Overloaded Training Increases Exercise-Induced Oxidative Stress and Damage

Stephane Palazzetti[1], Marie-Jeanne Richard[2], Alain Favier[2], and Irene Margaritis[1]

Abstract/Résumé

We hypothesized that overloaded training (OT) in triathlon would induce oxidative stress and damage on muscle and DNA. Nine male triathletes and 6 male sedentary subjects participated in this study. Before and after a 4-week OT, triathletes exercised for a duathlon. Blood ratio of reduced vs. oxidized glutathione (GSH/GSSG), plasma thiobarbituric acid reactive substances (TBARS), leukocyte DNA damage, creatine kinase (CK), and CK-MB mass in plasma, erythrocyte superoxide dismutase (SOD) activity, erythrocyte and plasma glutathione peroxidase (GSH-Px) activities, and plasma total antioxidant status (TAS) were measured before and after OT in pre- and postexercise situations. Triathletes were overloaded in response to OT. In rest conditions, OT induced plasma GSH-Px activity increase and plasma TAS decrease (both $p < 0.05$). In exercise conditions, OT resulted in higher exercise-induced variations of blood GSH/GSSG ratio, TBARS level (both $p < 0.05$), and CK-MB mass ($p < 0.01$) in plasma; and decreased TAS response ($p < 0.05$). OT could compromise the antioxidant defense mechanism with respect to exercise-induced response. The resulting increased exercise-induced oxidative stress and further cellular susceptibility to damage needs more study.

---

[1]Laboratoire Physiologie des Adaptations, Performance Motrice et Santé, Faculté des Sciences du Sport, Université de Nice-Sophia-Antipolis, 261 Route de Grenoble, BP 3259, 06205 Nice Cedex 3, France; [2]Laboratoire de Biologie du Stress Oxydant, Centre Hospitalo-Universitaire A. Michallon, BP 217X, 38043 Grenoble Cedex 9, France.

Nous avons émis l'hypothèse selon laquelle une surcharge de l'entraînement (OT) en triathlon provoquerait un stress oxydant et un endommagement cellulaire au niveau musculaire et de l'ADN. Neuf triathlètes masculins et 6 sujets sédentaires masculins ont participé à cette étude. Les triathlètes ont réalisé un duathlon avant et après 4 semaines de OT. Les analyses suivantes ont été effectuées avant et après OT, de même que avant et après le duathlon: rapport glutathion réduit/oxydé (GSH/GSSG), substances réactives à l'acide thiobarbiturique (TBARS) plasmatique, endommagement de l'ADN leucocytaire, créatine kinase (CK) et CK-MB masse plasmatiques, activité de la superoxyde dismutase (SOD) érythrocytaire, activités de la glutathion peroxydase (GSH-Px) plasmatique et érythrocytaire, statut antioxydant total (TAS) plasmatique. Les triathlètes étaient en état de surcharge après les 4 semaines d'entraînement. Au repos, OT a induit une augmentation de l'activité de la GSH-Px plasmatique et une diminution du TAS plasmatique (p < 0,05 pour les deux). En situation d'exercice, OT a provoqué une augmentation de la variation du rapport GSH/GSSG, du niveau de TBARS (p < 0,05 pour les deux) et de CK-MB masse (p < 0,01) plasmatique; et une diminution de la réponse du TAS (p < 0,05). La surcharge de l'entraînement pourrait compromettre le mécanisme de défense antioxydant au regard de la réponse induite par l'exercice. L'augmentation du stress oxydant et de la vulnérabilité de cellules à l'endommagement, en réponse à l'exercice en surcharge, nécessite d'autres investigations.

Introduction

Physical exercise leads to increased metabolic demand which in turn generates free radicals. During physical exercise, the major source of free radicals is thought to be the mitochondria of active muscles (Sen, 1995). The increased production of reactive oxygen species (ROS) may overwhelm the defense system and initiate oxidative stress, which can induce various cellular damages affecting lipids, proteins, and nucleic acids (Sen, 1995). The exercise-induced inflammatory response can also help trigger ROS production (Camus et al., 1993). It is known that heavy acute endurance exercise induces DNA damage in leukocytes (Niess et al., 1998), but nothing is known about chronic stimulation. To prevent exercise-induced oxidative stress, the organism is well equipped with an antioxidant defense system including enzymes such as catalase, superoxide dismutases (SOD), and glutathione peroxidases (GSH-Px), and nonenzymatic substances such as vitamins and reduced glutathione (Sen, 1995).

