a compensatory reaction to the increase in pro-oxidants produced during exercise. Another study observed a decline in GSH (both total and reduced) within 15 min after a stepwise field exercise test of 7000 m in warmblood eventing horses [11]. The authors theorize the reason why the GSH-T in the current study did not follow the same pattern as in previous studies could be due to the added stress of competition and jumping efforts during competition in combination with galloping at approximately 9.2 to 9.4 m/s. The added stress creates a higher demand for the mobilization of GSH, hereby causing the increase after cross-country followed by a compensatory decline during the recovery period of about 24 hrs.

A study involving endurance horses during a competitive race designed for research purposes evaluated antioxidant status with supplementation of vitamin E alone or in combination with vitamin C [7]. The 27% increase in RBC GPx observed in the last two stages of the 80 km race in both treatment groups likely reflected a response to utilize reduced glutathione during the radical scavenging process (reduced glutathione donates an electron to reduce a wide variety of hydroperoxides using GPx as a catalyst). It also reflected the consumption of pro-oxidants generated during exercise. This same trend was found in the current study with GPx in horses competing in both divisions increasing about 18% over baseline. A similar increase was found with GSH-T in both studies, with horses in our study increasing at XC (CCI2* = 60%; CCI3* = 27%) and with endurance horses in the previous study peaking after the first 21 km and then slowly decreasing to below baseline by the recovery sample [7]. In general, blood and plasma GSH-T reflects recent fluctuations in these concentrations in muscle cells [12, 13]. The typical pattern observed is an increase in concentration after the onset of exercise that continues for a period of time during recovery then drops below baseline by about 18 to 24 hr. This pattern was observed here and in previous studies of horses engaged in intense and endurance exercise [2, 5, 14].

Previous studies on the eventing horse have focused more on performance measures, including plasma LAC and CORT, serum CK, AST, and ion and water loss, rather than on antioxidant status [15–19]. This exercise challenge did elicit many other biochemical changes similar to that observed in other studies on the event horse. These measures are indicators of the rigor or intensity of the exercise being performed. They also help the researcher determine the level of fitness of the horse. Linden et al. [15] found similar blood concentrations as found in the current study of Hct, TP, and LAC in horses before and after the cross-country

phase of a low level event (similar to a CCI1*). The CORT levels found in the current study were about 40 to 70% higher than what was observed in the previous study [15]. The similar LAC concentrations indicate the horses are probably more fit and able to handle the higher intensity of exercise than the lower level of exercise in the previous study. However, the higher CORT concentrations observed here may be due to the fact that our study was conducted on horses exposed to a more rigorous acute exercise and a higher level of competition than in the prior study. This higher concentration of CORT may be indicative of more physiological stress placed on the horses during this high level of competition. This can also be seen with a recently published study using horses subjected to a lower level of jumping competition, where the postexercise sample had two to three times less LAC, CK, and AST concentrations than the CCI3* horses in the current study [10].

4.2. Division Effects. Several studies have investigated horses competing at different levels during three-day events and found varying results in terms of heart rate, temperature, LAC, CK, and AST responses [17–20]. One study investigated CCI1* and CCI2* horses competing in the long-format version of three-day eventing including roads, tracks and steeplechase phases during the same day of competition as the cross-country jumping phase [19]. The LAC, CK, and AST concentrations were similar between divisions, which the authors stated was not expected due to the considerable differences in speeds and distances of the two divisions. The LAC results after cross-country jumping were higher in the current study, despite the samples before exercise being similar in both studies. This could be due to the additional phases needed to complete before cross-country jumping. The current study did not find differences in LAC or AST concentrations between the CCI2* and CCI3* divisions, similar to what was found previously, probably due to the degree of fitness and athletic ability of the higher level horses. As for the CK in the previous study [19], there were no differences found between divisions, which does not agree with the results found in the current study; the CCI3* horses had higher levels of the enzyme after cross-country jumping (2.5 times higher CK than PRE). Also AST was 1.5 times higher numerically with only a trend for a division difference ($P = 0.07$). However, the numerical values were similar between studies. No recovery samples were taken in the previous study [19], so no comparisons can be made; however, it is important to point out that the AST concentrations remained higher in the CCI3* horses 18 to 24 hr after cross-country jumping. Another study looking at CCI2* horses competing either with or without roads, tracks and steeplechase also found no differences between these divisions for LAC, CK, AST, and other electrolyte measures [20]. Overall, these physiological measures are markers of exercise intensity and muscular effort during the exercise; however, they vary with horses physical fitness, age, gender, season of the year, and so forth. The current study shows that these subjects have fallen within wide variation of normal ranges for exercise at this level of intensity despite the differences between divisions.

Division effects were also found with GSH-T. One theory to explain the higher concentration in the CCI2* horses after cross-country jumping is that they were less conditioned or physically fit in order to handle that level of exercise stress compared to CCI3* horses therefore required higher concentrations of antioxidants. However, the CK response was lower for the CCI2* horses, indicating that CCI2* course placed a lower demand on the muscle cells of those horses compared to CCI3* horses. In a study looking at endurance races of varying intensity, there was higher GSH-T in the horses competing in the more intense endurance race, which is the opposite of what was found in the current study [21]. However, the endurance races did take place during different years and had drastically different environmental conditions; the more intense race had hotter and more humid ambient conditions could also have contributed to the higher GSH-T concentrations. The horses competing in the more intense endurance race also had higher CK concentrations, which reflect what we observed in the present study. Another theory for the higher concentrations of GSH-T in the CCI2* horses as compared to the CCI3* horses could also be due to dietary effects. Diet plays an important role in antioxidant status in humans as well as horses. As mentioned below in more detail, the CCI2* horses were found to be receiving more antioxidant supplements and had longer access to fresh green pasture (number one source for antioxidants in horses) than the CCI3* horses. Whether these amounts are enough to definitely increase antioxidant status is unknown, but it is a possibility given the current results.

4.3. Correlations. A positive correlation between serum AST and plasma TOC, like that observed in this study, supports the hypothesis that free radicals produced during exercise change membrane permeability of muscle cells [1]. This same correlation has been observed in previous studies looking at the effects of endurance competition on horses [21, 22]; however, the current study only found TOC to be correlated (positively) with AST. Even though most of the correlations presented could be used to infer that measures of oxidative stress and antioxidant status are a poor predictor of muscle enzyme leakage because it only explains a small amount of the variation, the authors feel that physiologically there was enough relevance to warrant the discussion.

Plasma LAC correlated positively with GSH-T, GPx, RET and CORT, which can be explained by the fact that all these measures peaked at XC and returned or was lower at POST. As for CORT, we found a positive correlation with RET, GSH-T, and CK, which again all increase with exercise, but a negative correlation with TOC. This negative correlation is a little harder to explain physiologically. Due to structural cellular changes during exercise like the increase in hematocrit due to splenic contraction and expulsion of erythrocytes in horses and an increase in muscular mitochondria that uptake additional vitamin E, any change in vitamin E plasma concentrations remains constant because the production anddegradation are overshadowed by the redistribution. The current study did not have an effect of exercise on plasma TOC, which is further evidence against the belief that TOC is released during lipolysis associate with

exercise. Other studies in human subjects have never been shown to be true and leave vitamin E bioavailability factors not well defined [23]. When taking into account the direction of responses, it makes more sense due to the fact that TOC does not increase at the XC sample and return to baseline or lower by POST as with the other measures as mentioned above.

Correlations with GPx and AST in the current study were negative, which is similar to one of the endurance competitions compared in a previous study [21]. However, the previous study in endurance horses found a positive correlation with a second endurance competition compared. These differences in the previous study were thought to be due to the difference in ambient conditions, terrain of the competition and level of difficulty. The present study showed a correlation similar to that of the more intense endurance competition in the previous study [21]. That endurance race was also an early season competition, with more horses that may not have been fully conditioned for the exercise required by the race. This could have been similar to the competition in the present study, which took place in the spring. Explanation of each correlation is difficult due to the transient nature of each marker and the amount of variability in each though worth reporting to share the comparative aspect of the results.

4.4. Diet Effects. Because feeding management of horses could not be controlled prior to competition, the effects of diet on antioxidant status could not be evaluated. However, a nutritional survey was conducted with the results summarized and reported elsewhere [8]. As mentioned earlier, the two most notable findings that could have possibly influenced antioxidant status of horses were the differences in hours of pasture turnout prior to arriving at the competition venue and antioxidant supplement use between CCI2* and CCI3* horses. In the current study, CCI2* horses were turned out for more hours each day prior to the competition compared to CCI3* horses (12.3 ± 1.3 versus 9.1 ± 1.3 hr, resp.). Since pasture is a good source of beta-carotene, the precursor to vitamin A that may be an explanation for why the CCI2* horses had higher BC levels before and after cross-country jumping. Another notable finding was that horses in both divisions received an average of 4 supplements per day and that more CCI2* horses received supplements specifically formulated as antioxidant supplements as compared to CCI3* horses (42.1% versus 8.7%, resp.). Antioxidant supplements fed typically contained vitamins E, C, and selenium, which, due to the antioxidant interaction and recycling, could partially explain the higher concentrations GSH-T in the CCI2* horses as compared to the CCI3* horses. Despite the difference in antioxidant supplements, we did not observe a difference in the number of CCI2* or CCI3* horses fed multi-vitamin and mineral supplements (21.0% versus 21.7%, resp.) or all-in-one supplements (5.3% versus 8.7%, resp.), which often contain vitamin E, C, and selenium as well. Given the horses' good BCS, sound feeding management programs, and supplement administration, it is likely that many of the horses were consuming antioxidants in adequate amounts to combat the oxidative stress experienced as a result of the three-day event. However, if specific recommendations for antioxidant supplementation for three-day event horses were to be developed, the antioxidants fed to the CCI2* horses should be investigated further.

5. Conclusions

In conclusion, the cross-country jumping phase of a three-day event is a challenge to the horse's antioxidant systems, with the CCI3* competitions being a more rigorous exercise bout than the CCI2*. The increase or upregulation of markers of antioxidant status is potentially due to increased scavenging of ROS created by oxidative stress experienced by horses during intense exercise. The ROS could also cause increased muscle membrane permeability as observed by the increase in muscle enzyme concentrations in the blood. Horses that are more fit may be better able to handle the stress of a CCI3* three-day competition, but the increased intensity also places a great deal of stress on the muscle cells more than in lower levels of competition. It is also likely that levels of antioxidants in the diet could enhance the horse's ability to cope with the physical demands of the cross-country jumping phase of a three-day event. The question now becomes how much oxidative stress is too much and how much supplementation is really necessary to combat this stress or if by combating the stress we are disturbing the natural adaptation mechanisms necessary to allow the body to cope with physical and psychological stresses.

Acknowledgments

The authors thank the Jersey Fresh event organizers, J. Cory and L. Mathews, and Technical Delegate, R. Muller, for making this study possible. They also thank the FEI head veterinarian, D. Williamson, DVM., the competitors who allowed us to collect data on their horses during the competition, and the volunteers who helped with data collection. Many thanks are due to Laura Gladney, Program Associate, Rutgers University for her edits.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2012, Article ID 756132, 12 pages
doi:10.1155/2012/756132

Review Article

Oxidants, Antioxidants, and the Beneficial Roles of Exercise-Induced Production of Reactive Species

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Received 16 March 2012; Accepted 2 April 2012

Academic Editor: Michalis G. Nikolaidis

This review offers an overview of the influence of reactive species produced during exercise and their effect on exercise adaptation. Reactive species and free radicals are unstable molecules that oxidize other molecules in order to become stable. Although they play important roles in our body, they can also lead to oxidative stress impairing diverse cellular functions. During exercise, reactive species can be produced mainly, but not exclusively, by the following mechanisms: electron leak at the mitochondrial electron transport chain, ischemia/reperfusion and activation of endothelial xanthine oxidase, inflammatory response, and autooxidation of catecholamines. Chronic exercise also leads to the upregulation of the body's antioxidant defence mechanism, which helps minimize the oxidative stress that may occur after an acute bout of exercise. Recent studies show a beneficial role of the reactive species, produced during a bout of exercise, that lead to important training adaptations: angiogenesis, mitochondria biogenesis, and muscle hypertrophy. The adaptations occur depending on the mechanic, and consequently biochemical, stimulus within the muscle. This is a new area of study that promises important findings in the sphere of molecular and cellular mechanisms involved in the relationship between oxidative stress and exercise.

1. Introduction

In recent years, there has been major progress in the redox biology of exercise making this review paper highly relevant to the area of Sport Science. Studies have shown that physical exercise increases the production of reactive species and free radicals. Although in excess the unbalance between these oxidant molecules and the body's antioxidants can lead to detrimental effects in our organism, reactive species produced during exercise have an essential role in muscle adaptation to exercise, as studies show.

This review starts by explaining the different modes of exercise, and then offers an overview of the exercise-induced production of reactive species. This is followed by a description of the body's antioxidant network, and specific details of enzymatic and nonenzymatic antioxidants are given. The efficacy of dietary antioxidant supplementation is also discussed. Finally, the review presents important findings on how reactive species during exercise lead to favourable exercise-induced adaptations, such as enhancement in the antioxidant enzyme activity, angiogenesis, mitochondria biogenesis, and muscle hypertrophy. Our approach provides a general understanding of the subject and presents interesting new findings; when necessary, reference is made to more specific reviews.

2. Definition of Exercise

Exercise can be defined as any planned structured activity that leads to increase in energy expenditure and heart rate. There are different modes of exercise in relation to intensity (aerobic and anaerobic), to muscle contraction (isometric,

concentric, and eccentric), and to frequency (acute and chronic). It is necessary to explain each of these modes of exercise so the reader can better understand the influence of exercise in reactive species production and its consequence in angiogenesis, hypertrophy, and mitochondria biogenesis.

2.1. Differences between Aerobic and Anaerobic Exercise. The main physiological difference between aerobic and anaerobic exercise is the energy source. On one hand, aerobic exercise can be characterized by the use of aerobic metabolism during the physical effort. In this case, the aerobic metabolism primarily generates energy from fat, and with the use of oxygen it produces energy, without much accumulation of lactic acid in the blood. On the other hand, anaerobic exercise is characterized by short periods of high to maximal efforts when energy is supplied via the anaerobic metabolism, that is, without the use of oxygen, and this results in high accumulation of lactic acid in the blood [1, 2]. Examples of aerobic-endurance exercises are jogging, running, swimming, rowing, and cycling when performed around 50–75% of participants' maximal aerobic capacity ($VO_{2\,max}$) and for relatively prolonged periods of time. Examples of anaerobic exercises are sprints (while swimming, biking, or running), long jumps, competitive weightlifting, or anything lasting up to 2 minutes and performed above the participants' lactate threshold (e.g., >75% $VO_{2\,max}$ for trained individuals).

2.2. Differences between Isometric, Concentric, and Eccentric Exercise. Muscle contraction occurs when the central nervous system transmits a signal to the muscle fibre. Providing that there is enough energy and calcium availability, the muscle fibres generate tension and the muscle may shorten, lengthen, or remain with the same size. Concentric contraction refers to the shortening of the muscle with the production of force. Examples of a concentric exercise includes the curling phase of a situp, where the two ends (origin and insertion) of abdominal muscles are moving closer together, contracting and shortening [1, 3]. Eccentric contraction corresponds to the "stretching" of the muscle from a concentric or static position while tensioned. An example of an eccentric exercise is the down phase of a biceps curl, where the opposite force generated by the dumbbell produces a force greater or equal to the one produced to elongate the two ends of the biceps to the initial position [1]. An isometric exercise consists in a muscle contraction taking place without motion in the affected joints, such as when an athlete holds a weight bell static at 90 degree in a biceps curl exercise.

2.3. Differences between Chronic and Acute Exercise. Acute exercise can be characterised by single bouts of exercise that produce temporary metabolic and cardiovascular responses lasting from few minutes up to several hours. Chronic exercise is when exercise bouts are repeatedly performed inducing a training response, where physiological and metabolic adaptations become more visible and long lasting [4]. Both aerobic and anaerobic exercises may be performed either chronically or acutely.

2.4. Components of Fitness. Components of fitness include the duration, the frequency, the intensity, and the type of activity of a training programme; these variables need to be considered carefully to certify that the aims of the programme are successfully attained. It is important to notice that the training strategies to improve, for example, aerobic performance are different from the ones required for strength fitness improvements, and each activity has specific needs that will influence the use of particular fitness components [1]. The type of sport or activity will dictate which kind of exercise should be incorporated to ensure a perfect improvement in physiological performance. Training aerobically is highly recommended for the development of long-distance running performance, but the same training plan does not apply for competitive weightlifting. Therefore, it is important to define the type of sport before applying the components of fitness in training.

It is also crucial to train at a frequency that will provide a stimulus which brings positive physiological adaptations, and balance it with enough recovery time in order to improve performance. Some activities will require higher frequency than others, compensated with adjustments on the intensity of exercise. Intensity is determined by the training target and the current fitness level of the athlete. This is manipulated, for example, by working at a different percentage from their maximal aerobic capacity (%$VO_{2\,max}$) or repetition maximum (1RM), normally in a progressive way, while the time (duration) of the training session should be controlled in relation to the intensity [1, 3]. Increasing the duration of an exercise session influences the component of fitness and, when well administered, can lead to positive gains in—amongst other aspects—performance.

Different sports and activities will use different energy systems and will recruit different muscle fibres, demanding therefore different training strategies by manipulating the components of fitness. Sprint runners will require higher anaerobic capacity and exercise intensity as well as less exercise bouts compared to long-distance runners. In addition, sports, such as tennis, involve eccentric movements on the lower body, so exercise prescription for that should aim at improving eccentric strength by using similar movements and intensity as the game. It is, therefore, clear that the components of fitness are interconnected and the manipulation of all of them, in a combined manner, is necessary for an improvement in physiological performance [1].

In this review, the effects of aerobic and anaerobic exercise when performed acutely or chronically will be discussed in depth, as will the differences between reactive species production in exercises that use different muscle contraction.

3. Production of Reactive Species during Exercise

3.1. Reactive Species and Free Radicals. Reactive species and free radicals are molecules that, due to their molecular instability (e.g., unpaired electron), promote oxidation reactions with other molecules, such as proteins, lipids, and

DNA, in order to become stabilized [5–7]. Many reactive species are oxygen centred (O_3, H_2O_2, etc.) and are, thus, denominated reactive oxygen species (ROS). Some ROS are also free radicals, such as superoxide anion (O_2^-) and nitric oxide (NO), because they have an unpaired electron [6]. Free radicals and reactive species are essential to our wellbeing, having various regulatory roles in cells. For example, ROS are produced by immune cells—neutrophils and macrophages—during the process of respiratory burst in order to eliminate antigens [8]. They also serve as stimulating signals of several genes which encode transcription factors, differentiation, and development as well as stimulating cell-cell adhesion, cell signaling, involvement in vasoregulation and fibroblast proliferation, and increased expression of antioxidant enzymes [9, 10]. The latter example is observed when individuals perform chronic exercise and will be further discussed in Section 4.

Our body has an elaborate network of antioxidants that acts as a defence system neutralizing free radicals and reactive species. This process allows for the maintenance of homeostasis. Nevertheless, oxidative stress and impaired cellular function may occur if there is an exacerbated increase in the body's oxidant concentrations, overwhelming the available antioxidants. It may also occur if there is depletion in the available antioxidants due to disease or poor diet [11]. In fact, chronic oxidative stress has been suggested as being the cause or consequence of many acute and chronic human diseases [7, 12, 13], for example, obesity, cardiovascular diseases, cancer, acute lung injury, and multiple sclerosis.

Directly measuring the production of free radicals and reactive species is very difficult due to their high reactivity and low steady-state concentration. Nevertheless, the electron spin resonance (ESR) technique is a direct method of detection of species that have an unpaired electron, generally meaning that it is a free radical [14]. However, this technique, and a couple of other direct measurements, require very expensive equipment and have complicated methods. Thus, for the assessment of oxidative stress indirect methods are mainly used. For the measurement of oxidative stress biomarkers in human muscle tissue, expired air, urine, blood, and nasal lavage can be used [15–19]. According to Powers and Jackson [10], reliable markers of this process need to have the following characteristics: be chemically unique, be chemically detectable and have relatively long half-lives, increase or decrease during the oxidative stress process, and not be affected by other cell cycles in order to avoid confounding factors. In addition, oxidative stress is also commonly measured by analyzing the shift in the body's antioxidant system and the activity of specific antioxidant enzymes [5].

3.2. Exercise-Induced Oxidative Stress. Under basal conditions the skeletal muscle produces superoxide anions and NO at a low rate. However, during contractile activity, this rate is drastically increased. In fact, aerobic exercise is associated with an increase in oxygen uptake both by the whole body and especially by the contracting muscle group. Sen [20] reported an increase of 10–15-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 aerobic exercise.

Although the direct evidence for ROS production during exercise is limited [14, 21, 22], mainly due to limitations of the methods used, there is an abundance of the literature providing indirect support that oxidative stress might occur during aerobic and anaerobic exercise (for review on exercise-induced oxidative stress, see Fisher-Wellman and Bloomer [5]). When analysing the available literature, there are some different findings regarding the oxidative-stress rates as the result of exercise performance. This is comprehensible because a variety of factors can influence the oxidative rate, such as the muscle groups recruited, the modes of contraction, the exercise intensity, the exercise duration, and the exercising population. Therefore, the exercise models used in studies deserve critical evaluation.

3.3. Mechanisms of Increased Free-Radical Production with Exercise. The causes of increased free radical and ROS production during exercise have not been totally clarified. Although various mechanisms have been identified, there is still a lack of understanding of how each one of them contributes to the total amount of oxidative stress produced. In addition, these mechanisms may act synergistically, and different types of exercise probably elicit different pathways of free radical production [23]. For instance, although the general consensus is that, during exercise, reactive species production occurs mainly by contracting muscle (skeletal and heart), other mechanisms, such as, inflammatory processes and increased release of catecholamine, that may occur with exercise, also play an important role in the generation of reactive species. The main mechanisms for reactive species production are described below.

Electron Leak at the Mitochondrial Electron Transport Chain. This theory has led to the interpretation that a substantial increased free radical generation is to be expected during exercise due to a "leak" of electrons in the respiratory chain in the mitochondrial inner membrane of the contracting muscle cells. This would occur due to the inadequate coupling of the electron transfer between the complexes I and III (Figure 1) [23, 24]. It seems that complex I—namely, the iron-sulfur clusters—releases the reactive oxygen species—superoxide anion—only towards the mitochondrial matrix, whereas complex III—the ubiquinol oxidation site—releases superoxide into both matrix and outside the inner membrane [24, 25]. Despite this theoretical appeal, there is little direct evidence that mitochondrial superoxide anion production is increased during exercise. In addition, there are studies with isometric exercise where the oxygen pressure (PO_2) in the mitochondria was proven to be low but still demonstrated an increase in the oxidative stress [26]. Bailey et al. [21] also demonstrated that free radical outflow of a contracting muscle was associated with decreases in intracellular PO_2 rather than with conditions of increased oxygen flux. These studies reinforce the fact that it is unlikely that an increase in mitochondrial oxygen flux is the only,

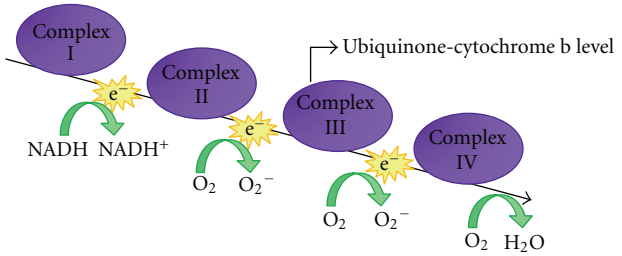

Figure 1: The mitochondrial respiratory chain. Electrons are transferred from complexes I, II, and III to IV. However, inadequate coupling of electron transfer can cause leakage, generating superoxide anions at different complex levels.

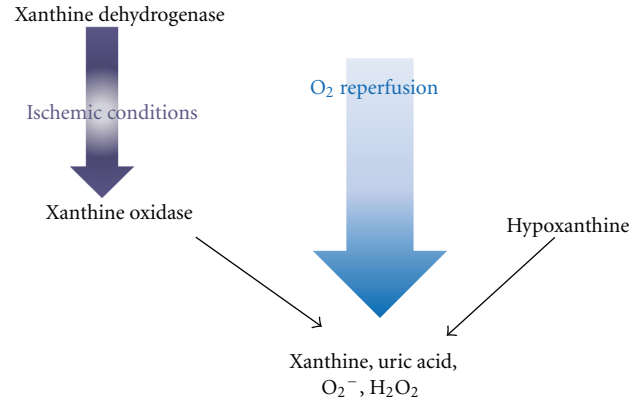

Figure 2: A suggested mechanism for the production of free radicals upon reoxygenation of ischemic or hypoxic tissues.

or main, cause for increases in radical production during exercise [23].