The excessive production of ROS during exercise severely hampers the antioxidant defenses and causes changes in cellular homeostasis (Child et al., 1998; Marzatico et al., 1997; Mena et al., 1991; Vasankari et al., 1997). Because of repetitive stimulation, well-controlled aerobic training can overstimulate the genes and thus enhance the activity of various antioxidant enzymes (Mena et al., 1991; Robertson et al., 1991; Tessier et al., 1995) and the glutathione status (Robertson et al., 1991). This enhanced activity consequently reduces the magnitude of exercise-induced stress and cellular damage during exercise (Margaritis et al., 1997).

However, an excessively stimulated adaptive effect (exercise) associated with insufficient recovery can damage antioxidant proteins, deplete cellular scavenger stores, and may provoke a transitory or prolonged lack of physiological and/or biochemical adaptations with or without associated clinical signs (Fry et al., 1991). Thus this imbalance between training loads and recovery, which may lead to an overtraining state, could also be involved in the failure of antioxidant systems to

adapt to training. Consequently, imbalance between ROS production and antioxidant response could cause chronic oxidative stress and cellular damage. However, to date nothing is known about the effects of overloaded training (OT) on exercise-induced oxidative stress. It can be expected that the oxidative stress consequences of OT in rest conditions would be similar to the acute effects of exercise. We sought to determine whether an OT in triathlon (1) induces oxidative stress, muscular impairment, leukocyte DNA damage, and lipid peroxidation both during the training and rest periods, and (2) increases oxidative stress, muscle damage, and lipid peroxidation during exercise.

Materials and Methods

SUBJECTS

Nine well-trained male triathletes (age 31.9 ± 7.0 yrs; height 172.4 ± 9.2 cm; body mass 67.7 ± 7.7 kg; body fat $11.7 \pm 3.3\%$; $\dot{V}O_2$max 66.0 ± 3.9 ml·kg^{-1}·min^{-1}) and six male sedentary subjects, the control group (age 29.0 ± 7.2 yrs; height 179.4 ± 3.4 cm; body mass 80.2 ± 14.5 kg; body fat $21.1 \pm 5.7\%$; $\dot{V}O_2$max 42.8 ± 6.5 ml·kg^{-1}·min^{-1}), participated in this study. Triathletes were long-distance competitors, who must continually manage social, occupational, family, and sport activities. Experimental procedures were approved by the Committee for the Protection of Persons in Biomedical Research (No. 99002), and all subjects gave written informed consent after receiving an explanation about the purpose, possible risks, and stress associated with the study. All were nonsmokers, had no history of medical disorders, and had not taken antioxidant supplements for at least 6 months prior to the study. They were instructed to refrain from making any drastic changes in diet and to abstain from anti-inflammatory or analgesic drugs throughout 8-week study.

PROTOCOL

Experimental Procedures for Well-Trained Male Triathletes.   Referring to the competition program of long-distance triathletes, training was overloaded (OT) during 4 weeks following a 4-wk-normal training (NT) phase. Each triathlete underwent maximal functional assessments in swimming, cycling, and running (see below) before NT. Before and after OT, the triathetes completed a duathlon test (5 km-run, 20 km-bike, 5 km-run) preceded 2 days earlier by a maximal treadmill test. Each functional assessment or duathlon test was separated by a day off and was performed at the same time of day. Urinary and venous blood samples were collected before and after OT. Only venous blood samples were collected in postexercise conditions before and after OT.

Maximal Multistage Swim Test.   To evaluate maximal swimming velocity and define training intensities in swimming, triathletes underwent a maximal multistage swim test (Lavoie et al., 1985) in an indoor 25-m pool (constant air and water temperature 27–28 °C).

Maximal Cycle Ergometer Test.   In the electrically braked cycle ergometer (Ergo-Metrics 900 S, Jaeger, Wuerzburg, Germany) test, triathletes were asked to maintain a constant pedal rate of 70 rpm. The initial load was 80 W for 5 min, followed by 40-W increases every 2 min until exhaustion.