Ischemia Reperfusion and Activation of Endothelial Xanthine Oxidase. The enzyme xanthine oxidase is known to be involved in the pathophysiology of the ischemia-reperfusion syndrome and can lead to tissue damage that may occur after an exhaustive bout of exercise [27]. The following mechanisms describe this process. During exercise, blood flow is shunted from many organs and tissues and redirected to the working muscles; this ischemic condition triggers the conversion of the enzyme xanthine dehydrogenase to xanthine oxidase; when the exercise ceases and the tissues are reoxygenized, xanthine oxidase produces superoxide (O_2^-) and H_2O_2 as byproducts of the degradation of hypoxanthine into xanthine and subsequently into uric acid [7], Figure 2. Although this has been shown to happen in few studies, more research is necessary to determine the role that endothelial xanthine oxidase plays in exercise-induced ROS production in humans.

Neutrophils and the Inflammatory Response. As a consequence of an exercise bout, tissue damage or an increase in the inflammatory cell pool may lead to a rise in reactive species production from nonmuscle source. When neutrophils or other phagocytic cells are activated, they release ROS. Despite the fact that this inflammatory response is critical to the removal of damaged proteins and infections, ROS and other oxidants released from these cells can also cause secondary damage, such as lipid peroxidation. Exercise can elicit muscle injury accompanied by the activation of neutrophils [28]. Bøyum et al. [29] showed not only an increase in neutrophil number following aerobic exercise (cycling for 65 min at 75% $VO_{2\,max}$) but also an increase in their respiratory burst activity measured as chemiluminescence. An increase in neutrophil counts has also been described in short duration (less than 20 min) resistance exercise [30]. This reinforces the fact that there is an increase in plasma neutrophils even when oxygen consumption during physical activity is only moderately increased.

NADPH Oxidase Structure. The reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex was originally identified and characterized in phagocytes, where it plays an essential role in nonspecific host defense against microbial organisms [31]. Nevertheless, this complex is also found at many sites in skeletal muscle, cellular endothelium, and plasma membrane [10]. NADPH oxidase is normally quiescent, but, when it becomes activated, during muscle contraction or when recruited for antimicrobial and proinflammatory events, it can generate large amounts of $O_2^{\bullet -}$ that can be converted in H_2O_2 by the antioxidant superoxide dismutase [31].

Autooxidation of Catecholamines. Adrenaline, noradrenaline, and dopamine are often referred to collectively as catecholamines. With exercise, there is an increase in the plasma concentration of these substances [32]. The oxidation of catecholamines can produce superoxide anion, H_2O_2, and other nonoxygen-derived species in a complicated series of reactions. This can lead to a depletion of cellular antioxidant concentration in the blood, such as glutathione, altering the redox (oxidation-reduction) balance [7, 10].

Despite the need for a better comprehension of how these mechanisms of ROS generation interact, the mechanism by which they affect the function of exercising muscles is well established. At rest, the muscles' low concentration of ROS is critical for force generation. During muscle contractile activity there is a rise in ROS production. On one hand, production of reactive species during both aerobic and anaerobic nonexhaustive exercise has been shown to be important for the adaptation of the muscle fibres [33]—this process will be discussed in Section 5. Nevertheless, during strenuous exercise, the production of ROS can be higher than the buffering capacity of the antioxidants of the muscles. As ROS accumulates in the contracting muscles, the oxidation of proteins and lipids might cause, amongst other things, inhibition in force production, contributing to the development of acute fatigue [10, 34]. In addition, this exaggerated increase in ROS levels in response to strenuous exercise can also lead to oxidative DNA modification, inhibit locomotory and bactericidal activity of neutrophils, reduce the proliferation of T lymphocytes and B lymphocytes,

inhibit natural killer cells, damage cell membrane, and other cellular compounds [35, 36].

4. The Antioxidant System and Exercise

Due to the potential role that reactive species and free radicals have in lipid, protein, and DNA damage, it is not surprising that a network of antioxidant defense mechanism is present in the body. In general, antioxidants are often reducing agents, which exist both intracellularly and extracellularly and have the capacity to react with free radicals and reactive species, minimizing their actions and, thus, delaying or preventing oxidative stress [10].

Antioxidants can be both synthesized *in vivo* and absorbed through diet. They can be divided into two groups: enzymatic and nonenzymatic. The main enzymatic antioxidants include superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT). Each of these enzymes is responsible for the reduction of a different ROS, and they are located in different cellular compartments. (1) SOD: there are 3 isoforms of this antioxidant, two of them are present within cells, whereas the other one is located in the extracellular space. Specifically in skeletal muscle cells, the highest percentage of SOD (65–85%) is found in the cytosol, and the remaining (15–35%) is present in the mitochondria of the muscles. SOD catalyses the reaction of superoxide radicals into oxygen and hydrogen peroxides (H_2O_2). (2) GPX: located in both the cytosol and the mitochondria of cells, it is responsible for the removal of a wide range of hydroperoxides—from complex organic hydroperoxides to H_2O_2—thus, it may protect membrane lipids, proteins, and nucleic acids from oxidation. GPX is also present in muscle cells, but its activity varies depending on the muscle fibre type, with the greatest activity present in slow twitch muscle fibres (type I) which have higher oxidative capacity. (3) CAT: it is extensively distributed within the cells, and its main function is to degrade H_2O_2 into H_2O and O_2. Nevertheless, it has a lower affinity for H_2O_2 compared with GPX. Similarly to the latter, CAT can be found in higher concentration in type I muscle fibres (for further details on these enzymes, refer to Powers and Jackson [10]).

The nonenzymatic antioxidant group includes glutathione, vitamin C, vitamin E, carotenoids, uric acid, and others. Similarly to the enzymatic antioxidants, these are present in different cellular compartments and elicit distinct antioxidant properties which maximize their effectiveness [11]. Below are more details on the nonenzymatic antioxidants—glutathione, vitamin C, and vitamin E.

Glutathione. Reduced glutathione (GSH) is a water-soluble low-molecular-weight tripeptide formed from the amino acids glutamate, cysteine, and glycine. These three amino acids can be obtained from food intake. All types of cells are capable of synthesizing GSH. This synthesis process occurs through two sequential reactions and requires the action of two enzymes: for the first reaction, γ-glutamylcysteine and GSH synthetases for the consecutive reaction (Figure 3). Levels of the produced GSH act as a feedback for the control

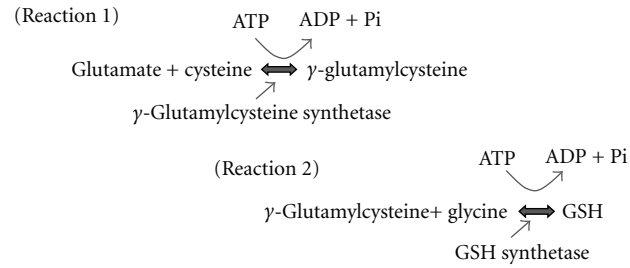

Figure 3: GSH synthesis illustrated by two reactions. Enzymes that catalyze the reactions are γ-glutamylcysteine synthetase and GSH sythetase.

of reaction 1 (see Figure 3), this helps maintain its adequate production [37]. GSH can also be obtained directly from food, but only small amounts of the intact tripeptide are absorbed by the gut; most are broken down into amino acids, which are then used in the synthesis cycle. Thus, a healthy diet with a balanced protein intake is essential for an adequate GSH homeostasis in the body [38, 39].

GSH exerts various essential functions in the body. Amongst these functions is its major antioxidant role. It efficiently scavenges ROS and free radicals preventing an increase in the oxidative stress process. In these reactions, the reduced GSH is oxidized, via the enzyme glutathione peroxidase, to form glutathione disulfide (GSSG). Note that GSSG is formed by two GSH molecules linked via a disulfide bond due to oxidation of the thiol (SH) groups. Once oxidised, GSSG can be reduced back to its original GSH form by the enzyme GSSG reductase and nicotinamide adenine dinucleotide phosphate (NADPH). Nevertheless, when there is a high level of oxidative stress, NADPH becomes depleted and there is an intracellular accumulation of GSSG. This excess GSSG can either be exported out of the cell or it can form a mixed disulfide. Despite what happens to the oxidized glutathione, depletion of cellular GSH can be observed when an intense oxidative stress process occurs [40].

Measuring the plasma level of GSH or its oxidized form (GSSG) is a widely accepted method of detecting oxidative stress and can be reported as redox potential, GSH or GSSG concentration, or GSH/GSSG ratio. It is not only a good indicator of systemic oxidative status but also a useful indicator of disease risk [41], and, therefore, it has been used in various studies to indicate the free radical production during exercise [19, 42–44]. However, these studies do present some divergent results which could possibly be explained by the difference in subject fitness, exercise protocol, and method of determining the glutathione concentration.

Vitamin C. It is a water-soluble vitamin and refers to both ascorbic acid and dehydroascorbic acid (DHA). Ascorbic acid is the main form of the vitamin found *in vivo*. This vitamin, also referred to as ascorbate, is found in relatively high levels in different tissues throughout the body. Ascorbate has clearly been shown to play an essential role in connective tissue biosynthesis, and its deficiency results in scurvy, a disease which leads to the deterioration of collagen

production and results in fragile blood vessels and impaired lesion healing. This disease is reversible once the individual restarts the ingestion of ascorbate. Vitamin C is also a strong reducing agent, due to its facility in donating electrons, with important antioxidant properties [45]. It can inactivate a variety of reactive species minimizing damage to body tissues.

During oxidation reactions, only small amounts of ascorbate are lost because, once it is oxidized, it can be reduced back to ascorbic acid by reductants such as glutathione, nicotinamide adenine dinucleotide (NADH), and NADPH. Similarly, vitamin C is also known to regenerate other antioxidants, such as vitamin E and glutathione, back to their reducing state; thus, maintaining a balanced network of antioxidants [46].

When ingested, vitamin C is absorbed in the intestines either by active transport, if the availability of the vitamin is low, or by simple diffusion if it is present in high concentrations. If ingested in excess, it is degraded in the intestines and can cause diarrhea and intestinal discomfort. Besides the absorption and degrading mechanisms occurring in the intestines, the kidneys are also responsible for conserving or eliminating unmetabolized ascorbic acid to help maintain the body's ascorbic acid balance [45].

Vitamin E. Is a lipid-soluble vitamin also referred to as α-tocopherol. There are a variety of vitamin E molecules that differ in structure. The various forms of this vitamin differ significantly in their metabolic functions and bioavailability. In humans, over 90% of vitamin E encountered in the body is α-tocopherol. However, not all the α-tocopherol forms are maintained in the plasma. The α-tocopherol, which is encountered naturally in food together with synthetic forms of the α-tocopherol (the isomers RRR-, RSR-, RRS-, and RSS-α-tocopherol), can be maintained in the human plasma and tissues. When ingesting α-tocopherol supplements, the RRR-α-tocopherol form is preferable [45, 47].

This vitamin has been shown to have beneficial effects in relation to some diseases, it has also been associated with a decreased risk in cardiovascular disease, and it can help slow the progress of degenerative diseases, such as atherosclerosis. In contrast, in patients suffering from impairment in intestinal fat absorption, α-tocopherol deficiency has been associated with neuronal degeneration [48]. Nevertheless, a recent review study [49], which thoroughly analyzes over 47 antioxidant supplementation research papers on all-cause mortality, has unexpectedly shown the negative effect of some supplements. Vitamin E, for example was one of the antioxidants that was associated with an increase in mortality, while Vitamin C did not show a similar effect. It is difficult to determine the specific biochemical and physiological mechanisms that may have led to this result. More investigation is, therefore, necessary to answer questions that such research generates. In addition, these results concerning synthetic antioxidants should not be transferred to effects that fruit and vegetables have on human health.

Similarly to vitamin C, vitamin E has important antioxidant properties. Due to its capacity for scavenging ROS and free radicals, particularly peroxyl radical ($ROO^{\bullet}$), it exerts the important function of protecting cellular membranes and plasma lipoproteins against lipid peroxidation. This is possible because vitamin E has a great affinity for reducing peroxyl radicals, preventing their interaction with the membrane phospholipids or lipoproteins [45]. Indeed, vitamin E can be considered one of the major inhibitors of lipid peroxidation *in vivo* [7]. Once oxidized, it can be regenerated back to its reduced state by vitamin C, as mentioned previously. Nevertheless, research shows that increased levels of α-tocopherol radicals that are not converted back to the reduced form can act as prooxidants, initiating oxidative stress processes by themselves. In addition, an environment that lacks other antioxidants or that has a high level of oxidative stress will contribute to vitamin E acting as a prooxidant [46].

The evidence that exercise leads to an increase in free radicals and reactive species has led researchers to analyse the efficacy of dietary antioxidant supplementation in order to attenuate the muscle oxidative stress generation and thus improve muscular performance and immune function. An example that can be mentioned is the supplementation with vitamin E. As mentioned at the start of this section, vitamin E has been found to protect cellular membranes from lipid peroxidation. Hence, it is logical to assume that this vitamin could protect muscle cells against exercise-induced damage. Early studies, analyzing the effects of vitamin E supplementation and exercise, investigated its effect on performance. Most of the studies, however, report no benefit of vitamin E neither for muscle strength nor for endurance performance [50]. Furthermore, it has been hypothesized that vitamin E supplementation could have a protective effect against the contraction-induced muscle damage oxidative stress that may occur after an intense exercise bout. This rationale is based on the knowledge that this vitamin can stabilize muscle membranes by interacting with its phospholipids which would, this way, provide some protection against the increase in oxidative stress or muscle damage observed after certain types of exercise [50]. Yet results of the various studies have been quite contradictory, and it is not within the scope of this review to discuss these issues in depth, for further details refer to the review of Jackson et al. [51].

Interestingly, recent work has shown that the increase in reactive species during exercise leads to favourable exercise-induced adaptations. Pertaining literature documents well that both aerobic and anaerobic training causes an enhancement in the antioxidant enzyme activity in various tissues [33, 52, 53]. This is an adaptation process that happens because the free radicals, produced during muscle contraction, act as signalling molecules. This stimulates the gene expression and, hence, increases production of antioxidant enzymes and modulates other oxidative stress protection pathways, such as enhancing the activity of DNA repair enzymes in skeletal muscles [33, 54]. This strengthens the body's antioxidant network system which, consequently, minimizes the oxidative stress process [33]. This stimuli

associated with enhanced antioxidant protection occurs not only in the muscles but also systemically so vital organs, such as liver and brain, also go through this beneficial adjustment [55].

This adaptation process, resultant from regular exercise stimulus, can be explained by the hormesis theory. This theory postulates that chemicals and toxic substances may have a low-dose stimulation high-dose inhibitory effect. That means they can provide positive responses when present in small amount [56]. In this regard, the type and duration of training are key for a significant upregulation of the endogenous antioxidants with long-duration high-intensity endurance training being more effective [57]. Niess et al. [58] reported that trained individuals presented less DNA damage after an exhaustive bout of exercise compared to untrained men. Other evidence for this training adaptation was reported by Miyazaki et al. [59], which showed that free radical production was reduced after 12 weeks of endurance training. More specifically, they reported a decrease in the neutrophil superoxide anion production and attenuation in the lipid peroxidation process. The contrary is also true as sedentarism not only reduces various physiological functions but also decreases the body's oxidative stress protection mechanisms. Consequently, there may be an increase in the occurrence of oxidative stress associated with diseases such as cancer, atherosclerosis, cardiovascular, and neurodegenerative diseases [55].

5. Does ROS Play an Important Role in Exercise-Induced Adaptation Response?

As previously mentioned, oxidative stress is often used to indicate a condition in which accumulation of reactive species has damaging effects in many tissues and organs [7]. However, emerging pieces of evidence have shown that oxidative stress plays a critical role in muscle homeostasis and exercise metabolism of skeletal muscle. This suggests that reactive species are not merely damaging agents inflicting random destruction to cell structures and functions, but that they are also—at least within physiological concentration—useful signalling molecules that regulate growth, proliferation, differentiation, and may be responsible for some adaptations in exercised tissue and in nonexercised tissues, such as the brain [55, 60]. Therefore, physical exercise induces acute increased production of reactive species in skeletal muscle, which can work as intracellular signaling molecules [61, 62]. But, how do reactive species act as physiological signaling molecules in exercise-induced remodeling tissue and who are the most important players in this game?

5.1. Angiogenesis. Angiogenesis is the process through which activated endothelial cells branch out from an existing capillary [63]. The growth of new blood vessels is an important natural process required for healing wounds and for restoring blood flow to tissues after injury or insult [64]. It is known that endurance training induces angiogenesis responses on muscle tissue [63]. For endurance athletes, this adaptation is very important due to the increased need of oxygen supply to all muscle mass. The process of angiogenesis is controlled by a number of mediators that are released in the tissues surrounding the small vessels [64]. Physical exercise can activate this process contributing with the release of some mediators. Significant variation (physiological or physiopathological) in mechanical forces that occur *in vivo* cannot be countered by acute regulation of vessel wall diameter, leading to phenotypical modulation of the endothelial cells and vascular cells, producing structural modifications of the arterial wall. As such, vascular remodeling is a fundamental basis of normal vessel growth and exercise-induced adaptation.

Recently, the role of ROS in high flow-induced vascular remodeling was demonstrated in a mouse model of artery-jugular vein (AVF) where ROS production was enhanced in arteries exposed to chronic high flow, both 1 and 3 weeks after opening of the AVF [65]. NADPH oxidase is identified as the major generator of shear stress-induced ROS in the AVF vascular wall. Generation of ROS and NO, derived from endothelial oxide nitric synthase (eNOS) activation, led to matrix metalloproteinases (MMP) activation, in AVF, and modulated flow-induced vascular enlargement. MMPs collectively cleave most, if not all, of the constituents of the extracellular matrix. Observations made in p47phox$^{-/-}$ mice and eNOS$^{-/-}$ mice also provided direct evidence that endogenous ROS and NO modulated not only the activity of MMPs but also their production in arteries exposed to elevated blood flow. Lehoux [66] showed that long-term structural adaptation to altered blood flow is mediated by ROS. In addition, Lehoux et al. [67] demonstrated that high intraluminal pressure-induced MMP-9 in carotid arteries contributed to increased vessel distensibility. This factor points to the role of matrix degrading enzymes in the early stages of vascular remodeling.

As is well known, the key to angiogenesis is the vascular endothelial growth factor (VEGF), but the real relationship between exercise and angiogenesis is not completely understood. Initial studies on this topic were conducted in skeletal muscle. An upregulation of VEGF, following chronic muscle stimulation or a single bout of moderately intense treadmill running, occurs in the skeletal muscle of rats [68]. In the case of treadmill running VEGF mRNA was upregulated in the active muscle of rats approximately 2- to 4-fold by the end of the exercise bout and remained elevated for the following 4 h. Eight hours after exercise, the VEGF mRNA levels had returned to that of resting muscle.

According to Gustafsson et al. [69], upregulation of VEGF mRNA also occurs during exercise in healthy humans. Similarly, exercise upregulates other elements that are important in the angiogenesis process, for example, the mRNA of the VEGF receptors 1 and 2 (VEGFR1 and VEGFR2 resp.) are increased after muscle activity [70]. Therefore, it seems that aerobic exercise can amplify the angiogenic cascade.

Another important and recently investigated player in the angiogenesis exercise-induced process is PGC-1α (peroxisome-proliferator-activated receptor-c coactivator-1α). Arany et al. [71] have shown that the PGC-1α is a potent metabolic sensor and regulator, induced by a lack of nutrients and oxygen. In addition, PGC-1α powerfully

regulates VEGF expression and angiogenesis in cultured muscle cells and skeletal muscle *in vivo*. Exercise can induce a temporary ischemia in muscular tissue [72]. Ischemia leads to a profound metabolic challenge with potentially catastrophic consequences. For example, PGC-1$\alpha^{-/-}$ mice showed a striking failure to reconstitute blood flow to the limb, in a normal manner, after an ischemic insult, whereas transgenic expression of PGC-1α in skeletal muscle is protective.

West et al. [73] described a novel mechanism of angiogenesis that is independent of hypoxia-triggered VEGF expression. The products of lipid peroxidation are generated as a consequence of oxidative stress and are recognized by toll-like receptors (TLR) promoting angiogenesis *in vivo*, thereby, contributing to accelerate wound healing and tissue recovery. Capillarity in active skeletal muscle is significantly increased by endurance exercise training, and, according to Bloor [63], any increase in muscle capillarity is important in improving blood-tissue exchange properties. This is because a greater capillary network would (1) increase the surface area for diffusion, (2) shorten the average diffusion path length within the muscle, and (3) increase the length of time for diffusive exchange between blood and tissue. Therefore, the persistence of newly formed vasculature in an inflammatory context leads to the maintenance of tissue remodeling. More studies are necessary to define the relationship of immune cells and other molecules and receptors, such as VEGF, PGC1-α, and TLR2, during the neovasculature process in response to exercise. This would be of relevance for creating training strategies for optimal tissue repair.

5.2. Mitochondrial Biogenesis. Regular aerobic training produces an adaptation in skeletal muscle termed mitochondrial biogenesis [74], that is, increase in the number and size of mitochondria [75]. The mechanism behind this adaptation is not clear, but recent studies suggest a possible role for ROS in this process. Although considerable focus has been placed on the damage created by production of ROS, it is also known that ROS can activate signaling pathways involved in phenotypic adaptations. Researches demonstrate that ROS induce mitochondrial network branching and elongation. Pesce et al. [76] showed that mtDNA copy number increased with rising levels of ROS in aging skeletal muscle, and this increase, in mtDNA, was accompanied by an induction in mitochondrial mass. This response appeared to be mediated by PGC-1α and nuclear respiratory factor 1 (NRF-1) since the expression of both increased following exogenous ROS treatment [77]. Recently, Irrcher et al. [78] demonstrated that ROS can lead to an increase in PGC-1α promoter activity and expression via both adenosine monophosphate-activated protein kinase-dependent (AMPK) and AMPK-independent pathways. These pathways most likely account, in part, for the increase in mitochondrial biogenesis observed in the presence of ROS.

5.3. Skeletal Muscle Hypertrophy. The literature suggests that reactive species have important roles in the regulation of cell signaling and changes in gene expression [79, 80], contributing, amongst other processes, to the control of skeletal muscle size. *In vitro* evidence indicates that ROS are capable of affecting the efficiency of muscular tissue differentiation and successful differentiation of satellite-derived myoblasts (progenitor cells that originate muscle cells) into functioning. Integrated myotubes is a fundamental prerequisite for muscle regeneration, a repair process which is of primary importance in maintaining muscle function [61].

ROS modulate the signaling of various growth factors via redox regulation. However, the role of ROS in insulin-like growth factor-1 (IGF-1) signaling is not fully understood. IGF-1 is a hormone with a similar molecular structure to insulin. It is a very important player in cell proliferation, differentiation, and survival, performing various tissue-specific functions [81]. IGF-1 induces myocyte (also known as a muscle cell) hypertrophy, particularly in skeletal muscle [82]. *In vivo*, muscle-specific transgenic mice overexpressing IGF-1 exhibit increased muscle mass, and mice specifically lacking IGF-1 receptor (IGF-IR) in their muscles have smaller muscles and reduced number of myofibers [83]. Hadayaningsih and coworkers [84] recently investigated whether ROS regulate the signaling and biological action of IGF-1 in C2C12 myocytes in mice. They found that IGF-1 induces ROS in C2C12 myocytes; while treatment with H_2O_2 significantly enhanced IGF-I-induced phosphorylation of the IGF-1 receptor (IGF-1R), phosphorylation was markedly attenuated when cells were treated with antioxidants. The downstream signaling pathway Akt/mTOR-p70S6K (a well-known hypertrophic pathway) was subsequently downregulated, and, furthermore, blocking Nox4—which is reported to produce ROS in insulin signaling—attenuated IGF-1-induced IGF-1R phosphorylation, indicating that Nox4 is involved in the regulation of IGF-1 signaling. Importantly, antioxidant treatments inhibited IGF-1-induced myocyte hypertrophy, demonstrating that ROS are necessary for inducing IGF-1 myocyte hypertrophy *in vitro*. These results indicate that reactive species play an essential role in the signaling and the biological action of IGF-1 in C2C12 myocytes, clearly showing that IGF-1 is an essential regulator of myocyte hypertrophy. As strength exercise stimulates skeletal muscle hypertrophy and local IGF-1 expression, this suggests the involvement of IGF-1 in exercise-induced myocyte hypertrophy.

Another molecule that appears to have an important role in the hypertrophic response to specific kind of exercise is interleukin-6 (IL-6). Recent scientific literature identifies IL-6 as an essential regulator of satellite cell-(muscle stem cell-) mediated hypertrophic muscle growth. IL-6 is locally and transiently produced by growing myofibres and associated satellite cells, and genetic loss of IL-6 blunted muscle hypertrophy *in vivo* [85]. Kosmidou et al. [86] have shown that ROS stimulate IL-6 release from skeletal myotubes, therefore, identifying a novel cellular source which could be responsible for the excess amounts of inflammation-responsive cytokine produced in strenuous-exercise models. Their results also suggest that the ROS-stimulated increase in IL-6 release is transcription dependent and involves p38 and

NFκ-B activation. Although p38-MAPK emerges as a pivotal molecule orchestrating sequential events in the myogenic pathway, many details of p38-MAPK-induced myogenesis are yet to be elucidated. Whether different p38 MAPK family members specifically regulate the expression of particular subsets of genes, at different stages of differentiation, and whether they possess inducing or repressing activities are still to be determined [87].

A balance between reactive species production and antioxidant defense systems appears to represent a central key of many adaptive responses in skeletal muscle as well as hypertrophy and atrophy. It looks like, if the production of ROS is ideal (little higher than basal levels), the adaptive response is hypertrophy; but if the levels of ROS are increased many folds above basal levels and antioxidant defense capacity, there is an atrophic response such as Duchenne muscular dystrophy [88].

6. Conclusions

As has been shown, a sophisticated signaling-transcription network within muscle fibres mediates exercise-induced skeletal muscle adaptation, and there is strong evidence that ROS has important role in this scenario. ROS participates in angiogenesis, mitochondrial biogenesis, and hypertrophy that have an important role in exercise prescription and athletic performance. In relation to acute exercise and free radical production, both anaerobic and aerobic exercise may result in augmented production of free radicals, but acute oxidative stress may not always be seen since ROS production is positively dependant of the load (intensity $\times$ duration) of exercise. It has been suggested that high ROS produced by acute bouts of exercise may be detrimental to the immune system; however, chronic exercise produces physiological adaptations capable of upregulating one's antioxidant system. In this regard, the type and duration of the training are key for a significant upregulation of the endogenous antioxidants with long-duration high-intensity endurance training having been shown to be more effective.

7. Future Perspectives

The past 3 decades has brought enormous progress to our understanding of the impact of reactive species on exercise adaptative response. The muscular sources of ROS have been identified, and many of the signaling pathways that are modified by ROS have been studied; however, much remains to be discovered regarding the function of ROS in the specific adaptative response as angiogenesis, hypertrophy, and mitochondrial biogenesis. There is now a better understanding of the regulation of ROS sources during exercise, but that still needs to be further explored. Additional work is necessary to elucidate the specific role of reactive species in migration processes like rolling, adherent, and transmigrating cells; mechanistic adaptation processes need to be investigated. Finally, there are many researchers working to extend our knowledge of the molecular and cellular mechanisms involved in the relationship between oxidative stress and exercise.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2012, Article ID 185867, 14 pages
doi:10.1155/2012/185867

Research Article

The Antioxidant Effects of a Polyphenol-Rich Grape Pomace Extract *In Vitro* Do Not Correspond *In Vivo* Using Exercise as an Oxidant Stimulus

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Received 22 January 2012; Accepted 26 March 2012

Academic Editor: Chad M. Kerksick

Fruits, such as grapes, are essential food of the Mediterranean diet. Grape extracts have potent antioxidant and chemopreventive properties *in vitro*. Numerous studies have examined the effects of plant extract administration on redox status at rest in animals and humans but their results are controversial. However, there are no studies comparing the *in vitro* and *in vivo* effects of plant extracts on oxidative stress using exercise as an oxidant stimulus. Thus, the aim of this study was to investigate whether a polyphenol-rich grape pomace extract of the *Vitis vinifera* species possesses *in vitro* antioxidant properties and to examine whether these properties apply in an *in vivo* model at rest and during exercise. Our findings indicate that the tested extract exhibits potent *in vitro* antioxidant properties because it scavenges the $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radicals and inhibits DNA damage induced by peroxyl and hydroxyl radicals. Administration of the extract in rats generally induced oxidative stress at rest and after exercise whereas exercise performance was not affected. Our findings suggest that the grape pomace extract does not behave with the same way *in vitro* and *in vivo*.

1. Introduction

Reactive oxygen and nitrogen species are involved in physiological processes such as signal transduction [1] and adaptations during exercise [2]. However, when reactive species are excessively produced, they may cause muscle damage [3] and fatigue [4]. Strenuous exercise leads to overproduction of reactive species and consequently to oxidative stress [5–7]. A very important contributor of reactive species during exercise is the enzyme xanthine oxidase [2], which catalyzes the oxidation of hypoxanthine to xanthine to uric acid. Xanthine oxidase uses molecular oxygen as the electron acceptor during purine degradation thereby resulting in superoxide radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) production [8]. The role of xanthine oxidase is dual as it results not only in reactive species production but also in generation of uric acid, one of the most potent antioxidant molecules in plasma [9, 10].

Various studies have examined the antioxidant effects of plant extracts using *in vitro* tests. Their findings have mostly shown that grape extracts are strong free-radical scavengers *in vitro* [11–13]. Apart from grape extracts, it has also been observed that extracts from legumes are potent antioxidant agents *in vitro* [14–16]. Generally, in the vast majority of

the relevant literature, extracts derived from different plants possess antioxidant properties *in vitro* judged by their capacity to scavenge free radicals.

In a number of studies, plant extracts possessing antioxidant properties *in vitro* have been administered in rodents and humans before exercise to examine whether these effects also apply *in vivo*. These studies mainly examined the effects of plant extracts supplementation on oxidative stress in blood and other tissues, yet the findings are controversial. More specifically, it has been demonstrated that administration of several plant extracts protected tissues from exercise induced oxidative stress [17–19]. In contrast, other studies in humans have examined the effects of plant extract administration on redox status at rest and reported antioxidant [20] or prooxidant effects [21, 22]. The effects of the vast majority of plant extracts on redox status are usually attributed to specific compounds they are consisted of. These compounds are polyphenols, which are secondary metabolites of plants. They protect plants against harmful environmental conditions and are divided in two main categories, namely, flavonoids and nonflavonoids [23].

The aforementioned data indicate that the potential antioxidant function of a plant extract *in vivo* cannot be safely extrapolated from *in vitro* tests, since they do not take into account (among others) the metabolic transformations and interactions that clearly affect the bioavailability and biological action of polyphenols. It is a common practice to use antioxidants as a way to enhance exercise performance. In order to make practical recommendations for the use of antioxidants, it is important to use both *in vitro* and *in vivo* models. Research from our laboratory has demonstrated that several grape extracts of the *Vitis vinifera* species possess potent antioxidant properties *in vitro* as they scavenge several free radicals, such as 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$), 2,2′-Azino-bis-(3-ethyl-benzthiazoline-sulphonic acid ($ABTS^{\bullet+}$), superoxide ($O_2^{\bullet-}$), hydroxyl ($OH^{\bullet}$), and peroxyl ($ROO^{\bullet}$) radicals [24–26]. It would be interesting to examine whether the *in vitro* properties of a grape extract also apply to an *in vivo* model, particularly considering the lack of studies in which the effects of the same extract in an *in vitro* and an *in vivo* model in the context of exercise are investigated. Thus, the aim of this study was to examine whether a polyphenol-rich grape pomace extract possesses *in vitro* antioxidant properties and whether the *in vitro* properties of the extract translate to an *in vivo* model when the extract is administered before exhaustive aerobic exercise in rats.

2. Material and Methods

This study is divided in two parts. In the first part, a polyphenol-rich grape pomace extract was examined for its possible antioxidant properties *in vitro* employing several assays. In the second part, the effects of the extract on redox status in an *in vivo* model using exercise as an oxidant stressor were investigated.

2.1. In Vitro Experiment

2.1.1. Preparation of the Grape Pomace Extract. The grape pomace used belongs to the species *Vitis vinifera* and to the variety Batiki Tyrnavou (red grapes grown in Central Greece). The raw material was dried in a shady, well-ventilated place and extracted with ethanol (96%) at 50°C for 4 hours. After filtration, the solvent was evaporated under reduced pressure, and the residue (grape pomace extract) was kept at −20°C by the time of analysis for the investigation of its polyphenolic content.

2.1.2. LC-HRMS Analysis of the Extract. For the characterization of the polyphenols content of the grape pomace extract an LC-HRMS method was developed and applied. For the analysis an Accela LC system (ThermoFinnigan, San Jose, USA) consisted of an HPLC pump, a degasser, an autosampler and a PDA detector were employed. Particularly for the HRMS analysis, an Orbitrap spectrometer (ThermoFinnigan, San Jose, USA) hyphenated to the HPLC-DAD system was used. The orbital trap allowed mass resolution around 30 000 and mass measurement accuracy close to 2 ppm to be achieved. The MS system was equipped with an ESI ionization probe and the analysis was performed in positive and negative mode. A Hypersil GOLD column (Thermo Scientific) (100 × 2.1 mm, 3 μm) was used for the analysis. A fast gradient elution method was developed and applied. The mobile phase used consisted of 0.1% aqueous formic acid (solvent A) and acetonitrile (solvent B), at a flow rate of 400 μL/min, at room temperature. The elution conditions used were initial A-B (95 : 5); in 5 min A-B (90 : 10); in 10 min A-B (80 : 20); in 15 min A-B (70 : 30); in 22 min A-B (50 : 50); in 25 min A-B (40 : 60); in 35 min A-B (5 : 95), hold until 40 min, back to initial conditions in 5 min; equilibration for 10 min. The chromatograms were recorded at 220, 280, and 365 nm by monitoring spectra within a wavelength range of 190–700 nm. Mass spectra were recorded in a range from *m/z* 100 to 1500. The ESI source was operated at a sheath gas flow of 30 arb, auxiliary gas flow of 10 arb, ion spray voltage of 3.5 kV, and a capillary temperature of 40°C. For all the high-accurate *m/z* measurements, the mass tolerance was set to 5 ppm. Measurements outside that range were rejected. Identification of compounds was accomplished by comparing the retention time (Rt), UV spectrum, HRMS, spectra of the peaks in the sample to those of standard compounds (Extrasynthese, Lyon, France). Xcalibur 2.0.7 SP1 software was used for the operation and processing of the data.

2.1.3. Assessment of Extract Total Polyphenol Content. The total polyphenol content of the grape pomace extract was determined using the Folin-Ciocalteu reagent [27]. Folin-Ciocalteu reagent (0.5 mL) and distilled water (5 mL) were added to the sample (0.1 mL). It was incubated for 3 min at room temperature (RT) and was subsequently mixed with 25% w/v solution of sodium carbonate (1.4 mL) and distilled water (3 mL). Following 1 h incubation at RT in the dark, the absorbance was measured at 765 nm. Blank contained Folin-Ciocalteu reagent and distilled water without the extract. The optical density of the sample (0.1 mL) in 25% w/v solution of sodium carbonate (1.4 mL) and distilled water (8 mL) at 765 nm was also measured. The total polyphenol content was determined by a standard curve of absorbance values

in correlation with standard concentrations (0, 50, 150, 250, 500 μg/mL) of gallic acid. The total polyphenol content is presented as mg of gallic acid per g of extract.

2.1.4. DPPH$^\bullet$ Radical Scavenging Assay. Free-radical scavenging capacity of the extract was evaluated using the DPPH$^\bullet$ radical [28]. Briefly, the reaction was carried out in 1 mL of methanol containing freshly made DPPH$^\bullet$ (100 μM) in methanol and the extract at different concentrations (2–50 μg/mL). The contents were vigorously mixed, incubated at RT in the dark for 20 min, and the absorbance was measured at 517 nm. In each experiment, the tested sample alone in methanol was used as blank and DPPH$^\bullet$ alone in methanol was used as control.

2.1.5. ABTS$^{\bullet+}$ Radical Scavenging Assay. ABTS$^{\bullet+}$ radical scavenging capacity of the extract was determined according to Cano et al. [29] with slight modifications. Briefly, the reaction was carried out in 1 mL of distilled water containing ABTS (1 mM), H_2O_2 (30 μM) and horse radish peroxidase (6 μM). The solution was vigorously mixed, incubated at RT in the dark for 45 min until ABTS$^{\bullet+}$ radical formation, and the absorbance was measured at 730 nm. Then, 10 μL of different extract concentrations (2–50 μg/mL) were added in the reaction mixture and the decrease in absorbance at 730 nm was read. In each experiment, the tested sample in distilled water containing ABTS and H_2O_2 was used as blank, and the ABTS$^{\bullet+}$ radical solution with H_2O was used as control.

2.1.6. Calculation of % Radical Scavenging Capacity. The radical scavenging capacity (RSC) of the extract was expressed as the percentage of DPPH$^\bullet$ or ABTS$^{\bullet+}$ elimination calculated according to the following equation:

$$\%\ \text{RSC} = \left[\frac{(\text{Ac} - \text{As})}{(\text{Ac})}\right] \times 100. \quad (1)$$

Ac is the absorbance of control and As is the absorbance of the sample. In order to compare the radical scavenging efficiency of the samples, IC_{50} values were also calculated, expressing the concentration of the extract that scavenges DPPH$^\bullet$ or ABTS$^{\bullet+}$ radical by 50%. All experiments were carried out in triplicate on three separate occasions.

2.1.7. Peroxyl Radical-Induced DNA Strand Scission Assay. The assay was performed using the protocol of Chang et al. [30]. ROO$^\bullet$ were generated from thermal decomposition of 2,2′-azobis(2-amidinopropane hydrochloride, AAPH). The reaction mixture (10 μL), containing Bluescript-SK+ plasmid DNA (1 μg), the extract at different concentrations (1–100 μg/mL), and AAPH (2.5 mM) in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4), was incubated in the dark for 45 min at 37°C. The reaction was terminated by the addition of loading buffer (3 μL, 0.25% bromophenol blue and 30% glycerol) and analyzed in 0.8% agarose gel electrophoresis at 70 V for 1 h. The gels were stained with ethidium bromide (0.5 μg/mL), distained with water, photographed by UV translumination using the Vilber Lourmat photodocumentation system (DP-001.FDC, Torcy, France), and analyzed with Gel-Pro Analyzer version 3.0 (Media Cybernetics, Silver Spring, USA).

2.1.8. Hydroxyl Radical-Induced DNA Strand Scission Assay. OH$^\bullet$-induced plasmid DNA relaxation assay was performed according to the method of Keum et al. [31] with slight modifications. OH$^\bullet$ were generated from UV photolysis of H_2O_2. The reaction mixture (10 μL) was consisted of Bluescript-SK+ plasmid DNA (1 μg), Tris-HCl (10 mM, 1 mM EDTA), the extract at different concentrations (100–1600 μg/mL), and H_2O_2 (40 mM). The reaction mixture was irradiated with a 300 W UV lamp (OSRAM) for 3 min at the distance of 50 cm. The reaction was terminated by the addition of loading buffer (3 μL, 0.25% bromophenol blue and 30% glycerol) and analyzed in gel electrophoresis as described previously. Additionally, Bluescript-SK+ plasmid DNA was also treated with the extract alone at the highest concentration used (1600 μg/mL) in order to test its effects on plasmid DNA conformation.

2.1.9. Inhibition of Free-Radical-Induced DNA Damage. Induction of DNA strand breaks by ROO$^\bullet$ and OH$^\bullet$ was evaluated by the conversion of supercoiled Bluescript-SK+ plasmid double-stranded DNA to open circular conformation analyzed in agarose gel electrophoresis. Preventive activity of the extract was assessed by inhibition of conversion of supercoiled (unnicked) conformation to open circular (nicked). The percentage inhibition of radical-induced DNA strand cleavage by the extract was calculated using the following equation:

$$\%\ \text{inhibition} = \left[\frac{\left(S_p - S\right)}{\left(S_p - S_0\right)}\right] \times 100. \quad (2)$$

S_0 is the percentage of supercoiled conformation in the negative control sample (plasmid DNA alone), S_p is the percentage of supercoiled conformation in the positive control sample (plasmid DNA with the radical initiating factor), and S is the percentage of supercoiled conformation in the sample containing plasmid DNA, the extract, and the radical initiating factor. In order to compare the efficiency of preventive capacity of the extract, IC_{50} value was evaluated showing the concentration needed to inhibit relaxation of supercoiled conformation induced by ROO$^\bullet$ and OH$^\bullet$ by 50%. All experiments were carried out in triplicate on three separate occasions. Bluescript-SK+ plasmid DNA was isolated from a large-scale bacterial culture.

2.2. In Vivo Experiment

2.2.1. Animals. Fourty adult male Wistar rats (9 weeks old, weighing 285 ± 5 g, mean ± SEM) were purchased from the Hellenic Pasteur Institute. Rats were housed under controlled environmental conditions (12-hour light/dark cycle, temperature 18–21°C, humidity 50–70%) in cages of three. Commercial rat chow and tap water were provided *ad libitum*.

Table 1: Polyphenolic composition of the Batiki Tyrnavou grape pomace extract.

Polyphenols	Rt (min)	m/z $[M\pm H]^{\pm}$
Flavan-3-ols		
Catechin	3.83	291.0863 $[M+H]^+$ 289.0707 $[M-H]^-$
Epicatecin	5.93	291.0863 $[M+H]^+$ 289.0707 $[M-H]^-$
Epicatechin-3-gallate	9.25	441.0816 $[M-H]^-$
Anthocyanidins		
Cyanidin	9.25	609.1467 $[M-H]^-$
Malvidin	11.74	331.0812 $[M+H]^+$
Delphinidin	13.55	301.0359 $[M-H]^-$
Petunidin	15.99	315.0516 $[M-H]^-$
Anthocyanins		
Myrtillin	9.53	463.0888 $[M-H]^-$
Kuromanin	10.63	447.0939 $[M-H]^-$
Oenin	6.72	491.1201 $[M-H]^-$
Peonidin-3-O-glucoside	5.76	463.1235 $[M+H]^+$
Phenolic acids		
Gallic acid	0.78	169.0147 $[M-H]^-$
Caftaric acid	26.18	311.0398 $[M-H]^-$
Flavonols		
Quercetin	9.56	301.0357 $[M-H]^-$
Kaempferol	15.79	285.0407 $[M-H]^-$
TPC (total polyphenol content)		648 (mg gallic acid/g extract)

The project was reviewed and approved by the institutional review board and the appropriate state authority.

Rats were randomly divided into four experimental groups as follows: (a) saline-administered and sacrificed 1 h after administration, (b) saline-administered, exercised 1 h after administration and sacrificed immediately after exercise, (c) grape pomace extract-administered and sacrificed 1 h after administration, and (d) grape pomace extract-administered, exercised 1 h after administration and sacrificed immediately after exercise.

2.2.2. Grape Pomace Extract Administration. A single dose of 300 $mg \cdot kg^{-1}$ body weight of grape pomace extract was administered intraperitoneally 1 h before the acute swimming protocol. This was in the range of commonly administered doses of plant extracts in similar experimental protocols [19, 21, 22, 32]. The polyphenolic composition of the tested extract, which is rich in catechin, is presented in Table 1. It has been previously referred to that 1 hour is enough for polyphenols, such as catechin, administered within extracts to reach their maximal concentration in blood [23].

2.2.3. Swimming Familiarization. Rats were allowed to acclimatize for 7 days in the animal facility before the beginning of the exercise protocol. The animals were then familiarized to swimming for a period of five days before the actual swimming protocol was implemented. During the first day of familiarization the rats remained in the water tank for 10 min free of load. The next two days the rats swam for 10 min with a load equal to 1% of their body weight adjusted at the base of their tails. The last two days the load increased to 2% of animal's body weight. Finally, the rats were rested for three days before the swimming protocol took place.

2.2.4. Swimming Protocol. Rats subjected to exercise individually swam until exhaustion in deep water tanks (diameter: 1.0 m, depth: 0.7 m) at a water temperature of 33–36°C, as previously described [5]. Constant load equal to 4% of the rats' body weight was adjusted at the base of their tail in order to achieve uninterrupted swimming. An animal was considered to have reached exhaustion when it was unable to constantly keep its nose out of the water. Swimming was selected as an exercise modality because, unlike treadmill running, it induces minor muscle damage [33]. Thus, any effects of swimming on oxidative stress are only partly attributed to muscle damage, which increases production of reactive species.

2.2.5. Blood and Tissue Collection. Rats were sacrificed by decapitation following short exposure to ether. Blood was collected in EDTA tubes and centrifuged immediately at 1,370 g for 10 min at 4°C to allow plasma isolation. The packed erythrocytes were lysed with 1 : 1 (v/v) distilled water,

inverted vigorously, and centrifuged at 4,020 g for 15 min at 4°C. Tissues were quickly removed and snaped-frozen in liquid nitrogen. Plasma, erythrocyte lysate and tissues were then stored at −80°C until biochemical analysis. In preparation for tissue biochemical analysis, tissue samples were initially ground using mortar and pestle under liquid nitrogen. One part of tissue powder was then homogenized with two parts (weight/volume) of 0.01 M phosphate buffered saline pH 7.4 (138 mM NaCl, 2.7 mM KCl, and 1 mM EDTA) and a cocktail of protease inhibitors (1 μM aprotinin, 1 μg/mL leupeptin and 1 mM PMSF) was added. The homogenate was vigorously vortexed and a brief sonication treatment on ice was applied. The homogenate was then centrifuged at 12,000 g for 30 min at 4°C and the supernatant was collected.

2.2.6. Oxidative Stress Markers. Xanthine oxidase activity, TBARS concentration, protein carbonyls concentration, reduced glutathione (GSH) concentration, catalase activity, and total antioxidant capacity (TAC) were measured as previously described [5, 34]. Each assay was performed in triplicate. Blood and tissue samples were stored in multiple aliquots at −80°C and thawed only once before analysis. All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2.7. Statistics. Data of the *in vitro* experiment were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple pair-wise comparisons. Data of the *in vivo* experiment (oxidative stress markers) were analyzed by two-way (treatment × time) ANOVA. Pairwise comparisons were performed through Bonferroni t-test. Performance data were analyzed using independent Student's t-test. The level of statistical significance was set at $P < 0.05$. All results are expressed as mean ± SEM. Data were analyzed using SPSS, version 13.0 (SPSS Inc., Chicago, Ill).

3. Results

3.1. In Vitro Experiment

3.1.1. LC-HRMS Analysis of the Grape Pomace Extract. The ethanolic extract of grape pomace was analyzed using a fast HPLC-HRMS method, in positive and negative mode. Using purchased standard compounds (purity ≥ 95%), several flavonoids and especially flavan-3-ols, anthocyanins and anthocyanidins, were identified. The well-known flavan-3-ols of wine, catechin, and epicatecin were traced at Rt = 3.83 min, and Rt = 5.93 min, respectively. Both isomeric compounds were detected in positive and negative mode based on their pseudomolecular ions $[M+H]^+$ at m/z 291.0863 and $[M-H]^-$ at m/z 289.0707, respectively. Additionally, epicatechin-3-gallate was identified at Rt = 9.25 min based on its pseudomolecular ion $[M-H]^-$ at m/z 441.0816. Moreover, the anthocyanidins cyanidin, malvidin, delphinidin, and petunidin were detected at 9.25 min, 11.74 min 13.55 min, 15.99 min based on their corresponding pseudomolecular ions $[M-H]^-$ at m/z 609.1467, $[M+H]^+$ at m/z 331.0812, $[M-H]^-$ at m/z 301.0359, and $[M-H]^-$ at m/z 315.0516, respectively. Likewise, the anthocyanins myrtillin, kuromanin, oenin, and peonidin-3-O-glucoside were traced. In particular, Myrtillin was detected mainly in negative mode, where its pseudomolecular ion $[M-H]^-$ at m/z 463.0888 and Rt = 9.53 min was observed. At Rt = 10.63 min, kuromanin was detected through its peudomolecular ion $[M-H]^-$ at m/z 447.0939 while at Rt = 6.72 min oenin was detected through its peudomolecular ion $[M-H]^-$ at m/z 491.1201. The peonidin-3-O-glucoside was also identified based on its pseudomolecular ion $[M+H]^+$ at m/z 463.1235 at Rt = 5.76 min.