Maximal Treadmill Test. Triathletes underwent a continuous, incremental running test on a motorized treadmill (2500 ST, Gymrol, Andrezieux Boutheon, France). The test began with a warm-up at 10 km per hour (2% slope) for 5 min; running speed was then increased by 2 km per hour every 2 min up to 14 km per hour, and then by 1 km per hour until exhaustion.

Data Analysis. During cycle ergometer and treadmill tests, ventilatory and gas exchange responses were measured on a breath-by-breath basis via an automatic spiroergometric system (Vmax 29, Sensor Medics, Rungis, France). Throughout the test, heart rate (HR) was monitored continuously and recorded using an electrocardiograph monitor (Hellige, SMS 182, Freibourg in Breisgau, Germany) and a telemetric system (Polar Accurex Plus, Polar Electro Oy, Kempele, Finland). The criteria used for determining $\dot{V}O_2max$ were a plateau in $\dot{V}O_2$ despite an increase in load or running speed, a respiratory exchange ratio (RER) above 1.1, and a heart rate over 90% of the age-predicted maximal HR. The data obtained from these tests were used to determine the intensities of training loads applied in cycling and running.

Training Program. Normal training was quantified based on swimming, cycling, and running distances completed the month prior to the study, in order to maintain the same training loads until the beginning of overloaded training. During OT, 100% individual capacity and the increase in training volumes (swimming, cycling, and running) were determined based on the highest triathlon distances completed in one week during the last 2 years of training. In swimming, intensities of training loads were calculated on the basis of maximal swimming velocity. In cycling and running, intensities of training loads were calculated on the basis of individual $\dot{V}O_2max$.

Five intensity levels were identified: Level 1 = 55 to 65% of $\dot{V}O_2max$; Level 2 = 65% of $\dot{V}O_2max$ to –5% of ventilatory anaerobic threshold; Level 3 = –5% of ventilatory anaerobic threshold to ventilatory anaerobic threshold; Level 4 = ventilatory anaerobic threshold to +5% of ventilatory anaerobic threshold; Level 5 = +5% of ventilatory anaerobic threshold to 100% of $\dot{V}O_2max$. Intensity levels increased from NT to OT. Endurance training principles (long-distance training, interval training, and fartleck) were applied in swimming, cycling, and running for NT and OT. During overloaded training the triathletes performed "multi-linking" (run-bike-run-bike-run).

Quantification of Training Loads in Triathlon. Individual training loads were quantified by a modified version of the method of Morton et al. (1990):

$$W(t) = D \ I \ K$$

where W(t) is the assessment of amount of training undertaken during a training session (arbitrary units), D is the duration (h) of training session, I is the relative intensity of training session (% of $\dot{V}O_2max$), and K is the weighting factor (ae^{bI}) applied to increase the magnitude of quantity of training nonlinearly at higher training intensities. The ae^{b} is determined from exponential regression analysis between ventilatory equivalent of oxygen ($\dot{V}_E/\dot{V}O_2$) and percentage of $\dot{V}O_2max$. Total training loads (arbitrary units) in swimming, cycling, and running are summarized in Figure 1.
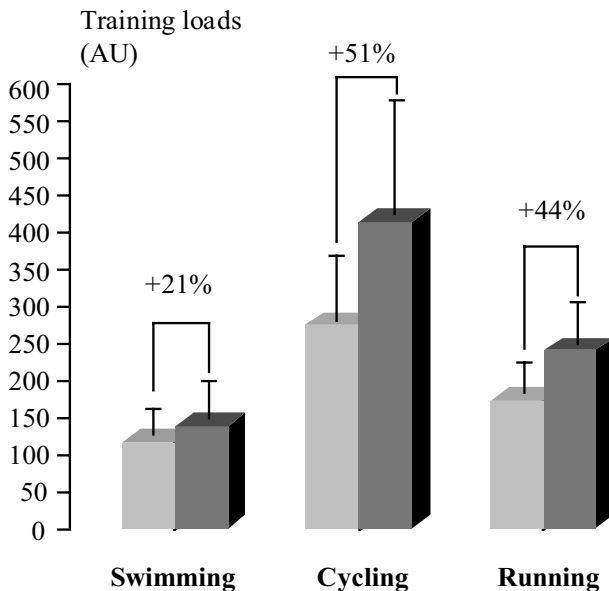
Training loads
(AU)

Figure 1. Quantification of total training loads in swimming, cycling, and running in NT (light bars) and OT (darker bars). Training loads are expressed in arbitrary units (AU).