Apart from the above-mentioned flavonoids, two phenolic acids, gallic acid (Rt = 0.78 min) and caftaric acid (Rt = 26.18 min), were also identified based on their pseudomolecular ions $[M-H]^-$ at m/z 169.0147 and $[M-H]^-$ at m/z 311.0398, respectively. Finally, the flavonols quercetin at Rt = 9.56 min and kaempferol at Rt = 15.79 min were detected and identified with the same way and in particular based on their peudomolecular ions $[M-H]^-$ at m/z 301.0357 and $[M-H]^-$ at m/z 285.0407. The last four compounds have been previously isolated and identified in our laboratory and their purity, determined by HPLC-DAD analysis is estimated from 86 to 98%. It is important to note that due to the capabilities of Orbitrap analyzer, all the m/z measurements were highly accurate, and specifically the calculated Δm for all the compounds under investigation was found from 0.5 to 3.2 ppm.

3.1.2. Total Polyphenol Content. Total polyphenol content of the extract was evaluated and found equal to 648 mg of gallic acid per g of extract.

3.1.3. Radical Scavenging Capacity of the Extract. The tested extract exerted significant capacity to scavenge the $DPPH^\bullet$ and $ABTS^{\bullet+}$ radicals. The results are expressed as IC_{50} values. The lower the IC_{50} value, the higher the antioxidant capacity of the extract. The IC_{50} data for the $DPPH^\bullet$ and $ABTS^{\bullet+}$ radicals are 25 and 5.5 mg/mL, respectively (Figure 1).

3.1.4. Protective Activity of the Extract against Free Radical-Induced DNA Damage. The tested extract exhibited significant protective activity on DNA. Particularly, it inhibited DNA damage induced by $ROO^\bullet$ and $OH^\bullet$ radicals (Figure 2). The IC_{50} values for $ROO^\bullet$ and $OH^\bullet$ radicals are 1.5 and 500 mg/mL, respectively.

3.2. In Vivo Experiment

3.2.1. Exercise Performance. Swimming performance was measured in 20 animals of the exercised groups. Half of the rats were treated with saline and the other half with the extract. No difference in performance between the saline-treated and extract-treated groups was observed. The swimming time to exhaustion for the saline- and extract-treated animals was 46.1 ± 2.0 and 45.1 ± 1.4 min, respectively.

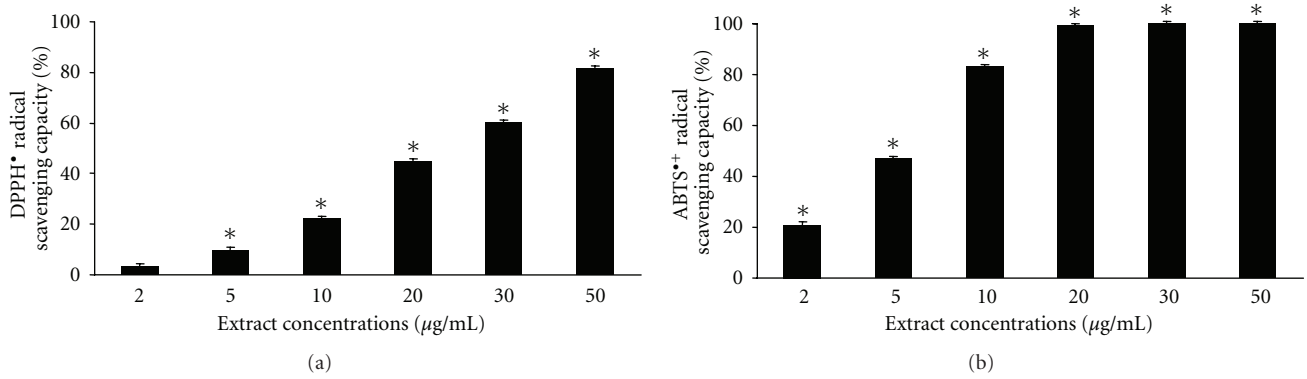

Figure 1: DPPH• and ABTS•+ radical scavenging capacity of the grape pomace extract. *Significantly different from the control value ($P < 0.05$).

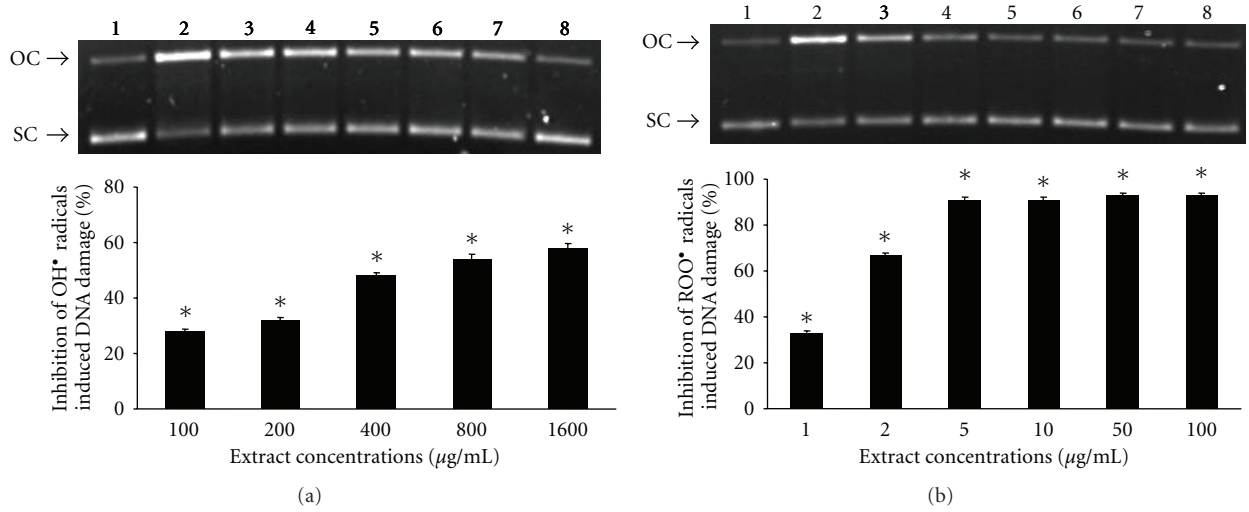

Figure 2: Protective activity of the grape pomace extract on DNA strand scission induced by OH• and ROO•. (a) Extract antioxidant activity against OH•. Bluescript-SK+ plasmid DNA was exposed to UV plus H_2O_2 (lane 2) or to UV plus H_2O_2 in the presence of 100 μg/mL, 200 μg/mL, 400 μg/mL, 800 μg/mL and 1600 μg/mL of the extract, respectively (lanes 3–7) or to 1600 μg/mL of the extract alone (lane 8). (b) Extract antioxidant activity against ROO•. Bluescript-SK+ plasmid DNA was exposed to ROO• alone (lane 2) or to ROO• in the presence of 1 μg/mL, 2 μg/mL, 5 μg/mL, 10 μg/mL, 50 μg/mL and 100 μg/mL of the extract, respectively (lanes 3–8). Lane 1 represents Bluescript-SK+ plasmid DNA without any treatment. *Significantly different from the control value ($P < 0.05$). OC: open circular conformation of the plasmid, SC: supercoiled conformation of the plasmid.

3.2.2. Oxidative Stress Markers

Plasma. In xanthine oxidase (Figure 3(a)), significant main effects of treatment and time were found. In post hoc within-group comparisons, xanthine oxidase activity significantly increased after exercise in the saline-treated group only. In post hoc between-group comparisons, xanthine oxidase activity was significantly lower in the extract group compared to the saline group, both at rest and at postexercise. In TAC (Figure 3(b)), significant main effects of treatment and time were found. In the within-group comparisons, TAC increased after exercise in both saline- and extract-treated groups. In the between-group comparisons, TAC was higher in the extract group compared to the saline group at postexercise only. In protein carbonyls (Figure 3(c)) significant main effects of treatment, time, as well as interaction of treatment × time were found. In the within-group comparisons, protein carbonyl concentration increased after exercise in both saline and extract-treated groups. In the between-group comparisons, protein carbonyl concentration was higher in the extract group compared to saline group at rest only. In TBARS (Figure 3(d)) significant main effects of treatment and time were found. In the within-group comparisons, TBARS concentration increased after exercise in the saline-treated group only. In the between-group comparisons, TBARS concentration was higher in the extract-treated group compared to the saline-treated group at rest only.

Erythrocytes. In protein carbonyls (Figure 4(a)), significant main effects of treatment and time were found. In the within-group comparisons, protein carbonyl concentration increased after exercise in both saline, and extract-treated groups. In the between-group comparisons, protein carbonyl

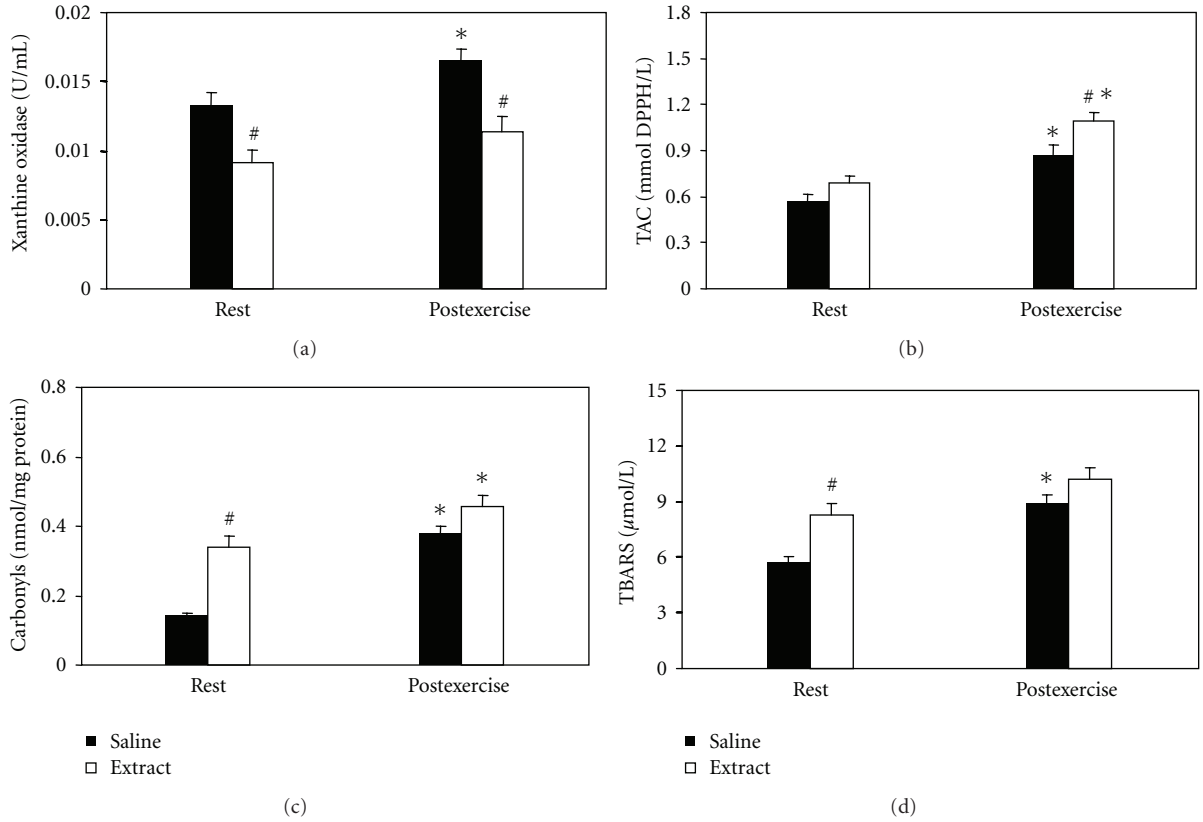

Figure 3: Effects of the grape pomace extract on oxidative stress markers in plasma at rest and postexercise. *Significantly different from the rest value within either the saline or the extract group ($P < 0.05$). #Significantly different between the saline- and the extract-treated groups at the same time point ($P < 0.05$).

concentration was higher in the extract group compared to saline group at rest only. In TBARS (Figure 4(b)), significant main effects of treatment and time were found. In the within-group comparisons, TBARS concentration increased after exercise in both saline- and extract-treated groups. In the between-group comparisons, TBARS concentration was higher in the extract group compared to the saline group at postexercise only. In GSH (Figure 4(c)) an interaction of treatment × time was found. In the within-group comparisons, GSH concentration decreased postexercise in the saline group only. In the between-group comparisons, GSH concentration was lower in the extract group compared to the saline group at postexercise only. In catalase (Figure 4(d)), neither significant main effects nor interaction were found.

Gastrocnemius Muscle. In xanthine oxidase (Figure 5(a)), significant main effect of time and interaction of treatment × time was found. In post hoc within-group comparisons, xanthine oxidase activity significantly decreased at postexercise in the saline-treated group only. In post hoc between-group comparisons, xanthine oxidase activity decreased in the extract-treated group at rest and increased in the same group compared to the saline-treated group at postexercise. In TAC (Figure 5(b)), protein carbonyls (Figure 5(c)) and GSH (Figure 5(e)), neither significant main effects nor interaction were found. In TBARS (Figure 5(d)), an interaction of treatment × time was found. In within-group and between-group comparisons, TBARS concentration increased at postexercise in the saline-treated group only. In catalase (Figure 5(f)), significant main effects of treatment and time were found. In within-group and between-group comparisons, catalase activity increased at postexercise in the extract-treated group only.

Heart. In xanthine oxidase (Figure 6(a)), and GSH (Figure 6(e)), neither significant main effects nor interaction were found. In TAC (Figure 6(b)), main effect of treatment was found. In protein carbonyls (Figure 6(c)), main effect of time and interaction of treatment × time was found. In post hoc within-group comparisons, protein carbonyl concentration increased at postexercise in the saline-treated group only. In post hoc between-group comparisons, protein carbonyl concentration increased at rest in the extract-treated group only. In TBARS (Figure 6(d)), main effects of treatment and time were found. In within-group comparisons, TBARS concentration at postexercise increased in both saline-treated and extract-treated groups. In between-group comparisons, TBARS concentration increased in saline-treated group at rest and at postexercise. In catalase (Figure 6(f)), main effect of time was found.

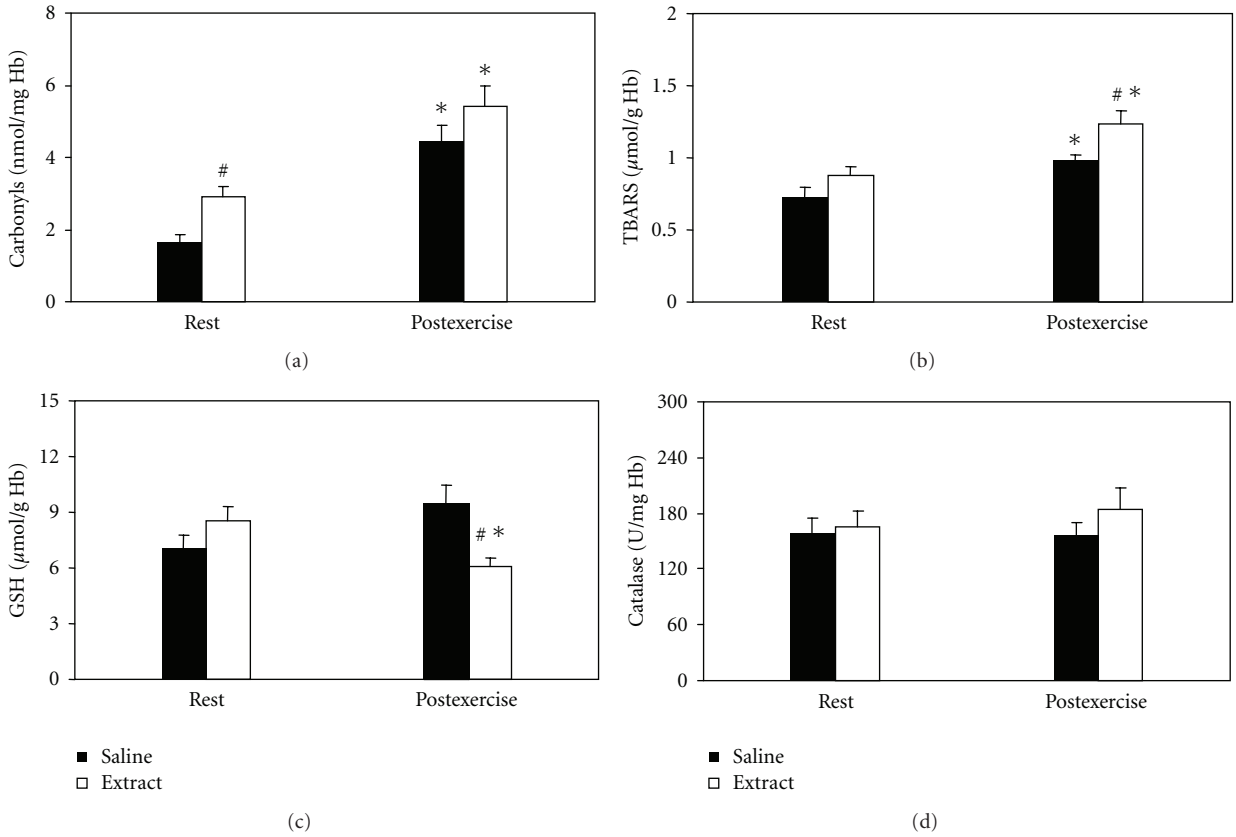

Figure 4: Effects of the grape pomace extract on oxidative stress markers in erythrocytes at rest and postexercise. *Significantly different from the rest value within either the saline or the extract group ($P < 0.05$). #Significantly different between the saline- and the extract-treated groups at the same time point ($P < 0.05$).

Liver. In xanthine oxidase (Figure 7(a)), TAC (Figure 7(b)) and catalase (Figure 7(f)), neither significant main effects nor interaction were found. In protein carbonyls (Figure 7(c)), main effect of time was found. In TBARS (Figure 7(d)), main effect of treatment and interaction of treatment × time were found. In post hoc within-group comparisons, TBARS concentration at postexercise increased in extract-treated group. In post hoc between-group comparisons, TBARS concentration increased in extract-treated group compared to the saline-treated group at postexercise. In GSH (Figure 7(e)), main effects of treatment and time were found. In within-group comparisons, GSH concentration at postexercise decreased in saline-treated group. In between-group comparisons, GSH concentration decreased in extract-treated group compared to the saline-treated group at rest.

4. Discussion

Over the last decades, various plant extracts have gained a lot of interest because of their beneficial effects on human health. Vegetables and fruits are substantial part of the Mediterranean diet. Grapes, in particular, are thought to possess health-related properties. It has been established that grape consumption is related to the prevention of chronic diseases such as cardiovascular diseases [35] and cancer [36]. The biological importance of grape extracts is mainly attributed to the antioxidant properties of the polyphenolic compounds they possess [35, 37]. This is the main reason why polyphenolic compounds and plant extracts have been increasingly used as part of the diet or as nutritional supplements. Nevertheless, polyphenols may also act as prooxidants as they may induce free-radical production mainly via Fenton reaction [38, 39].

The rationale of the present study was to compare the effects of a polyphenol-rich grape pomace extract on redox status using both *in vitro* and *in vivo* models. The tested extract was initially examined for its possible antioxidant capacity. The results demonstrated that the extract has potent antioxidant and chemopreventive properties *in vitro* as it scavenges free radicals ($DPPH^{\bullet}$ or $ABTS^{\bullet+}$) and prevents DNA damage induced by $ROO^{\bullet}$ and $OH^{\bullet}$ radicals. It is established that $ROO^{\bullet}$ are the major factors initiating the cascade reactions of lipid peroxidation [40]. Thus, the preventive activity of the extract against the detrimental effects of $ROO^{\bullet}$ on DNA in a relatively low concentration implies that it might participate in protection against lipid peroxidation. Furthermore, the extract could be considered as a chemopreventive agent as $ROO^{\bullet}$ and lipid peroxidation cause mutations in DNA and are crucial for the initiation of carcinogenic process [41]. The protective effect of the extract

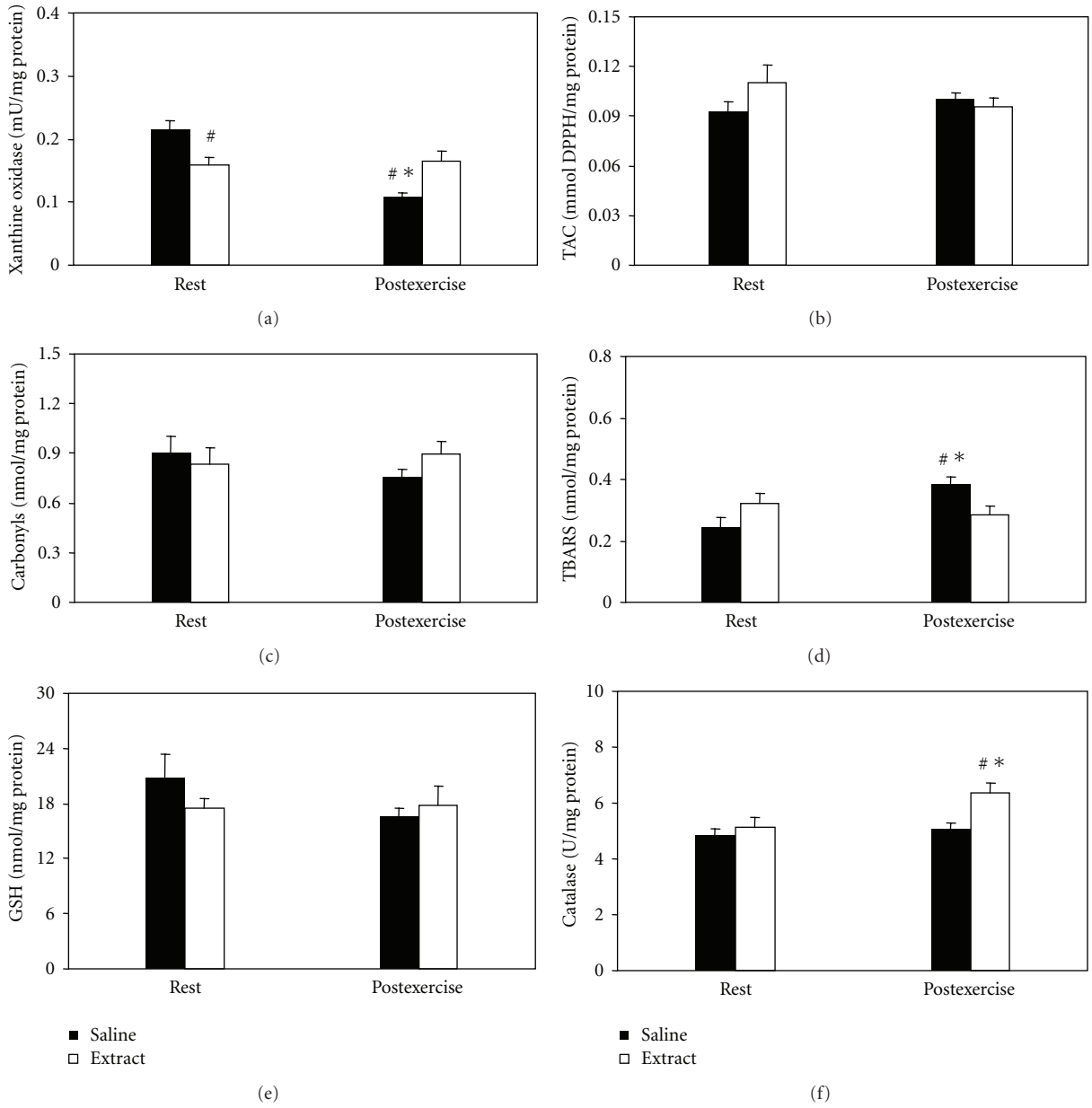

Figure 5: Effects of the grape pomace extract on oxidative stress markers in gastrocnemius muscle at rest and postexercise. *Significantly different from the rest value within either the saline or the extract group ($P < 0.05$). #Significantly different between the saline- and the extract-treated groups at the same time point ($P < 0.05$).

on the DNA damage induced by $OH^{\bullet}$, despite the fact that it was observed in much higher concentration that against $ROO^{\bullet}$, is of high importance. It is known that $OH^{\bullet}$ are highly reactive and can easily cause mutations in DNA [42]. Given that UV radiation is one of the main producers of $OH^{\bullet}$, it could be considered that the extract possesses preventive properties *in vitro* against the effects of UV radiation. These findings are in accordance with the potent *in vitro* antioxidant and chemopreventive properties of other grape extracts of the *Vitis vinifera* species [24–26].

Thereafter, the intention of this study was to examine if the *in vitro* antioxidant properties of the extract apply in an *in vivo* experimental model using exercise as an oxidant stimulus. Swimming was chosen as an experimental model because it causes limited muscle damage and the requirement for antioxidant activity is much less due to a dramatic reduction in inflammatory processes related to muscle damage and repair. The grape pomace extract was administered in rats before exhaustive swimming and generally induced oxidative stress at rest. This is evident by the increased concentration in plasma and erythrocyte protein carbonyls, plasma TBARS, heart protein carbonyls and TBARS, as well as the decreased concentration in liver GSH in the extract-treated rats compared to the saline counterparts at rest.

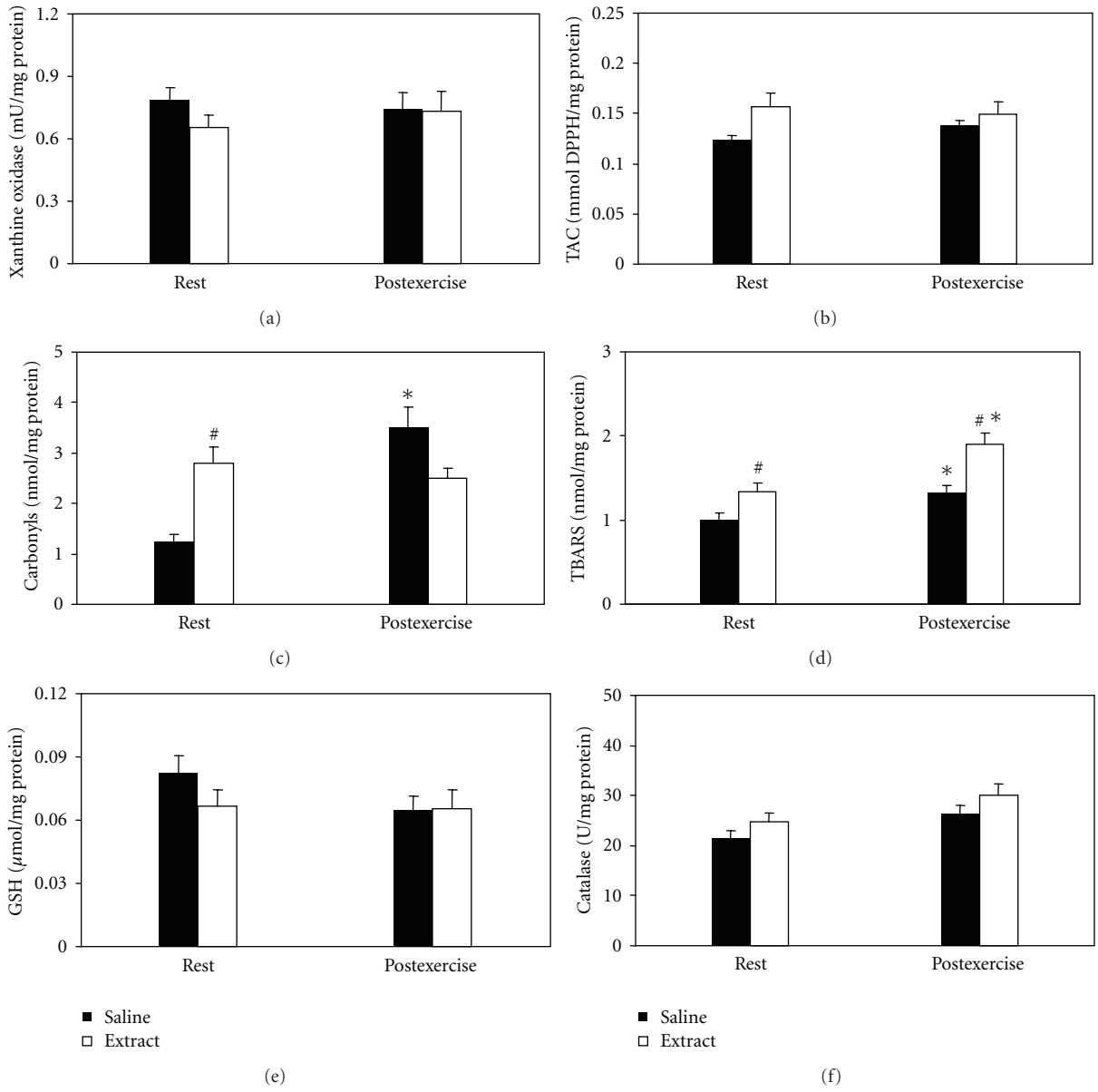

Figure 6: Effects of the grape pomace extract on oxidative stress markers in heart at rest and postexercise. *Significantly different from the rest value within either the saline or the extract group ($P < 0.05$). #Significantly different between the saline- and the extract-treated groups at the same time point ($P < 0.05$).

Exercise, as expected, enhanced one of the main pathways that contribute to free-radical production during exercise as seen by the increased activity of xanthine oxidase in plasma in saline group postexercise. Exercise alone induced oxidative stress as indicated by the increased protein carbonyl concentration in plasma, erythrocytes, and heart, the increased TBARS concentration in plasma, erythrocytes, gastrocnemius muscle, and heart, the increased TAC in plasma, and the decreased GSH concentration in liver in saline group postexercise. The effects of exercise alone on oxidative stress that are described in the present paper are in line with previous findings. Thus, it has also been found that exercise increases plasma, erythrocyte, and gastrocnemius muscle protein carbonyl concentration [2, 43–45] and lipid peroxidation in plasma, erythrocytes, and gastrocnemius muscle [43, 45, 46].

Extract administration inhibited xanthine oxidase activity in plasma postexercise. In a previous study of our research group, it has been demonstrated that the grape pomace extract used is an *in vitro* inhibitor of xanthine oxidase activity, and this could be a possible reason for the decrease in the activity of the enzyme [47]. It is important to mention that xanthine oxidase is one of the major contributors of reactive species during exercise. However, emphasis should also be given on the contribution of mitochondria, which are very much loaded during strenuous physical exercise because of the high energy demand. Furthermore, in some cases,

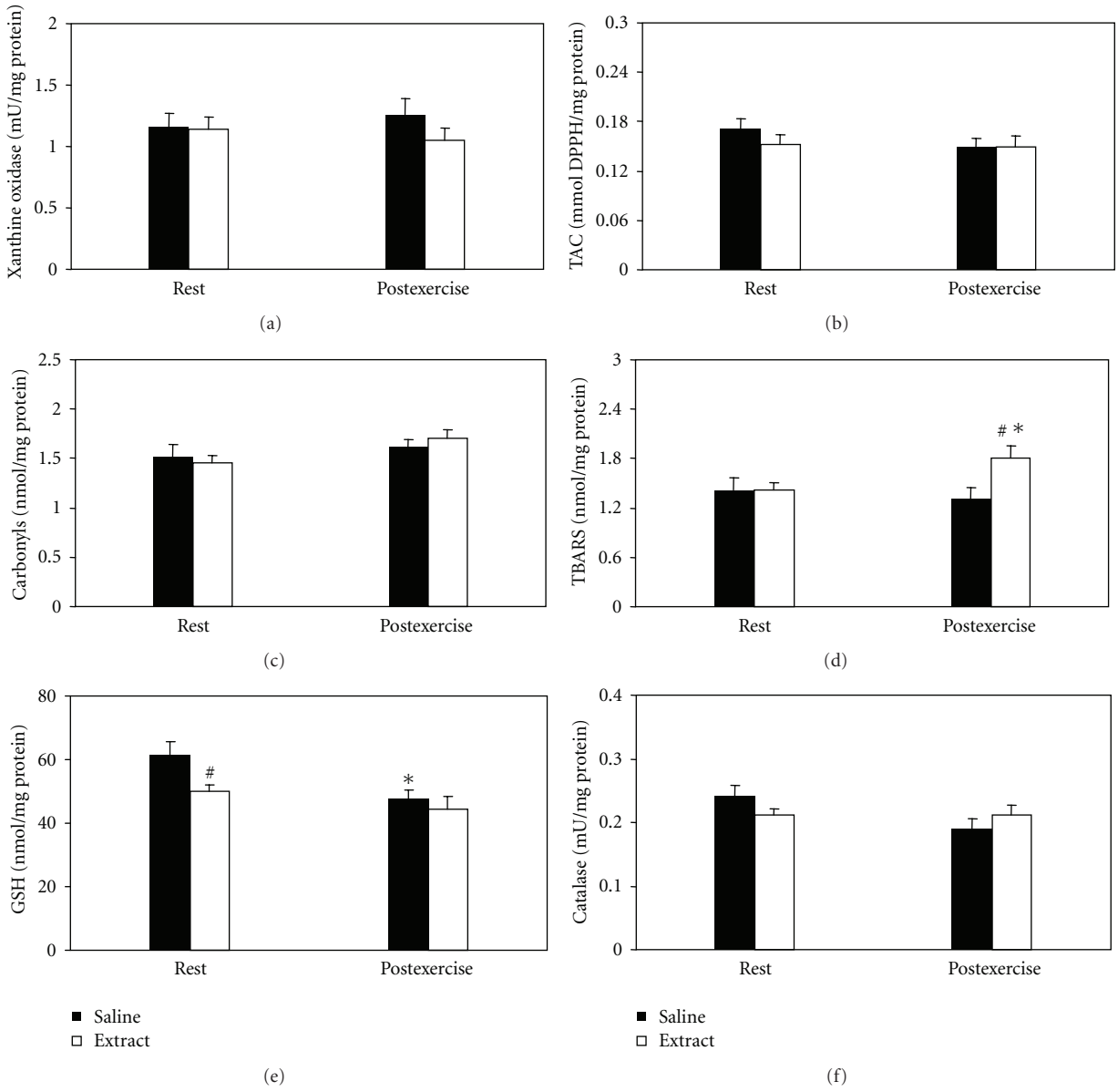

Figure 7: Effects of the grape pomace extract on oxidative stress markers in liver at rest and postexercise. *Significantly different from the rest value within either the saline or the extract group ($P < 0.05$). #Significantly different between the saline- and the extract-treated groups at the same time point ($P < 0.05$).

extract administration in combination with exercise induced oxidative stress further than that induced by exercise alone as shown by the increased TBARS concentration in erythrocyte and liver, catalase activity in gastrocnemius, and TAC in plasma, as well as the decreased GSH in erythrocytes. The prooxidant effects of plant extracts after exercise have also been referred to in previous studies. Specifically, artichoke-leaf extract administration did not limit oxidative damage to erythrocytes in competitive rowers subjected to strenuous training [21]. On the contrary, there is evidence indicating the *in vivo* antioxidant activity of several plant extracts administered before exercise [48–50]. It should be mentioned that timing is a variable that may influence antioxidant recommendations. For example, the outcome may differ if the extract is administered before exercise, after exercise or studied under chronic supplementation.

To our knowledge, there are no studies comparing the *in vitro* and *in vivo* effects of a plant extract on redox status before exercise. However, there are few *in vitro* versus *in vivo* studies measuring oxidative stress markers in response to other oxidative stress stimuli such as diabetes [51, 52], exposure in xenobiotics [1, 53], or reactive oxygen species [54]. These studies have shown that several different extracts exhibit antioxidant or chemopreventive properties both *in vitro* and *in vivo*. Nevertheless, other studies demonstrated that the antioxidant *in vitro* activity does not always apply to

in vivo models. In particular, black tea extract and its major polyphenolic antioxidant constituent, epigallocatechin gallate, protect against lipid peroxidation induced by the water-soluble radical generator AAPH *in vitro*. However, this is not the case when they are consumed by human subjects as they do not protect plasma from lipid peroxidation [55]. Furthermore, despite the high antioxidant capacity of individual apple polyphenols and apple extracts *in vitro*, ingestion of large amounts of apples and apple polyphenols by humans does not appear to result in equivalent *in vivo* antioxidant effects [56]. This disagreement is not surprising. Polyphenols when consumed are absorbed by the gastrointestinal tract, and their concentration in plasma does not reach concentrations higher than 1 μmol/L because of its rapid metabolism by tissues [57]. Administration of 2 g of catechin and 50 mg of gallic acid (the most abundant polyphenols in the grape pomace extract used in the present paper) resulted in 3.5 μmol/L and 1.8 μmol/L plasma concentrations, respectively [58]. The fact that the polyphenolic compounds are degraded in metabolites with smaller molecular weight is partly responsible for their different *in vitro* and *in vivo* effects on redox status [59]. Besides, polyphenols are metabolized as typical xenobiotics and such metabolism alters or decreases their antioxidant capacity [57]. These data raise serious concerns whether any potential antioxidant effects of polyphenols on redox status *in vivo* can be simply extrapolated from their antioxidant activities *in vitro*.

A main finding of the present study was that the administration of the grape pomace extract did not affect exercise performance, as indicated by the almost identical swimming time to exhaustion between the saline- and extract-treated groups. Several studies that examined the effects of antioxidant supplementation on exercise performance have reported controversial results. More specifically, exercise performance was not affected after administration of vitamin E, ascorbic acid and other antioxidants in humans and rats [60–64] or supplementation of black currant extract [20], artichoke extract [21], rhodiola rosea extract [22] in humans, or *panax ginseng* extract [65] in humans and selenium administration in rats [66]. On the contrary, performance was improved after administration of N-acetyl cysteine in humans [67], tocotrienols in rats [68], as well as vitamin E [69], *Pseudosasa japonica* leaves [70], and green-tea extract [71] in mice. Furthermore, it has been previously reported that antioxidant supplementation barely affects exercise performance by more than 10% [72]. In a previous study of our research group administration of allopurinol, a potent inhibitor of xanthine oxidase, markedly decreased performance and caused a 4-fold decrease in xanthine oxidase activity in plasma and gastrocnemius muscle [5]. As a consequence, there was an inhibition of uric acid production, one of the most potent antioxidant molecules in plasma [9, 10]. In the current study, the grape extract inhibited xanthine oxidase activity in the examined tissues by only about 30%. This differential effect of allopurinol and grape pomace extract on the reduction of xanthine oxidase activity might be a reason why performance was not affected after extract administration.

The data of the present study illustrate that the *in vitro* antioxidant activity of a grape pomace extract does not necessarily translate to *in vivo* antioxidant activity either at rest or after exercise. This finding suggests that the *in vitro* antioxidant activity of the particular grape pomace extract was not effective when applied to an *in vivo* system, at least when exercise is used as an oxidant stimulus. In the light of these findings, we suggest that the term "antioxidant" may be system-related. Therefore, the common practice of supplementing antioxidants before exercise should be examined with a more critical view and further be investigated. An alternative and also interesting suggestion is that the pro-oxidant effect of grape pomace extract might be beneficial because it triggers the antioxidant machinery of the body to respond with a more efficient way. Whatever the case it should be, the answer can bring new evidence in the oxidative stress field.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2012, Article ID 145418, 6 pages
doi:10.1155/2012/145418

Research Article

Changes of Myogenic Reactive Oxygen Species and Interleukin-6 in Contracting Skeletal Muscle Cells

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Received 13 January 2012; Revised 28 February 2012; Accepted 29 February 2012

Academic Editor: Michalis G. Nikolaidis

The aim of this study was to measure changes in myotube reactive oxygen species (ROS) and the production of interleukin (IL)-6 in electrically stimulated mouse C2C12 skeletal muscle cells. After five days of differentiation, myotubes were stimulated using an electrical stimulator set at 45 V at a frequency of 5 Hz, with a pulse width of 20 ms. Acute stimulations were performed for 45, 60, 75, 90, or 120 min in each dish. ROSs were detected in the extracted cells directly using a fluorescent probe. IL-6 mRNA expression in C2C12 myotubes and IL-6 concentration in C2C12 myotube supernatants were determined using real-time PCR and ELISA, respectively. Compared with control cells, ROS generation was significantly increased at 45 min after the onset of stimulation ($P < 0.01$) and continued to increase, reaching a maximum at 120 min. IL-6 mRNA expression and IL-6 concentration in C2C12 cells were significantly increased after 75 min ($P < 0.01$) and 120 min ($P < 0.05$) of electrical stimulation (ES) compared with the control cells. Our data show that a specific ES intensity may modulate ROS accumulation and affect IL-6 gene expression in contracting skeletal muscle cells.

1. Introduction

Interleukin (IL)-6 is a cytokine with varied biological effects, is expressed by mesenchymal, epithelial, and other cell types, and is unregulated in response to noxious stimuli, other cytokines, and growth factors. Numerous studies have demonstrated that IL-6 production is associated with muscle contraction and that the sympathoadrenal response to exercise plays only a minor role in the exercise-induced increase in plasma IL-6 [1]. Increases in the steady-state IL-6 mRNA level in skeletal muscle homogenates after long distance running suggest that skeletal muscle tissue might be a source of circulating IL-6 released during exercise [2]. Exercise does not induce an increase in plasma TNF-α level but induces a strong anti-inflammatory cytokine response with the appearance of IL-6 in the circulation being particularly upregulated and preceding that of other cytokines [3]. The IL-6 gene is inactive in resting muscles but is rapidly activated by muscle contraction. Additionally, IL-6 acts as an energy sensor, which is dependent on the glycogen content in muscle [4]. Low glycogen levels have been shown to induce IL-6 gene transcription in skeletal muscle during exercise.

More recent studies have shown that IL-6 release from muscle during exercise may be related to free radical metabolism, especially with reactive oxygen species (ROS) generation [5]. The generation of ROS is a normal occurrence during *in vivo* aerobic processes [6, 7]. Exercise is an effective stimulation method and is accompanied by an increased generation of free radicals, resulting in a measurable degree of oxidative modifications to various molecules [8, 9]. Although the increase in circulating levels of IL-6 after exercise is well documented, the cellular source and stimulus for its release remain elusive. A key limitation of these studies is that the experimental models were not physiological. Thus, *in vivo* studies are essential to define the role of IL-6 in skeletal muscle during exercise.

To examine whether skeletal muscle cells produce IL-6 and to identify possible stimuli for its release that are relevant to muscle contraction, we used differentiated C2C12 skeletal muscle cells (myotubes) as a skeletal cell model of electrical stimulation to imitate skeletal muscle contraction, as previously reported [10–12]. Previous data has shown that this pattern of stimulation can bring about a variety of functional

and structural changes in myotubes [13, 14]. It has been reported that control of myotube contraction using electrical pulse stimulation can induce large and rapid changes in the mRNA expression of genes encoding mitochondrial proteins. These mRNA increases result from a disruption in the equilibrium that exists between gene transcription and mRNA stability during nonadaptive steady-state conditions. Irrcher and Hood have shown that the contractile activity-mediated induction of transcription factor mRNA expression is highly divergent in C2C12 myotubes. This variability in transcription factor mRNA induction and turnover is likely important for the time-dependent, gene-specific transcription events that are responsible for many muscle phenotypic adaptations to contractile activity at the level of contractile proteins and mitochondrial biogenesis [15].

Thus, the purpose of this study was to investigate the changes in ROS level and the production of IL-6 generated by skeletal myocyte contraction. We hypothesized that ROS generation induced by skeletal muscle contraction may be one of the factors regulating muscle-derived IL-6 production and release.

2. Materials and Methods

2.1. Reagents and Cell Culture. C2C12 skeletal muscle cells were obtained from the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. The cells were cultured in growth medium (Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 μg/mL streptomycin) in a humidified incubator at 37°C with 5% CO_2. When myoblast cultures reached confluence, they were switched to DMEM containing 2% heat-inactivated horse serum supplemented with antibiotics (differentiation medium) for five days, and the C2C12 skeletal muscle cells were differentiated into myotubes.

2.2. Electrical Stimulation Protocol. The design of the electrical stimulation apparatus was derived by modification of the existing method [16, 17]. After five days of differentiation, myotubes were stimulated using an electrical stimulator, (TY-C type), set at 45 V at a frequency of 5 Hz. Stimulations were performed acutely for 45, 60, 75, 90, and 120 min, separately. Cell extracts obtained from each dish were prepared immediately after stimulation.

2.3. ROS Measurements. A fluorescent probe, 2′, 7′-dichlorofluorescin diacetate (DCFH-DA), was used for the assessment of intracellular ROS formation in cultured C2C12 myotubes [7]. This assay is a reliable method for the measurement of intracellular ROS such as hydrogen peroxide (H_2O_2), hydroxyl radical (OH•), and hydro peroxides (ROOH) [11, 16]. Myotubes were loaded with the probe and incubated for 20 min. After loading, the cells were washed in preparation for electrical stimulation, collected immediately, and then placed in the spectrofluorometer to determine the ROS production at the excitation and emission wavelengths of 522 nm and 488 nm, respectively. The peak value of luminescence as a function of time was taken after subtracting the value of the blank signal (luminescence of the illuminated medium).

2.4. The IL-6 mRNA Measurements. Total RNA was isolated with TriZol and reverse transcribed with Moloney murine leukemia virus (M-MLV) (Promega, USA). RT-PCR was performed using Taq DNA polymerase (Promega, USA). The sequences for the primers were as follows: for β-actin cDNA: 5′-CGT GAA AAG ATG ACC CAG ATC A-3′, 5′-CAC AGC CTG GAT GGC TAC GT-3′; IL-6 mRNA: 5′-TCC AGC CAG TTG CCT TCT TGG-3′, 5′-TCT GAC AGT GCA TCA TCG CTG-3′. The conditions used were 94°C for 1 min, 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 1 min, repeated for a total of 39 cycles, final extension was performed at 72°C for 10 min. The products were run on a 1.5% agarose gel and visualized under a UV lamp. The point where the two visualized bands (200 bp from the amplification of the native IL-6 cDNA and 400 bp from the competitor molecule amplification) were of equal intensity was considered as the isopoint of the reaction (where equal starting concentrations of IL-6 cDNA and competitor existed in the PCR mix). The cycle number was chosen for each primer pair that maintained approximately exponential amplification with the enriched sample.

2.5. The IL-6 Concentrations Measurements. C2C12 cells were grown in monolayers and allowed to differentiate after reaching confluence. They were then treated with ES for 45, 60, 75, 90, and 120 min, separately. After ES treatment, the supernatants were collected and centrifuged for 20 min at 1000 g/min in a tabletop microcentrifuge to remove floating cells. Following centrifugation, pellets were discarded and supernatants used for ELISA in accordance with the manufacturer's instructions. C2C12 cell monolayers in the multiwell plates were lysed with 1 N NaOH. Protein amounts per well were determined by the Bradford method and were used to normalize against the values obtained for cytokine release.

2.6. Analysis. Electrophoretic bands were observed under UV transillumination and immediately photographed using an imaging system (SYNGENE, USA). Bandscan 5.0 (USA) band analysis software was used for analysis of electrophoretic bands to calculate the brightness of each PCR amplification product.

Data are expressed as mean ± standard deviation (SD). The means were compared using a two-sided Student's t-test. Significant differences were determined using one-way ANOVA. Statistical analysis was conducted using SPSS13.0 (SPSS Inc.). Differences were considered significant at $P < 0.05$.

3. Results

3.1. Estimation of Cell Differentiation Fusion. The monolayer organization, as directly observed by inverted phase contrast microscopy, changes during the differentiation of myoblasts

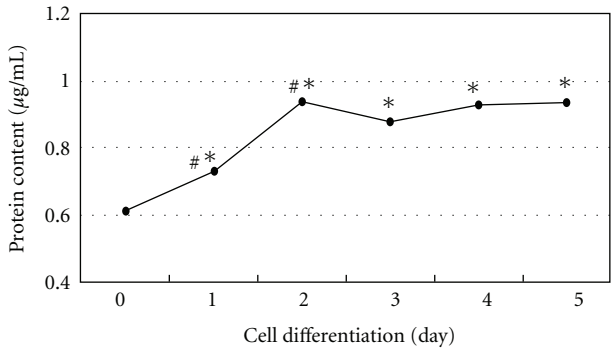

Figure 1: Total protein content of C2C12 myotubes during cell differentiation. When the C2C12 skeletal muscle cells were differentiated into myotubes, the absorbance of each well (OD) was measured by standard curve with a microplate reader at 450 nm wavelength, and then the protein content was calculated through the standard curve. $^{*}P < 0.05$, significant difference compared with the first day, $^{\#}P < 0.05$, significant difference compared with the previous day.