Training Logs. Triathletes kept a daily training log of swimming, cycling, and running volumes, and gave subjective ratings of training intensity (modified Borg scale) on a 15-point scale. In addition, each triathlete kept a record of morning heart rate upon awakening, as well as sleep patterns (i.e., hours of sleep, quality of sleep), body mass, occurrence of illness, and causes of stress (e.g., emotional disturbances).

Profile of Mood States. Every week each triathlete completed the Profile of Mood States questionnaire (POMS) (McNair et al., 1992). The POMS was administered to quantify the influence of training loads on mood state.

Dietary Records. During NT and OT, the triathletes kept daily dietary records. They were all instructed on proper nutritional recording, including estimating portion sizes. Dietary record analysis showed that the recommended daily amounts in macro- and micronutrients (vitamins A, C, and E; trace elements selenium, copper, and zinc) met French standards.

Duathlon Test. All duathlon tests were held outdoors between March and April in Nice, France. Outside temperature ranged from 17 to 22 °C. Triathletes underwent all testing in the same equipment conditions and drank the same energy beverage. Before each duathlon test, all triathletes undertook a 30-min warm-up by alternating jogging and stretching. Running trials were performed on a flat circuit alternating lawn and asphalt. Cycling was done on an exercise bike (EliteTravel, Fontaniva, Italy) over which was positioned the triathletes' own personal bike. Duathlon tests were performed at $84 \pm 2\%$ of $\dot{V}O_2max$.

Experimental Procedures for Sedentary Subjects.   Sedentary subjects underwent three continuous, incremental tests until volitional exhaustion during a maximal ergometer cycle test in baseline, after 4 weeks, and again 4 weeks later. In the electrically braked cycle ergometer (Ergo-Metrics 900 S, Jaeger) test, sedentary subjects were asked to maintain a constant pedal rate of 60 rpm. The initial load was 60 W for 5 min, followed by 30-W increases every 2 min until exhaustion. Data analysis is described above. Repeated functional assessments, performed in the evening, allowed us to establish sedentary status. Sedentary subjects recorded daily sleep patterns, any occurrence of illness and causes of stress, and completed the POMS questionnaire every week. Urinary and venous blood samples were collected all 4 weeks. Sedentary subjects served as environmental controls for biological parameters in rest conditions only.

Blood and Urinary Sampling Procedures.   Subjects reported to the laboratory after a day off and an overnight fast. The time of day for basal blood test was standardized to within 30 min for each subject, and all samples were taken between 6 and 8 a.m. Postexercise venous blood samples were obtained immediately after the duathlon tests, which took place the same day in late afternoon. Blood samples were collected by venipuncture from an antecubital vein. Four hundred µl of whole blood for glutathione analysis and 5 µl of whole blood for DNA damage analysis were immediately treated, as described later. The blood samples were centrifuged at 4000 rpm, 4 °C, for 10 min, and plasma or serum was divided into aliquots and frozen in dry ice prior to storage at –80 °C until assay. Urine was collected the night before the blood samples were drawn, using bottles containing 5 ml of concentrated hydrochloric acid. The subjects voided just before going to bed; thereafter, the total urine, including the early morning urine, was collected during a 6- to 8-hour nocturnal resting phase. Urine was stored at 4 °C and transferred to the laboratory.

Measurement of Catecholamine Metabolites.   Urinary catecholamine excretion was assayed by high-pressure liquid chromatography (HPLC) with electrochemical detection (Riggin and Kissinger, 1977).

Free Testosterone.   Serum free testosterone was determined via solid phase ^{125}I radioimmunoassay (Diagnostic Products, Los Angeles, CA).

Cortisol.   Serum cortisol was determined using an automated ELISA system (model ES300, Boerhringer Mannheim, Germany) with a cortisol kit (Boehringer Mannheim, No. 1098 578).

Oxidized and Reduced Glutathione.   Immediately after venipuncture, 400 µl of whole blood was transferred into a tube containing 3600 µl of 6% (v/v) metaphosphoric acid in water. The solution was mixed and centrifuged for 10 min at 4 °C. The acidic protein-free supernatants were stored at –80 °C until analysis.