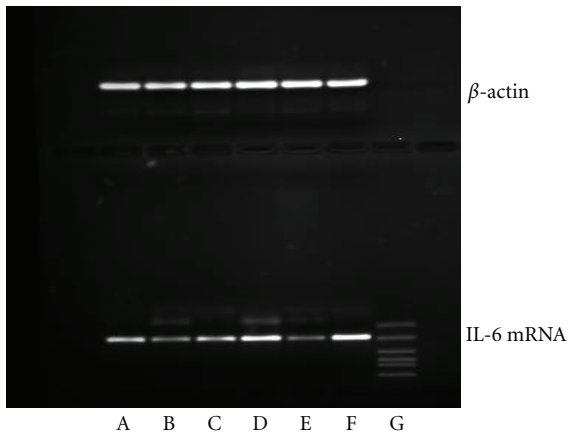

Figure 2: Gel photo of IL-6 mRNA expression from C2C12 myotubes following electrical stimulation. The products were run on a 1.5% agarose gel and visualized under a UV lamp. A: CN; B: ES 45 min; C: ES 60 min; D: ES 75 min; E: ES 90 min; F: ES 120 min; G: marker.

to myotubes. In the undifferentiated condition, myoblasts appear as fusiform or star-shaped cells, mostly flattened and closely adherent to the substrate. At the initial differentiation stage, intercellular spaces disappear, cells progressively align and occasionally elongate.

The bicinchoninic acid (BCA) assay was used to measure the synthesis and decomposition of C2C12 myotube intracellular protein content. Figure 1 shows the changes in total protein content, which significantly increased from the undifferentiated state to the final phase. Total protein content increased, reaching maximal values at the 3rd day of differentiation, and then steadily increased and showed minimal values in the late differentiation stage.

3.2. ROS Generation after Acute Electrical Stimulation. To ensure accurate quantification of ROS generation, we applied the fluorescent probe DCFH-DA to mark cells after ES and subsequently measured the optical density (OD) values using a fluorescent spectrofluorometer. Table 1 shows that ROS generation significantly increased at 45 min after the onset of stimulation ($P < 0.01$) and continued to increase, reaching a maximum at 120 min, compared with control cells.

Table 1: ROS generation during different electrical stimulation times.

	Mean ± SD		Mean ± SD
Control	48.94 ± 0.2547	ES75	$592.30 \pm 3.398^{*\#}$
ES45	$341.54 \pm 3.4034^{*\#}$	ES90	$401.52 \pm 4.6214^{*}$
ES60	$503.52 \pm 3.3929^{*\#}$	ES120	$631.74 \pm 3.5515^{*\#}$

The optical density (OD) values were measured using a fluorescent spectrofluorometer after ES. The peak value of luminescence as a function of time was taken after subtracting the value of the blank signal (luminescence of the illuminated medium).
$^{*}P < 0.01$, significant difference compared with the control group,
$^{\#}P < 0.05$, significant difference compared with the 90 min ES group.

Table 2: IL-6 mRNA expression during different electrical stimulation times.

	Mean ± SD		Mean ± SD
Control	0.5510 ± 0.1637	ES75	$0.7748 \pm 0.070^{\#*}$
ES45	0.5679 ± 0.05984	ES90	0.5701 ± 0.0114
ES60	0.6417 ± 0.1280	ES120	$0.7367 \pm 0.1266^{*}$

$^{*}P < 0.05$, significant difference compared with the control group,
$^{\#}P < 0.01$, significant difference compared with the control group.

3.3. IL-6 mRNA Expression and IL-6 Concentration during Different Electrical Stimulation Times. IL-6 mRNA expression in C2C12 myotubes changed after ES (Table 2). Figure 2 shows the gel photo of IL-6 mRNA expression from C2C12 myotubes after ES. The results show that IL-6 mRNA concentration in C2C12 myotube supernatants changed with differing times of ES.

IL-6 mRNA expression in C2C12 myotubes changed following electrical stimulation. IL-6 mRNA expression in C2-C12 myotubes was significantly increased after 75 min ($P < 0.01$) and 120 min ($P < 0.05$) of ES compared with the control group. Compared with the 90 min ES group, IL-6 mRNA expression in the 75 and 120 min ES groups was significantly increased ($P < 0.05$).

IL-6 protein concentration from C2C12 myotube supernatants was changed after ES. IL-6 concentration increased in C2C12 myotube supernatants with increasing ES time compared with the control group, with the peak values appearing at 75 and 120 min ($P < 0.01$). Compared with the 90 min ES group, IL-6 concentration in the 75 and 120 min ES groups was significantly increased ($P < 0.05$) (Table 3).

4. Discussion

Contractile activity is a potent stimulus for the induction of numerous cellular adaptations in skeletal muscle [18]. To assess alterations in response to contractile activity, we used an isolated cellular system in the absence of neural and

Table 3: IL-6 concentration during different electrical stimulation times.

	Mean ± SD (pg/mL)		Mean ± SD (pg/mL)
Control	81.83 ± 13.16	ES75	2800 ± 69.28*#
ES45	347.17 ± 59.28*	ES90	274.17 ± 13.58*
ES60	674.33 ± 51.99*#	ES120	675.33 ± 86.09*#

*$P < 0.01$, significant difference compared with the control group,
#$P < 0.05$, significant difference compared with the 90 min ES group.

humoral factors. The results show that electrical stimulation (45 V, 5 Hz, 20 ms) modulates ROS accumulation and affects the gene expression level of IL-6. However, these changes did not cause any significant cell damage, implying that the intracellular redox balance was maintained. Skeletal muscle myotubes displayed a large and rapid increase in ROS production during ES. ROS release after different contraction times *in vitro* has not been previously described. However, abundant *in vivo* evidence has demonstrated that ROS production is a stimulus for skeletal muscle adaptation to exercise training [8, 19]. Previous reports support the concept that exercise-induced ROS generation alters muscle gene expression and contributes to exercise-induced adaptations of skeletal muscle *in vivo* [20]. A common approach in many of these studies is to abolish the signaling effects of exercise-induced ROS production in skeletal muscle by treating animals with antioxidants. For example, two independent studies have recently concluded that antioxidant supplementation can impede important training adaptations in human skeletal muscle [21, 22]. Our previous studies have also demonstrated that ES of C2C12 myotubes can induce rapid increases in ROS, induce cellular dysfunction, and induce mitochondrial dysfunction [11, 21]. This change is inconsistent with results previously reported *in vivo* [8, 20].

ROS production in muscle cells elicited by ES is related to the voltage, frequency, and duration applied. Different intensities of ES can induce different changes in ROS production [7]. Silveira et al. used different ES intensities on muscle cells and found that intense ES (50 V, pulse width 1 ms, pulse interval 0.01 s) of muscle cells increased intra- and extracellular DCF fluorescence, a measure of oxidation, by 171% and 105%, respectively, compared with control nonstimulated cells. In contrast, moderate stimulation (50 V, pulse width 1 ms, pulse interval 0.1 s) did not significantly increase the extracellular DCF fluorescence [7]. Increasing the stimulation frequency increases skeletal muscle contraction strength, which leads to increases in skeletal muscle mitochondrial activity and superoxide generation. McArdle et al. used ES (30 V, 1 Hz, pulse width 2 ms) on differentiated rat satellite cells to study the time course of ROS release. The authors found that a short (15 min) contraction in satellite cells caused a rapid 40x increase in ROS [23]. This increase in ROS can cause skeletal muscle protein thiol modification, a transient decrease in SOD and catalase activity, and an increase in heat shock protein concentration. However, these changes did not cause any notable cell damage [24].

In this study, we used ES (45 V, 5 Hz) to simulate C2C12 myotubes. We found that ROS production significantly increased with ES compared with the control nonstimulated cells, and the change in ROS generation followed a triphasic curve pattern, with the peaks appearing in the 75 and 120 min ES groups. This is similar to our previous findings using the same mode of ES [11, 12]. Our previous results showed that exercise-induced ROS changes are related to mitochondrial oxygen consumption and the regulation of the antioxidant system. Many *in vivo* studies have also shown that oxidative stress in skeletal muscle is a hallmark of pathophysiological states [19]. ROS production was evident across the physiological range of membrane potentials and was relatively insensitive to membrane potential changes [25]. Abnormally high levels of ROS and the simultaneous decline of antioxidant defense mechanisms can lead to damage of cellular organelles and enzymes. We hypothesized that ROSs are removed mainly by cellular antioxidant systems, which include antioxidant vitamins, protein and nonprotein thiols, and antioxidant enzymes under physiological conditions. However, when ROS production is excessive, such as during prolonged aerobic exercise, cellular antioxidant systems may be overwhelmed, leading to extensive cell and tissue damage. The exercise-induced IL-6 production and secretion within skeletal muscle fibers suggests that IL-6 may play a role in energy supply and storage in muscle during and after exercise. The role is likely short term, physiological, and locally active within minutes or a few hours [26]. In the present study, we observed the changes in IL-6 mRNA expression and IL-6 protein content in C2C12 myotubes at different ES times (0, 45, 60, 75, 90, and 120 min). This is different from previous studies, which suggest that the IL-6 mRNA level in skeletal muscle is markedly increased during exercise [27–29]. IL-6 protein has been shown to accumulate in contracting muscle fibers and be released in large amounts into plasma. Furthermore, transcriptional activation of the IL-6 gene in the skeletal muscle of humans is highly elevated during prolonged exercise [5, 30]. Additional studies have shown that the time and intensity of exercise required to accumulate IL-6 protein within contracting muscle are not well defined. However, duration of exercise has been shown to be the single most important factor that determines the magnitude of the systemic IL-6 response [29]. Our results suggest that IL-6 mRNA expression in C2C12 myotubes changed with differing ES times. IL-6 mRNA expression in both C2C12 myotubes and supernatants was significantly increased in the 75 and 120 min ES groups, compared with the control group.

Few studies have addressed the relationship between myogenic ROS and IL-6 after exercise *in vivo*. Some studies have shown that supplementation with vitamins C and E attenuated the systemic IL-6 response to exercise in plasma and skeletal muscle [28, 31]. Antioxidants, such as vitamins C and E, have been shown to attenuate oxidative stress at rest as well as in response to exercise, thereby reducing the generation of ROS [32]. Using C2C12 myotubes incubated with H_2O_2 at different concentrations, Kosmidou et al. found that IL-6 content of cultured supernatants increased dependently after 24 h. The authors concluded that ROSs stimulate IL-6 production from skeletal myotubes by increasing transcriptional activation of the IL-6 gene through an NF-κB-dependent pathway [33]. In human bronchial epithelial cells,

ROS-stimulated IL-6 production is preceded by increases in IL-6 steady-state mRNA levels [34]. Keller et al. have suggested that induction of IL-6 expression in contracting myofibers may be largely responsible for the rise in circulating IL-6 concentration during prolonged exercise [30]. Therefore, we speculate that similar mechanisms are likely active in contracting skeletal muscle and that ROS generation may play a role in the signaling processes by which C2C12 myotubes adapt to changes in the pattern or duration of contractile activity.

It is well known that a variety of stresses can induce IL-6 upregulation. However, studies on the IL-6 response to electrical stimulation are scarce. To the best of our knowledge, this is the first report addressing the IL-6 response to ES. In the present study, we have shown that ES can cause changes in IL-6 mRNA expression, IL-6 protein content, and variable increases in ROS generation. We speculate that myogenic ROS generation is likely to mediate myogenic IL-6 generation. However, in this study, we only investigated the possible link between myogenic IL-6 and ROS after ES, not ROS produced by muscle vasoconstriction and subsequent IL-6 release from skeletal myotubes. Thus, we cannot exclude the possibility that other intramuscular metabolic changes induce myogenic ROS production and subsequent release of IL-6 during exercise. Further studies are needed to investigate this possibility.

Our results indicate that electrical stimulation (45 V, 5 Hz, 20 ms) may modulate ROS accumulation and affect the gene expression level of IL-6. Furthermore, the variation trend is consistent with the stimulation time. We speculate that ROS generated by skeletal muscle cell contraction may be one of the factors regulating muscle-derived IL-6 production and release.

Acknowledgments

This work was supported by Research Start-up Grants from Sun Yat-Sen University "Hundred Talent Program". X. Xu supervised the research design and is the cofirst author of this study.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2012, Article ID 835970, 9 pages
doi:10.1155/2012/835970

Research Article

A Combination of Lipoic Acid Plus Coenzyme Q10 Induces PGC1α, a Master Switch of Energy Metabolism, Improves Stress Response, and Increases Cellular Glutathione Levels in Cultured C2C12 Skeletal Muscle Cells

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Received 3 February 2012; Accepted 22 February 2012

Academic Editor: Michalis G. Nikolaidis

Skeletal muscle function largely depend on intact energy metabolism, stress response, and antioxidant defense mechanisms. In this study, we tested the effect of a combined supplementation of α-lipoic acid (LA) plus coenzyme Q10 (Q10) on PPARγ-coactivator α (PGC1α) activity, expression of glutathione-related phase II enzymes and glutathione (GSH) levels in cultured C2C12 myotubes. Supplementation of myotubes with 250 μmol/L LA plus 100 μmol/L Q10 significantly increased nuclear levels of PGC1α, a master switch of energy metabolism and mitochondrial biogenesis. The increase of nuclear PGC1α was accompanied by an increase in PPARγ transactivation, a downstream target of PGC1α, and an increase in mitochondrial transcription factor A mRNA centrally involved in mitochondrial replication and transcription. Furthermore, supplementation of myotubes with LA plus Q10 resulted in an increase of genes encoding proteins involved in stress response, GSH synthesis, and its recycling. In LA-plus-Q10-treated myotubes a significant 4-fold increase in GSH was evident. This increase in GSH was accompanied by increased nuclear Nrf2 protein levels, partly regulating γGCS and GST gene expression. Present data suggest that the combined supplementation of skeletal muscle cells with LA plus Q10 may improve energy homeostasis, stress response, and antioxidant defense mechanisms.

1. Introduction

The peroxisome proliferator-activated receptor γ (PPARγ)-coactivator α (PGC1α) has been characterized as a master switch of energy homeostasis [1]. PGC1α interacts with transcription factors such as PPARγ, which is a downstream target of PGC1α, thereby mediating adaptive thermogenesis, mitochondrial biogenesis, and fiber type switching of the skeletal muscle [1]. Gain-of-function studies indicate an increased resistance to induced fatigue in isolated skeletal muscle of transgenic mice overexpressing PGC1α [2]. An activation of PGC1α is associated with an increased expression of slow-twitch (type I) fibers which are characterized by an increased mitochondrial biogenesis and thus oxidative metabolism as main energy source [1]. Physical exercise may activate PGC1α [3], whereas aging is accompanied by a significant decrease of PGC1α expression in skeletal muscle [4]. This decrease in PGC1α may impair mitochondrial respiratory capacity, thereby increasing the production of reactive oxygen species which in turn results in glutathione (GSH) depletion and oxidative stress. Thus, strategies are warranted that activate PGC1α and increase GSH synthesis and its recycling thereby maintaining muscle function and integrity.

α-Lipoic acid (LA) is synthesized from octanoic acid in the mitochondria where it serves as a cofactor of mitochondrial α-ketoacid dehydrogenase [5]. Beside endogenous synthesis LA may also derive from exogenous dietary sources including supplements (reviewed by [6]). As a component of the electron transport chain in the inner mitochondria membrane, coenzyme Q10 also known as ubiquinone is centrally involved in cellular respiration and mitochondrial

biogenesis. Furthermore, it is well known for its free radical scavenging properties, which are partly mediated by electron transfer (reviewed by [7]). LA and Q10 may not work in isolation [8] although little is known regarding the potential synergistic activity of LA plus Q10 in terms of muscle cell physiology. Therefore, in this study we investigated the effect of a combined supplementation of LA plus Q10 on PGC1α levels, stress response, and glutathione synthesis in cultured myotubes.

2. Materials and Methods

R(+)LA and Q10 were obtained from Sigma-Aldrich Co. (Munich, Germany). LA was dissolved in DMSO (Carl Roth, Karlsruhe, Germany). Q10 stock solutions were prepared as described previously [9]. The stock solutions were stored at −80°C until usage.

2.1. Cell Culture. C2C12 cells (Institute for Applied Cell Culture, Munich, Germany) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose, 4 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 10% fetal calf serum (FCS), 100 U/mL penicillin, and 100 μg/mL streptomycin (PAA, Coelbe, Germany). Cells were grown in 5% CO_2 at 37°C under a humidified atmosphere. Before each treatment, C2C12 cells were differentiated to myotubes via serum starvation applying DMEM supplemented with 2% horse serum (Gibco, Life technologies, Carlsbad, USA) for 48 h–72 h. All cell-culture plasticware was purchased from Sarstedt (Numbrecht, Germany) unless otherwise stated. For all cell culture assays vehicle controls have been performed and did not affect any of the parameters measured.

2.2. Cytotoxicity Measurement. Cytotoxicity was determined via the neutral red assay [10]. The neutral red assay is based on the pH-dependent accumulation of neutral red in the lysosomes of viable cells. C2C12 cells were seeded in 24-well plates (Fisher Scientific, Schwerte, Germany) at a density of 100,000 cells/well, differentiated for 72 h, and treated with 250 μmol/L LA plus 100 μmol/L Q10 for 24 h in serum-containing medium, respectively. The culture medium containing the test substances was replaced with fresh serum-containing medium including 50 μg/mL of neutral red (Carl Roth). After incubation for 3 h, the medium was removed and the cells were extracted using a solution comprising 50 : 49 : 1 (v/v/v) ethanol, water, and glacial acetic acid. The absorbance was measured in a plate reader (Labsystems, Helsinki, Finland) at 540 nm.

2.3. Transient Transfection and PPARγ Luciferase Reporter Gene Assay. C2C12 cells were differentiated in 24-well plates. The cells were transiently transfected with three vectors: one vector containing the PPARγ-LBD (ligand-binding domain), one UAS-GAL4-vector containing the reporter gene firefly luciferase, and a normalization vector (pRL-TK; Promega, Mannheim, Germany) containing the *Renilla reniformis* luciferase gene. Transfection was performed using JetPei transfection reagent (Polyplus transfection, Illkirch Cedex, France) according to manufacturer's instructions. Following 24 h of transfection the test compounds as well as 1 μM rosiglitazone were applied in serum-containing medium and treated for 24 h. Subsequently, cells were lysed and luciferase activity was measured using the Dual-Luciferase reporter gene assay system (Promega) according to the manufacturer's protocol in a Tecan Infinite 200 microplate reader (Tecan Group Ltd., Crailsheim, Germany).

2.4. RNA Isolation and Real-Time PCR. C2C12 cells were precultured in 6-well plates at a density of 300,000 cells/well. Cells were differentiated and subsequently treated with 250 μmol/L LA plus 100 μmol/L Q10 and the respective compounds in coapplication for 16 h.

RNA for real-time PCR was isolated with TRIsure following the manufacturer's protocol (Bioline, Luckenwalde, Germany). Primers were designed by primer3 software with the following sequences: PGC1α forward: TGCCCAGATCTTCCTGAACT; reverse: TCTGTGAGAACCGCTAGCAA; TFAM forward: TAGGAAAATTGCAGCCCTGT; reverse: GCTGAACGAGGTCTTTTTGG; 18SrRNA forward: CCTGCGGCTTAATTTGACTC; reverse: AACTAAGAACGGCCATGCAC (MWG Biotech, Ebersberg, Germany). Real-time PCR was performed using Sensi-Mix one-step kit (Quantace, Berlin, Germany).

2.5. Agilent Microarray Analysis. Differential gene expression was determined via an Agilent MicroArray platform (Source BioScience, ImaGenes GmbH, Berlin, Germany). C2C12 cells were treated as described above. RNA for gene chip analysis was isolated using a Qiagen RNeasy Kit (Duesseldorf, Germany) according to the manufacturer's protocol. Agilent Quick Amp Labeling Kit One Color (Agilent Technologies, Santa Clara, USA) was used according to the manufacturer's instructions for generation and amplification of fluorescent cRNA. Yield and labeling of cRNA was controlled via NanoDrop measurement (Thermo Fisher Scientific). For hybridization, the Agilent gene expression hybridization kit was used according to the manufacturer's protocol. The microarray slides were washed (following the Agilent protocol) and subsequently scanned using an Agilent microarray scanner. Data were extracted using the Agilent Feature Extraction. Quality parameters (e.g., background detrend or grid) and abnormalities of each sample were monitored.

For microarray analysis DAVID Bioinformatics Resources 6.7 (Database for Annotation, Visualization an Integrated Discovery [11]; http://david.abcc.ncifcrf.gov/) was used. Therefore, gene IDs of the regulated genes (fold change > 2 or <−2) were uploaded. For subsequent functional clustering, KEGG (Kyoto Encyclopedia for Genes and Genomes) pathway maps were used.

2.6. In Silico Promoter Analysis. For promoter analysis, sequences of genes differentially regulated by LA plus Q10 treatment were uploaded to MatInspector Software (http://www.genomatix.de/) in order to identify putative binding sites for Nrf2.

2.7. Glutathione Measurement. C2C12 cells were treated with the test compounds for 24 h as described above. Subsequently, cells were washed and lysed in 10 mmol/L HCl and repeated freeze-thaw cycles. GSH was determined following the microtiter plate assay protocol according to Vandeputte et al. [12]. In brief, cell protein was precipitated by addition of 6.5% sulfosalicylic acid and centrifugation (2000 × g, 15 min). Cell samples were applied on the microtiter plates, mixed with stock buffer and a reagent mix comprising (final reaction concentration) 0.71 mmol/L DTNB and 0.24 mmol/L NADPH. The reaction was initialized by addition of glutathione reductase (1.2 IU/mL, Sigma Aldrich Co.). Kinetic measurement of the chromophore DTNB-GSH at 415 nm was performed in a plate reader (Labsystems, Helsinki, Finland). Total glutathione was determined using a GSH-standard curve (all reagents Carl Roth unless otherwise stated).

2.8. Western Blot Analysis of PGC1α and Nrf2 in Nuclear Extracts. For PGC1α and Nrf2 detection, cells were treated with the test compounds for 6 h. Subsequently, cells were washed with ice-cold PBS, scraped off, centrifuged, and nuclear extracts were subsequently prepared as described by Wagner et al. (2010) [13]. A quantity of 40 μg protein of each sample were mixed with loading buffer, incubated at 95°C for 5 min and separated on a 12% SDS-PAGE. Subsequently, the samples were transferred onto a PVDF (polyvinylidene fluoride) membrane and blocked with 3% (w/v) skim milk dissolved in TBS + 0.05% (v/v) Tween-20 (TBST) for at least 2 h and probed with antibodies against PGC1 (1 : 200; Santa Cruz Biotechnology Inc., Santa Cruz, USA), Nrf2 (1 : 200; Santa Cruz), TATA box binding protein (TBP; Santa Cruz; 1 : 200), or α-tubulin (Abcam, Cambridge, UK; 1 : 5000) at 4°C overnight. Following, the membranes were incubated with a secondary antibody (1 : 4000 anti-rabbit (Bio-Rad, Munich, Germany): Nrf2; 1 : 4000 anti-mouse (Bio-Rad): TBP, α-tubulin; 1 : 3000 anti-goat (Santa Cruz): PGC1) for 1 h, and the bands were visualized by using ECL reagent (Thermo Scientific) in a ChemiDoc XRS system (BioRad). Molecular weight of the protein bands was estimated using a Western C protein standard (Bio-Rad).

2.9. Statistical Analysis. Statistical analysis was conducted using SPSS 19 (IBM, Chicago, USA). Data were analysed for normality of distribution (Kolmogorov-Smirnov). In case of not normally distributed data the nonparametric Mann-Whitney U test was applied. Student's t-test or one-way analysis of variance (ANOVA) with a Dunnett-T (homogeneous variances) post hoc test was performed. Data are expressed as mean ± SEM. Significance was accepted at $P < 0.05$.

3. Results

In order to test for cytotoxicity of the test compounds C2C12 myotubes were incubated with 250 μmol/L LA plus 100 μmol/L Q10 (in the following abbreviated as LA plus Q10) for 24 h. Under the conditions investigated cell viability was not negatively affected by the LA plus Q10 treatment (Figure 1(a)).

PGC1α is an important regulator of skeletal muscle energy homoeostasis. Therefore, we determined PGC1α nuclear protein levels following 6 h of incubation with LA plus Q10. LA plus Q10 supplementation of C2C12 myotubes significantly enhanced nuclear PGC1α protein levels as summarized in Figure 1(b). Differences in PGC1α protein levels in response to the LA plus Q10 treatment were also reflected in terms of PGC1α gene expression (Figure 1(c)). In fact we found a 70% increase in PGC1α mRNA levels in LA-plus-Q10-treated myotubes as compared to untreated control cells.

PGC1α is centrally involved in the regulation of PPARγ activity, which is a downstream target of PGC1α. Therefore, we determined PPARγ transactivation in transiently transfected C2C12 myotubes using a luciferase reporter gene assay. Rosiglitazone (1 μmol/L; rosi) was used as positive control. Treatment of our C2C12 myotubes with LA plus Q10 significantly increased PPARγ transactivation by approximately 50% (Figure 1(d)). However, LA plus Q10 cotreatment was less potent than rosiglitazone as far as the induction of PPARγ transactivation is concerned.

Aerobic muscle turnover may be accompanied by induction of reactive oxygen species which are partly produced in the respiratory chain of the inner mitochondrial membrane. Therefore, we investigated if and to what extent the application of LA plus Q10 may affect cellular GSH which is an important endogenous antioxidant. By using Agilent gene chip technology we found several transcripts to be differentially regulated by LA plus Q10 in our C2C12 myotubes. We filtered those genes in terms of their role in GSH synthesis, metabolism, and recycling. Interestingly within the GSH pathway 12 genes were differentially regulated (P value < 0.001, Benjamini enrichment score < 0.05) due to the LA plus Q10 supplementation as summarized in Figures 2(a) and 2(b).

Both γGCS light and heavy chain were manifold upregulated by the LA plus Q10 treatment. Since γGCS is the key enzyme in GSH synthesis we further determined cellular GSH levels in our skeletal muscle cells. Importantly, the induction of γGCS gene expression was accompanied by a 4-fold increase in cellular GSH in the LA-plus-Q10-treated cells as compared to control (Figure 2(c)).

Glutathione reductase (GSR) is centrally involved in the NADPH-mediated reduction of GSSG to GSH, thereby providing GSH for further reactions. Following incubation with LA plus Q10, we observed a 3.2-fold increase in GSR gene expression (Figure 2(c)). Interestingly, not only GSR but also glucose-6-phosphate dehydrogenase 2 (G6PD) and phosphogluconate dehydrogenase (PGD) gene expression was induced by LA plus Q10. Both enzymes provide NADPH which is used by GSR for GSSG reduction.

Induction of glutathione-S-transferase (GST) may protect the skeletal muscle from lipid peroxides thereby improving stress response. In fact, it has been shown that GST inactivates lipid peroxides such as 4-hydroxynonenal due to conjugation with GSH [14]. We found in our myotubes various genes encoding GST (GST α1, GST α2, GST α3, GST

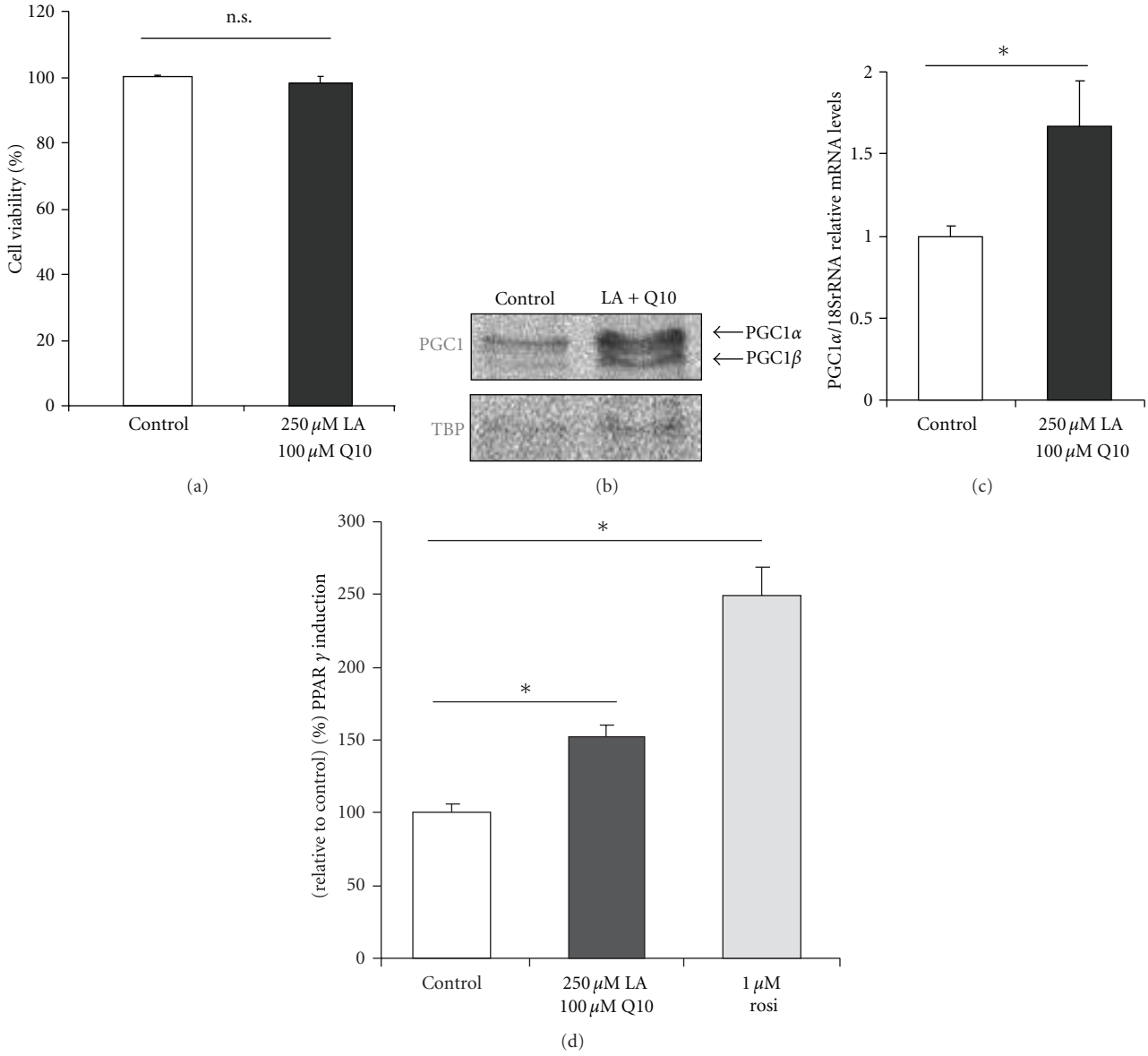


Figure 1: (a) Viability of differentiated C2C12 cells following incubation with 250 μmol/L LA plus 100 μmol/L Q10 for 24 h (data are mean + SEM). (b) PGC1 induction in C2C12 cells following incubation with 250 μmol/L LA plus 100 μmol/L Q10 for 6 h in nuclear cell lysates and (c) PGC1α mRNA levels in response to the LA plus Q10 treatment (data are mean + SEM of three independent experiments; *indicates significant difference to the control cells; $P < 0.05$; Student's t-test). (d) The increase in PGC1α is accompanied by an increased PPARγ transactivation as shown in transiently transfected C2C12 cells (data are mean + SEM of two independent experiments; *indicates significant difference to the control cells; $P < 0.05$; one-way ANOVA).

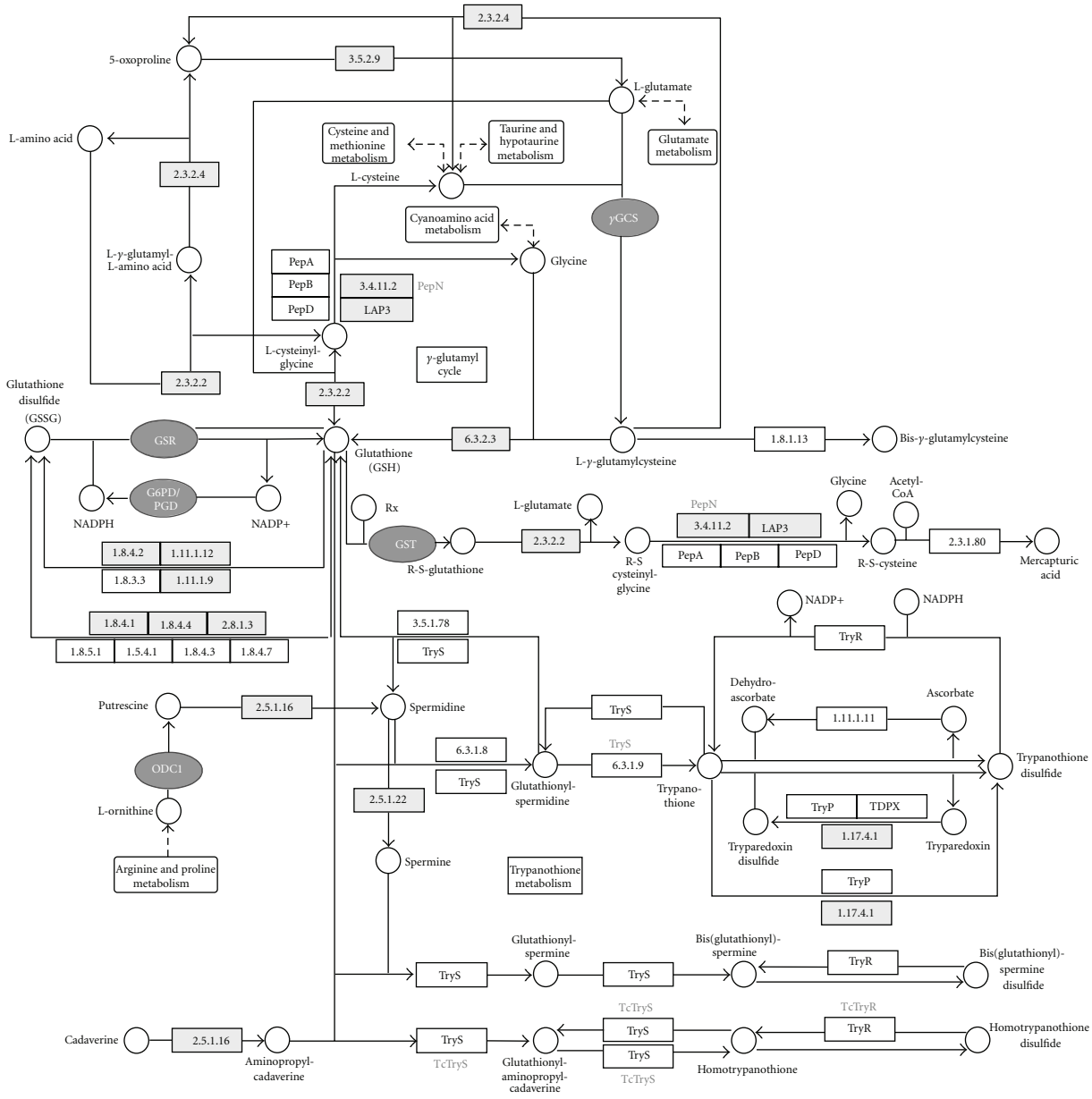
α4, GST π1, GST π2, GST μ6) to be upregulated by the cotreatment of LA plus Q10 (Figures 2(a) and 2(b)).

To identify binding sites for Nrf2 in murine promoter regions in LA-plus-Q10-regulated genes, the respective murine promoter sequences were uploaded to MatInspector. As summarized in Figure 2(b), several binding sites for Nrf2 have been identified in the promoter of the γGCS (GCLC (0–4 putative binding sites), GCLM (0–4)), GSR (1–5), GST (GST α1 (1-2), GST α2 (1), GST α3 (0–2), GST α4 (0/4), GST π1 (0–8), GST π2 (0–8), GST μ6 (0–2)) ODC1 (1), G6PD (1), and PGD (1–6) genes, respectively, (Figure 2(b)).

Treatment of C2C12 cells with LA plus Q10 increased TFAM mRNA levels by 70% ($P = 0.078$) as compared to controls (Figure 3(a)). Furthermore, LA plus Q10 supplementation resulted in an increase in nuclear Nrf2 levels as summarized in Figure 3(b).

4. Discussion

Under the conditions investigated LA plus Q10 significantly increased nuclear PGC1α levels in our C2C12 skeletal muscle cells. PGC1α partly controls PPARγ activation which is



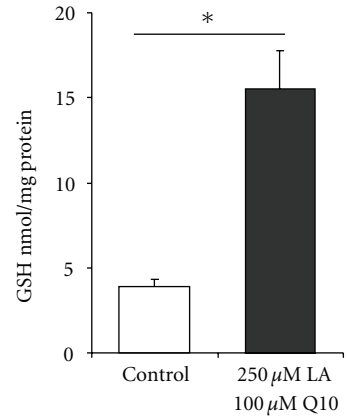
(a)

Gene	NCBI reference sequence ID	Fold change (relative to control)	Number of putative binding sites for Nrf2
GCLC*	NM_010295	2.96	0–4
GCLM*	NM_008129	4.44	0–4
GSR	NM_010344	2.29	1–5
G6PD	NM_019468	2.23	1
PGD	NM_001081274	2.83	1–6
GST $\alpha 3$	NM_001077353	7.67	0–2
GST $\alpha 1$	NM_008181	5.94	1-2
GST $\alpha 2$	NM_008182	4.24	1
GST $\pi 1$	NM_010358	3.21	0–8
GST $\alpha 4$	NM_010357	2.92	0/4
GST $\mu 6$	NM_008184	2.51	0–2
GST $\pi 6$	NM_181796	2.02	0–8
ODC1	NM_013614	2.05	1

*GCLC: γGCS catalytic subunit; GCLM: γGCS modifier subunit

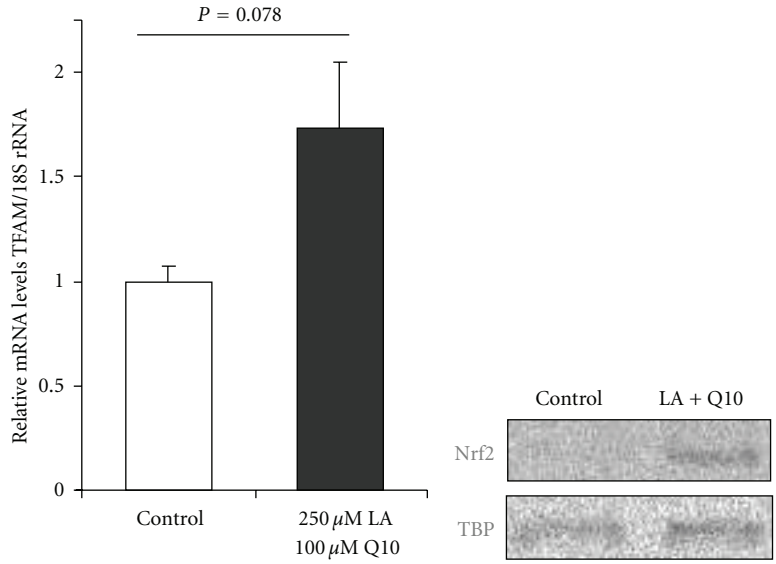
(b)

Figure 2: Continued.



(c)

Figure 2: (a) Treatment of C2C12 cells with 250 μmol/L LA plus 100 μmol/L Q10 for 16 h differentially affects genes encoding proteins involved in glutathione synthesis, recycling, and metabolism as well as glutathione-related phase II response genes including γ-glutamylcysteine-synthetase (γGCS), glutathione reductase (GSR), glucose-6-phosphate dehydrogenase (G6PD), phosphogluconate dehydrogenase (PGD), and glutathione-S-transferases (GST), according to DAVID and KEGG pathway analysis. (b) Indicates fold changes, gene IDs, and the respective putative Nrf2 binding sites in the alternative murine promoter sequences of LA-plus-Q10-regulated genes as determined via MatInspector. (c) Glutathione (GSH) concentration in C2C12 cells in response to LA plus Q10 treatment (data are mean + SEM of three independent experiments; *indicates significant difference to the control cells; $P < 0.05$; Student's t-test).



(a) (b)

Figure 3: (a) TFAM mRNA and (b) nucleic Nrf2 protein levels of C2C12 cells following incubation with 250 μmol/L LA plus 100 μmol/L Q10 for 16 h (mRNA data are mean + SEM of three independent experiments; Mann-Whitney U test).

centrally involved in cellular glucose uptake. In fact it has been previously shown that lipoic acid may stimulate glucose uptake and insulin sensitivity in adipocytes via a PPARγ-dependent signal transduction pathway [15, 16].

PGC1α is a key factor in mitochondrial biogenesis. PGC1α, in the presence of the nuclear respiratory factor 1 (NRF1), coactivates the mitochondrial transcription factor A (TFAM; also called mtTFA) which is essential for mitochondrial replication and transcription [17, 18]. Therefore, we determined TFAM levels in our LA-plus-Q10-treated myotubes. Interestingly, LA plus Q10 resulted in an increase in TFAM mRNA levels as compared to untreated controls (Figure 3(a)). LA plus Q10 cotreatment was more potent than the single treatment with LA and Q10 per se in increasing TFAM gene expression (data not shown). Further studies are needed to prove whether the increase in TFAM gene expression due to the LA plus Q10 cotreatment is also reflected in terms of increased nuclear TFAM protein levels as well as an increased cellular mitochondrial density, ATP synthesis, and oxygen consumption.

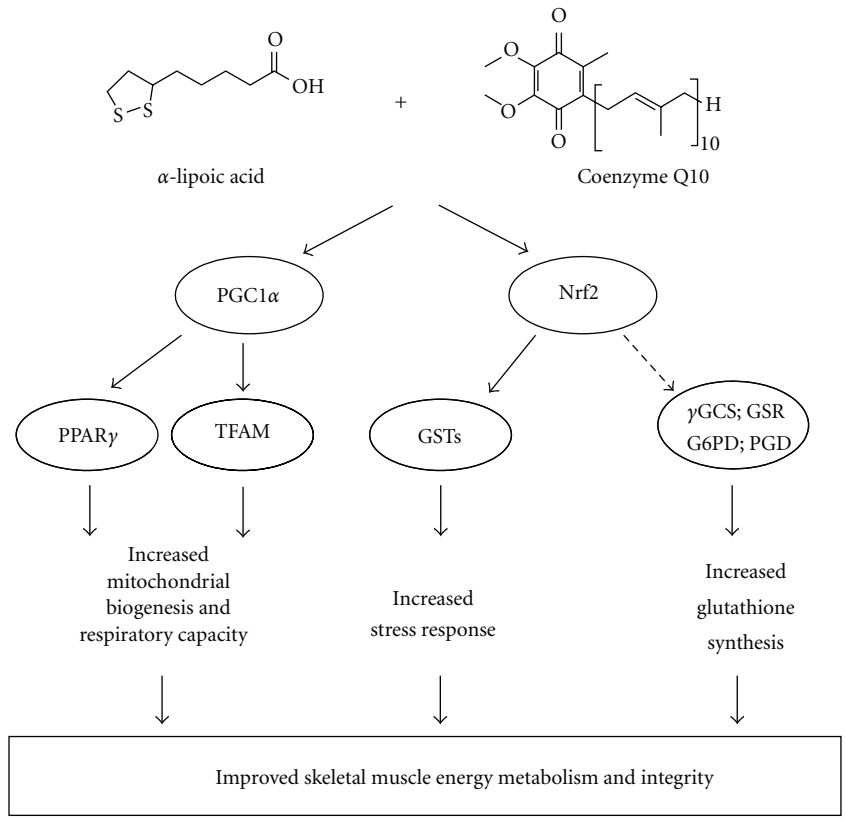


Figure 4: Molecular mechanisms by which the coadministration of LA plus Q10 may improve skeletal muscle energy metabolism and integrity. LA plus Q10 induce the transcription factor PGC1α which may activate TFAM. Furthermore, LA plus Q10 induce GSTs and genes encoding enzymes involved in GSH synthesis and recycling. Ultimately, LA plus Q10 may increase mitochondrial biogenesis, improve stress response, and increase cellular GSH levels.

We observed a significant increase in cellular GSH levels in response to LA plus Q10 treatment in our skeletal muscle cells. Similar to PGC1α cellular GSH levels decrease with age [19]. Thus, LA plus Q10 may prevent age-dependent GSH depletion. The underlying mechanisms by which LA plus Q10 increased cellular GSH may be partly related to an increased cysteine uptake as previously reported [20]. Furthermore, in the present study LA plus Q10 significantly increased gene expression of γGCS, the rate limiting enzyme of GSH synthesis. γGCS gene expression is under transcriptional control of nuclear factor erythroid-like 2 derived factor 2 (Nrf2), a Cap N'Collar basic leucine zipper transcription factor [21–23]. Interestingly, LA plus Q10 treatment also manifold upregulated numerous GSTs which are also Nrf2 target genes [24]. Therefore, we determined Nrf2 nuclear levels in our skeletal muscle cells in response to the LA plus Q10 treatment. Since LA plus Q10 treatment substantially increased nuclear Nrf2 levels, it seems plausible that the induction of GSH via γGCS as well as the induction of GST gene expression in our C2C12 cells may have occurred via a Nrf2-dependent mechanism. In fact, it has been recently suggested that LA, may act as an electrophile which targets cysteine residues of the Nrf2 inhibitor Keap1 [25].

Our gene expression analysis also reveals ornithine decarboxylase 1 (ODC1) as a LA plus Q10 sensitive molecular target (fold change = 2.1, see Figure 2(b)). Interestingly, ODC1 is induced by exercise in skeletal muscle [26]. The increase in ODC1 activity may in turn increase cellular levels of putrescine, spermine, and spermidine [27], thereby improving DNA stability [28], which warrants further investigations.

In this study we tested the combination of LA plus Q10 in terms of PGC1α activity, stress response, and cellular GSH levels. However, we conducted also experiments using LA and Q10 as single treatments in order to relate our results to any of the test substances administered (data not shown). While Q10 was mainly mediating PGC1α inducing activity, LA increased stress response and cellular GSH levels. As far as TFAM gene expression is concerned, neither of the single test compounds enhanced TFAM. Thus, TFAM induction may be due to synergistic interaction between LA and Q10 in coapplication. Accordingly, in order to simultaneously induce PGC1, stress response genes, and cellular GSH levels in skeletal muscle cells a combination of LA plus Q10 rather than a treatment with the single compound may be suggested.

Overall present data indicate that a combination of lipoic acid plus coenzyme Q10 induces PGC1α, improves stress response, and increases cellular glutathione levels in cultured C2C12 skeletal muscle cells as summarized in Figure 4.

We supplemented our myotubes with 250 μmol/L LA and 100 μmol/L Q10. LA concentrations, as used in this study, are by in large physiologically achievable [6, 29], whereas Q10 levels seem to be higher than those achievable in human plasma and skeletal muscle [30]. Thus, current finding regarding the role of LA and Q10 in terms of PGC1α activity, stress response, and antioxidant defense in cultured skeletal muscle cells in vitro should be verified in appropriate in vivo models.

Abbreviations

γGCS:	γ-Glutamylcysteine-synthetase
GST:	Glutathione-S-transferases
G6PD:	Glucose-6-phosphate dehydrogenase 2
GSH:	Glutathione
GSR:	Glutathione reductase
LA:	α-Lipoic acid
Nrf2:	Nuclear factor erythroid-like 2 derived factor 2
ODC1:	Ornithine decarboxylase 1
PGC1α:	Peroxisome proliferator-activated receptor γ (PPARγ)-coactivator α
PGD:	Phosphogluconate dehydrogenase
TFAM:	Mitochondrial transcription factor A
rosi:	Rosiglitazone.

Authors' Contribution

A. E. Wagner and I. M. A. Ernst contributed equally to this work.

Acknowledgment

The authors thank Gaby Steinkamp and Vivien Schmuck for excellent technical assistance.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2012, Article ID 741239, 7 pages
doi:10.1155/2012/741239

Research Article

Serum Oxidant and Antioxidant Status in Adolescents Undergoing Professional Endurance Sports Training

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Received 29 December 2011; Revised 11 February 2012; Accepted 16 February 2012

Academic Editor: Michalis G. Nikolaidis

This study evaluated the impact of professional training on serum oxidant and antioxidant status in adolescent endurance athletes and compared it with that of untrained individuals. Firstly, serum thiobarbituric-acid-reactive substances (TBARSs), xanthine oxidase (XO), catalase (CAT), reduced glutathione (GSH), superoxide dismutase (SOD), and total antioxidant capacity (T-AOC) were measured in 67 male runners, cyclists, and untrained adolescents. Seven-day dietary intakes were also assessed. Secondly, for age- and Tanner-stage-matched comparison, 36 out of the 67 subjects (12 for each group) were then selected and investigated. In cyclists, XO, GSH, and CAT were higher as compared with runners and controls. The CAT in runners, but not GSH and XO, was also higher than in controls. TBARS, T-AOC, and SOD did not differ among the study populations. Regarding the inter-individual relationships among serum redox statuses and dietary nutrient intakes, significant correlations were noted in CAT versus carbohydrates, protein, magnesium, and manganese; GSH versus carbohydrates, protein, fat, selenium, zinc, iron, and magnesium; XO versus cholesterol; CAT versus GSH. These findings suggest that the resting blood redox balance in the professional adolescent athletes was well maintained partly by the increase of individual antioxidant in adaptation to chronic exercise.

1. Introduction

It is known that physical activity plays a critical role in enhancing growth and development in both childhood and adolescence. The healthy adaptations to repeated exercise are featured by increased muscle and bone mass, appropriate body fat composition, angio- and arteriogenesis, and increased number of mitochondria [1]. However, the induced stress and the alterations in immune and inflammatory status in response to exercise are similar to those resulting from chronic diseases such as asthma and arthritis [2]. Such physiological responses are associated with catabolism rather than anabolism. Closely linked to these catabolic responses to exercise is the exercise-mediated oxidative stress, which develops when the production of reactive oxygen species (ROS) exceeds the antioxidant defences [3]. The excessive ROS-caused cytotoxicity, injury, and inflammation in body tissues are known to be associated with a kaleidoscope of pathologies, including cardiovascular and metabolic diseases [3].

Nevertheless, the signalling function of ROS also plays a role in mediating the physiological adaptations gathered from regular exercise, including myokine production that is essential in muscle adaptation to exercise and upregulation of antioxidant defence mechanisms [4, 5]. The regular training-induced adaptations in blood antioxidant capacity and the following attenuated exercise-induced oxidative stress have been clearly demonstrated in adult athletes [6–8]. However, the investigations of the specific training adaptations in highly trained adolescent athletes are scarce to the best of our knowledge. Year-round training in professional sports

beginning at a relatively young age is increasingly frequent in the youth, with training volume being comparable to that of adult athletes (i.e., 1.5 to 3 training hours per session, 2 sessions per day, 6 days per week). Recently, impaired oxidant-antioxidant balance was noted in adolescent endurance runners subsequent to a single-routine training session of exhaustive 21 km run [9]. Whether the resting oxidant-antioxidant levels in professional endurance-trained adolescents are comparable to those reported in their adult counterparts was not clear. As such, the aim of this study was to evaluate the impact of professional training on serum oxidant and antioxidant status in adolescent endurance runners and cyclists, and compare it with that of untrained individuals matched for sex, age, and Tanner stage. It is known that exercise mode specificities can promote different responses regarding oxidative stress [10], and that is why the redox status of adolescent runners and cyclists was examined separately. Since exogenous dietary substance may affect antioxidant defence system, the dietary intake of the athletes and the untrained individuals was also assessed.

2. Method

2.1. Subjects. In this study, totally 67 male adolescent endurance runners ($n = 27$, 15.8 ± 1.4 years), cyclists ($n = 20$, 15.7 ± 1.0 years), and untrained individuals ($n = 20$, 15.7 ± 0.4 years) had been invited to participate in the experiments. All the runners and cyclists performing at national level were trained professionally in a sports club in Liaoning province, China. The untrained adolescents were active, but did not participate in any sports training. All trained and untrained subjects had no familial history of cardiovascular disease or assumed related medication. More importantly, none of them received anti-inflammatory medication or nutritional supplements. Following an explanation of the purpose and constraints of the study, subjects and their tutors gave written informed consent for participating in this study. The local Ethical Committee for the Use of Human and Animal Subjects in Research provided ethical approval of the study.

For age- and Tanner-stage-matched comparison, 36 runners, cyclists, and untrained adolescents (12 for each group) out of the 67 subjects were selected for investigation. The physical characteristics of the selected subjects and the training background of the athletes including the training volume are shown in Table 1. The energy costs for training per day in each athlete were estimated on the bases of the metabolic equivalents (METs) corresponding to the specific training intensity expressed as average running/cycling speed that were reported in the Compendium of Physical Activities [11], the average training hours per day, and the body weight.

2.2. Procedures. Subjects of each group visited the laboratory in a single morning session. The laboratory visit of each group was arranged on separate days. Prior to the laboratory visit, the adolescents abstained from exercise training for 3 days. Upon arrival at the laboratory at 9 am after an overnight fasting, anthropometric measurement and Tanner staging assessment were carried out following a 10 min rest. After that, blood samples were collected with subjects in a seated position. 5 mL venous blood was drawn from the antecubital vein using venous puncture for serum redox analyses. One week preceding the abstinence of exercise training, the diets of consecutive 7 days of the subjects were recorded according to the guidelines for monitoring dietary consumption provided. Diet records were analyzed using the dietary and nutritional analysis system designed for Chinese athletes and general population (National Research Institute of Sports Medicine, China).

2.3. Measurements. In this study, we evaluated the oxidant and antioxidant status of the subjects by quantifying serum concentrations of thiobarbituric-acid-reactive substances (TBARS), reduced glutathione (GSH), and total antioxidant capacity (T-AOC), as well as the enzymatic activity of xanthine oxidase (XO), superoxide dismutase (SOD), and catalase (CAT). After blood sampling in vacuum tubes containing no additives, the serum was separated at 2,000 g for 20 minutes, aliquoted, and stored at -20°C for later analysis.

The levels of TBARS and GSH and enzymatic activity of XO, SOD, and CAT were measured using commercial assay kits (Nanjing Jiancheng Institute, China) on a spectrophotometer (DU7400, Beckman Co, Fullerton, USA), according to the instructions of manufacturer. Briefly, lipid peroxidation was evaluated using the thiobarbituric-acid-reactive substances method and was expressed as a TBARS concentration. This method was used to obtain a spectrophotometric measurement of the colour produced during the reaction of thiobarbituric acid and malondialdehyde (an indicator of peroxidation of polyunsaturated fatty acids in cell membranes subsequent to reactions with ROS) at 535 nm. The TBARS level was expressed as $\text{nmol} \cdot \text{mL}^{-1}$. The GSH level was determined colorimetrically at 412 nm following reaction with DTNB (5,5′-dithio-bis(2-nitrobenzoic acid)) and was expressed as $\text{nmol} \cdot \text{mL}^{-1}$. XO and SOD activities were measured by the xanthine-xanthine oxidase system, which is a superoxide anion generator, following the increase or decrease of absorbance, respectively. The activity of XO and SOD was expressed as $\text{U} \cdot \text{L}^{-1}$ and $\text{U} \cdot \text{mL}^{-1}$. CAT activity, expressed as $\text{U} \cdot \text{mL}^{-1}$, and was determined by the decrease of H_2O_2 absorbance at 240 nm. T-AOC was measured by the ferric-reducing ability of plasma (FRAP) assay of Benzie and Strain [12]. The stable colour of the Fe^{2+}-*o*-phenanthroline complex (produced with reducing agents in plasma by reducing Fe^{3+} to Fe^{2+}, which reacts with the substrate ophenanthroline) was measured at 520 nm. T-AOC was expressed in $\text{U} \cdot \text{mL}^{-1}$, where 1 unit is defined as an increase in absorbance (A_{520}) of 0.01 per min at 37°C.

The inter- and intra-assay coefficients of variation of the above-mentioned biochemical analyses are as follows: TBARS, 5.4% and 2.2%; GSH, 4.8% and 1.8%; XO, 9.2% and 4.5%; SOD, 8.7% and 5.0%; CAT, 11.4% and 6.2%; T-AOC, 8.5% and 4.6%, respectively.

2.4. Statistical Analysis. The Kolmogorov-Smirnov normality test revealed that the data for all the variables were normally distributed. One-way ANOVA was computed to

Table 1: Physical characteristics of study subjects, training years, and training volume of runners and cyclists are shown.

	Runners ($n = 12$)	Cyclists ($n = 12$)	Untrained ($n = 12$)
Age (yrs)	15.5 ± 1.3	15.3 ± 0.7	15.9 ± 0.5
Tanner Stage	3.25 ± 0.87	3.08 ± 0.29	2.75 ± 0.45
Weight (kg)	57.7 ± 6.3[a]	70.8 ± 4.3[a,b]	65.0 ± 8.9
Height (cm)	170.9 ± 5.4[a]	179.3 ± 4.1[a,b]	177.7 ± 5.6
Body fat (%)	9.90 ± 2.3[a]	11.3 ± 1.9[a]	15.8 ± 7.0
BMI	19.7 ± 1.2	22.0 ± 1.3[b]	20.6 ± 2.6
Years of training	2.2 ± 0.9	2.4 ± 0.6	—
Training hours/day	3	5	—
Training days/week	6.5	6	—
Energy costs for training ($Kcal \cdot d^{-1}$)	1,426.0 ± 654.1	2544.9 ± 155.8[b]	—

[a]Significant at $P < 0.05$ when compared with untrained group.
[b]Significant at $P < 0.05$ when compared with runners.
Values are mean ± SD.

Table 2: Serum thiobarbituric-acid-reactive substances (TBARSs), xanthine oxidase (XO), catalase (CAT), reduced glutathione (GSH), superoxide dismutase (SOD), and total antioxidant capacity (T-AOC) in adolescent runners, cyclists, and untrained subjects are shown.

	Runners ($n = 12$)	Cyclists ($n = 12$)	Untrained ($n = 12$)
TBARS ($nmol \cdot mL^{-1}$)	4.85 ± 0.76	4.81 ± 1.04	4.46 ± 1.11
XO ($U \cdot L^{-1}$)	16.1 ± 2.1	19.1 ± 1.4[a,b]	16.7 ± 1.3
GSH ($mg \cdot L^{-1}$)	15.1 ± 4.5	23.7 ± 9.6[a,b]	12.1 ± 2.9
CAT ($U \cdot mL^{-1}$)	1.89 ± 0.55[a]	2.61 ± 0.92[a,b]	0.53 ± 0.36
T-AOC ($U \cdot mL^{-1}$)	15.4 ± 1.6	15.6 ± 2.5	14.3 ± 2.2
SOD ($U \cdot mL^{-1}$)	56.7 ± 3.3	58.2 ± 2.9	61.8 ± 11.1

[a]Significant at $P < 0.05$ when compared with untrained group.
[b]Significant at $P < 0.05$ when compared with runners.
Values are mean ± SD.

examine the differences in TBARS, GSH, XO, SOD, CAT, and T-AOC across groups (runners, cyclists, control). Post hoc analyses using Newman-Keuls were performed for cases in which the main effect was significant. Relationships between variables were assessed using simple regression. All tests for statistical significance were standardized at an alpha level of $P < 0.05$, and all results were expressed as mean ± SD.

3. Results

In this study, cyclists displayed higher body weight and height as compared with the control group, whereas the runners were rather short and leaner. Nevertheless, % body fat was similar between the runners and cyclists, and both were lower than in the control group (Table 1). The training hours per day and training days per week among athletes in each group were identical, since cyclists and runners were trained in same teams. The absolute energy costs (i.e., METs) for training in cyclists were higher than that in runners (Table 1) while the intensity level of both exercise trainings was classified as "rigorous" [3].

For the resting serum oxidant status (Table 2), no significant difference was observed in serum TBARS among the runners, cyclists, and control group, while higher serum XO was found in cyclists as compared with runners and control group ($P < 0.05$). The serum XO between the runners and control group was not significantly different. For the resting serum antioxidant parameters, GSH and CAT were higher in cyclists as compared with runners and control group ($P < 0.05$). The GSH between runners and control group did not significantly differ, whereas the CAT in runners was significantly higher than in the control group ($P < 0.05$). There was no significant difference in serum T-AOC and SOD among the runners, cyclists, and control group ($P > 0.05$).

The average dietary intakes of the runners, cyclists, and control group across consecutive 7 days prior to the blood test are shown in Table 3. The absolute intakes of macronutrients (CHO, protein and fat) as well as the associated total energy intake were significantly higher in the cyclists as compared with those of the runners and control group ($P < 0.05$). The intake of CHO in runners was also higher than that in control group ($P < 0.05$). It is also noteworthy that the cholesterol intake in cyclists was higher than that of control group, while it was relatively lower in runners ($P < 0.05$). Similar to macronutrient intake, the intakes of micronutrient of zinc, iron, manganese, and magnesium

Table 3: Daily dietary intakes of study subjects are shown.

	Runners ($n = 12$)	Cyclists ($n = 12$)	Untrained ($n = 12$)
Total energy intake (Kcal)	2354.4 ± 234.7[a]	3163.3 ± 259.4[a,b]	2133.0 ± 289.0
Protein (g)	83.1 ± 8.95	102.4 ± 10.5[a,b]	86.9 ± 12.1
Protein (%EI)	14.2 ± 1.18[a]	13.0 ± 0.66[a]	16.5 ± 2.37
CHO (g)	366.0 ± 49.7[a]	508.4 ± 40.8[a,b]	296.2 ± 54.7
CHO (%EI)	62.1 ± 3.95[a]	64.3 ± 2.68[a]	55.6 ± 7.20
Fat (g)	62.1 ± 10.5	80.4 ± 12.0[a,b]	66.8 ± 22.2
Saturated fat (g)	9.37 ± 2.10	12.1 ± 4.27	10.0 ± 3.64
Monounsaturated fat (g)	18.3 ± 3.48	20.8 ± 5.03	17.8 ± 8.80
Polyunsaturated fat (g)	11.3 ± 2.34	14.6 ± 2.72	13.4 ± 8.67
Cholesterol (mg)	253.1 ± 52.7[a]	432.5 ± 96.0[a,b]	342.1 ± 119.9
Fat (%EI)	23.8 ± 3.74	21.8 ± 3.61[a]	28.0 ± 7.52
Fibres (g)	11.6 ± 2.36	11.8 ± 1.75	10.7 ± 3.08
Vitamin A (μg RE)	712.8 ± 247.3	754.0 ± 249.1	779.6 ± 468.3
Vitamin C (mg)	86.3 ± 40.2	86.6 ± 11.1	102.3 ± 43.3
α-Tocopherol (mg)	28.8 ± 6.86	33.3 ± 6.16	31.1 ± 19.4
Selenium (μg)	81.9 ± 13.0[a]	114.2 ± 17.9[b]	100.8 ± 28.4
Zinc(mg)	13.5 ± 1.95	16.1 ± 1.20[a,b]	13.6 ± 2.09
Copper (mg)	2.37 ± 0.38	2.92 ± 0.48	2.79 ± 1.12
Iron (mg)	27.6 ± 4.73	31.8 ± 4.05[a,b]	27.3 ± 4.33
Magnesium (mg)	339.5 ± 39.2	413.7 ± 38.0[a,b]	327.7 ± 46.0
Manganese (mg)	7.35 ± 1.22[a]	7.97 ± 0.98[a]	5.68 ± 0.88

[a]Significant at $P < 0.05$ when compared with untrained group.
[b]Significant at $P < 0.05$ when compared with runners.
EI: total energy intake, CHO: carbohydrate.
Values are mean ± SD.

were higher in cyclists ($P < 0.05$). The manganese intake in runners was higher than that of control group, while the intake of selenium was relatively lower ($P < 0.05$).

As regards the relationships between nutrient intakes and serum redox status within all the study population ($n = 36$), we observed that GSH was significantly correlated ($P < 0.05$) with CHO ($r = 0.55$), protein ($r = 0.49$), fat ($r = 0.36$), total energy intake ($r = 0.59$), selenium ($r = 0.37$), zinc ($r = 0.39$), iron ($r = 0.36$), and magnesium ($r = 0.36$). Significant correlations were also found between CAT and CHO ($r = 0.67$), protein ($r = 0.39$), total energy intake ($r = 0.64$), magnesium ($r = 0.43$), and manganese ($r = 0.54$). For serum XO, it was significantly correlated with cholesterol ($r = 0.57$). Among serum oxidant and antioxidant biomarkers, serum CAT and GSH were found to be significantly correlated ($r = 0.70$).

4. Discussion

This study evaluated the resting oxidant and antioxidant status of adolescent athletes who took part in professional training of either long-distance run or road cycling, with a training volume comparable to that of adult athletes (Table 1). In comparison to age- and Tanner-stage-matched untrained individuals, there was a trend of higher serum antioxidant status in athletes, whereas the serum oxidant status appeared to be similar. The elevated resting levels of antioxidant biomarkers in the adolescent athletes, likely secondary to the adaptations to regular endurance training, are in agreement with those reported in adult athletes [7, 13]. These findings in adolescent athletes involved in professional endurance sports training are the first to be described in the current scientific literature to the best of our knowledge.

In the present study, the resting serum TBARS of runners and cyclists appeared not significantly different as compared with that of an untrained group, although a relative higher serum XO was observed in the cyclists (Table 2). The higher serum XO in the cyclists may be attributed to their diet habit rather than chronic exercise-induced increase in the resting enzymatic activity level. The significant correlation of serum XO and cholesterol intake (Figure 1) within our study population as well as the apparent high cholesterol intake in the cyclists (Table 3) may partly explain this difference. This is also in line with a recent notion that diet-induced hypercholesterolemia is associated with increase in XO activity [14]. In this study, the lack of apparent oxidative stress at rest in athletes is in disagreement with that previously observed in adolescent swimmers. Santos-Silva et al. [15] have reported that adolescent swimmers trained 20 hours per week exhibited a higher ratio of resting plasma oxidative stress biomarkers and antioxidant capacity in comparison to that of their untrained counterparts. Similar changes were also observed in children involved in relatively low volume (1 hr session × 4 per week) of swimming training [16]. The absence of marked oxidative stress in athletes in the present study should not be attributed to insufficient

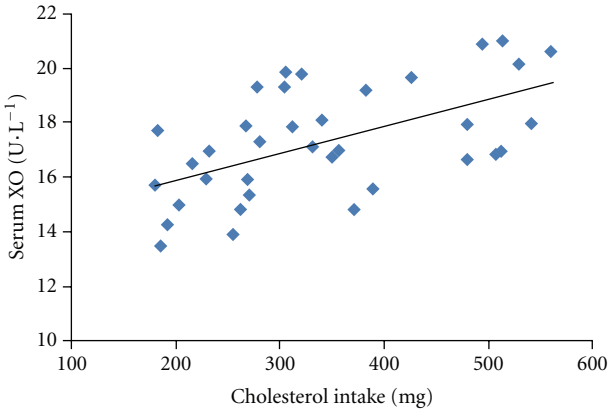

Figure 1: The linear relationship ($r = 0.57$, $n = 36$, $P < 0.05$) between cholesterol intake and serum xanthine oxidase (XO) is shown.

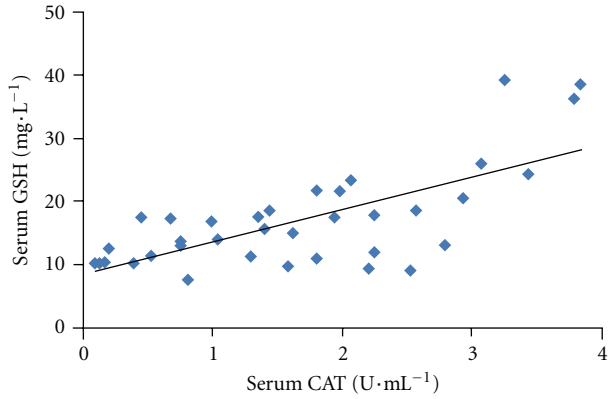

Figure 2: The linear relationship ($r = 0.70$, $n = 36$, $P < 0.05$) between serum catalase (CAT) and reduced glutathione (GSH) is shown.

stimulation of physical work to body as the training volumes of the runners and cyclists were much greater than those of the swimmers engaged in the previous studies. It has been reported that the performance of exhaustive one-leg stepping exercise with different contributions of concentric and eccentric contractions in a 1 : 1 versus 1 : 2 ratio of timing would result in distinct level of oxidative stress in adolescents [10]. This suggests that the nature of activity (eccentric versus concentric; land versus aquatic) may be an important factor for the generation of ROS.

Regarding the blood antioxidant status of the runners and cyclists, the relatively high serum GSH and CAT in comparison with those of untrained subjects reveal that augmented antioxidant capacity in adaptation to chronic exercise likely occurs in the athletes. Although the augmentation of antioxidant capacity with chronic exercise has been regularly reported in adult athletes, such training adaptation was equivocal in children and adolescents. Previous studies found that the antioxidant levels of trained adolescent swimmers were not different or even lower in comparison to that of age-matched untrained adolescents [15, 16]. In contrast, Carlsohn et al. [17] noted in adolescent athletes, but not in untrained controls, that antioxidant capacity increased markedly with age-associated increase in training effort. Kabasakalis et al. [18] found that children aged 10-11 years involved in 23-week intense swimming training (covered >2.5 km per session, ≥3 sessions per week) could improve their antioxidant capacity in the same manner as reported in adult athletes [19]. This implies that training effort/volume, rather than maturation, dominates the development of antioxidant defence system in children and adolescent athletes. This potential dose-dependent mechanism of adaptation to exercise-induced increase in ROS formation is further supported by our current findings that the serum GSH and CAT are relatively higher in cyclists (Table 2) than in runners, for whom the energy costs for training were much lower than in cyclists (Table 1).

Although resting serum CAT and GSH were greater in athletes, the serum T-AOC and SOD were similar between athletes and controls. These inconsistent changes in resting antioxidant biomarkers in adaptation to chronic exercise have also been observed in previous studies [18, 20]. The exogenous antioxidant intakes from habitual diets and the type of exercise and its intensity and duration applied during training are all factors that would influence blood antioxidant status in athletes and might partly explain the discrepancies [21]. In the present study, the dietary intakes of vitamin A, vitamin C, and α-tocopherol, which are deeply involved in antioxidant mechanism, did not differ among runners, cyclists, and controls (Table 3). However, macronutrients and other antioxidant nutrients including CHO and manganese were in a greater amount taken by athletes in comparison to controls. It was noted in our study population that serum CAT and GSH were correlated to the intakes of macronutrient of CHO, protein, and fat. Serum CAT was also correlated with the manganese intake and tends to be correlated with the intake of iron ($r = 0.32$, $P = 0.06$), which both function as cofactor for reduction of the antioxidant enzyme in blood [22]. For the GSH, it was correlated with various antioxidant nutrient intakes including magnesium, selenium, and zinc. Magnesium is essential in GSH synthesis [23], while selenium and zinc are associated with endogenous GSH production and maintenance [24, 25]. These findings suggest that the mechanisms for up-regulation of the endogenous antioxidants in adaptation to chronic exercise in athletes, other than dose-dependent mechanism, may also be associated with their habitual intakes of exogenous antioxidant nutrients. However, the adaptive endogenous processes are not well understood. In the present study, the serum CAT and GSH within study population are significantly correlated (Figure 2). This supports the previous concept that antioxidant defences in human act as a coordinated system, with various metabolites and enzymes having synergistic and interdependent effects on one another [26]. The counterbalance effects of each antioxidant on ROS damages may depend on the proper function of other members of the system [27]. The current findings of the correlation among the antioxidant biomarkers suggest that the maintenance of the resting blood redox balance in adolescent runners and cyclists participating in

professional training might partly result from the integrative effect of augmentation of individual antioxidant.

In summary, the resting blood redox balance was well maintained in the adolescent athletes participating in professional endurance sports training, with training volume comparable to that of adult athletes. The maintenance of the redox balance might partly result from the integrative effect of augmentation of individual antioxidant in adaptation to chronic exercise. Such adaptive endogenous processes in athletes might be associated with their habitual intakes of antioxidant nutrients. Notwithstanding the limited number of subjects studied, this investigation has however several strengths. First, although the number of blood redox biomarkers may still not thoughtfully reflect the specific adaptations to chronic exercise, our findings provide for the first time a reasonable information regarding distinct resting serum oxidant and antioxidant status in professional adolescent endurance athletes. Then, the high homogeneity of the study populations allows a very reliable comparison among adolescents engaged in different sports disciplines (e.g., cycling and running), as well as with untrained controls. For future research, further assessment of additional biomarkers (e.g., plasma F_2-isoprostanes, plasma antioxidant vitamins, Trolox-equivalent antioxidant capacity, uric acid) could give us more information about oxidant and antioxidant status of adolescents professional athletes.

Acknowledgment

This study was supported by a Research Grant from the General Administration of Sport of China (no. 10B001).

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