Total glutathione was determined by the method of Akerboom and Sies, slightly modified (Emonet et al., 1997) with 2-vinyl pyridine. An aliquot of the deproteinized extract was neutralized with a solution containing 0.4 M N-morpholinopropanesulfonic acid and 2 mM EDTA adjusted to pH 6.75 with 1 M KOH. Glutathione was determined using enzymatic cycling of reduced glutathione (GSH) by means of NADPH and glutathione reductase (GR) coupled with DTNB. The fraction of GSH was calculated as GSH = total glutathione –2 GSSG. To assay

oxidized glutathione, we masked GSH by adding 10 µl of 3-vinyl-pyridine to 500 µl of deproteinized extract adjusted to pH 6 with triethanolamine. The mixture was allowed to stand for 60 min.

Total CK Activity.   Plasma total creatine kinase (CK) activity was assessed by an automated enzymatic procedure, using a reflectance spectrophotometric method (Kodak Ektachem 700 analyzer).

CK-MB Mass.   The mass level of creatine kinase isoenzyme MB (CK-MB mass) was determined in plasma by immunoassay using the ELISA sandwich principle with fluorogenic marker.

Myoglobin.   Plasma myoglobin was assessed using a standardized spectrophotometric method (Turbiquant myoglobin).

Single-Cell Gel Electrophoresis Assay.   The single-cell gel electrophoresis technique (SCG or comet assay) was performed as described by Singh et al. (1988), with minor modifications (Emonet et al., 1998). A total of 150 microliters of 0.5% agarose diluted in Ca- and Mg-free PBS buffer was added to fully frosted microscope slides (Touzart et Matignon, Paris, France), immediately covered with coverslips, and kept for 10 min in a refrigerator to solidify. Then we removed the coverslips and added to the slides 5 µl of whole blood mixed with 60 µl of 0.6% low-melting-point agarose (LMPA; Biozym, Hessisch Oldendorf, Germany) diluted in Ca- and Mg-free PBS buffer (60 µl). The slides were covered again with a coverslip and kept in the refrigerator for another 10 min to solidify the LMPA. After removing the coverslips again, we immersed the slides in a jar containing cold lysing solution (2.5 mM NaCl, 100 mM EDTA, 10 mM TRIS, 1% Na-Sarcosinate, 1% Triton X-100, and 10% dimethyl sulphoxide were added fresh) and kept them at 4 °C for at least 16 hours.

We conducted electrophoresis using a freshly made alkaline buffer (10 N NaOH and 200 mM EDTA, pH 10.0). The cells were first exposed to this alkali buffer for 40 min to allow for DNA unwinding and expression of alkali-labile sites. All these steps were conducted under dim light to prevent any additional DNA damage. After electrophoresis (25 V, 300 mA, 30 min), we placed the slides horizontally and added Tris buffer (0.4 M Tris, pH 7.5) to neutralize the excess alkali. The slides were allowed to sit for 5 min and this neutralization step was repeated three times. Finally, we added 50 µl of ethidium bromide ($20 \, \mu g.ml^{-1}$) to each slide, which was covered with a coverslip and kept in a humidified box at 4 °C until analysis.

Slides were examined using an epifluorescence microscope, Zeiss Axioskop 20 (Carl Zeiss, Microscope Div., Oberkochen, Germany) equipped with a short arc mercury lamp HBO (50 W, 516–560 nm, Zeiss) and filters 5 and 15 (Zeiss) at 20x magnification. Fifty randomly selected comets on each slide were scored with a Pulmix TM 765 camera (Kinetic Imaging, Liverpool, UK), linked to an image analysis system Komet 3.0 (Kinetic Imaging). DNA damage was quantified using the tail moment. Tail moment is determined by the product of the tail distance, i.e., the distance between the center position of the head and the center of gravity of the tail, and the percentage of DNA in the tail relative to the total amount of DNA in the entire comet (head + tail) (Hellman et al., 1995).

Indices of Lipid Peroxidation.   Thiobarbituric acid reactants were evaluated in plasma by a Perkin Elmer Model LS 50 fluorometer (Perkin-Elmer Ltd,

Bucks, UK) with a malondialdehyde-kit (Sobioda, Grenoble, France) as previously described (Richard et al., 1992).

Metalloenzymes.   Plasma and erythrocyte selenium-dependent GSH-Px were evaluated using terbutyl hydroperoxide (Sigma Chemical Co, Via Coger, Paris) as substrate instead of hydrogen peroxide. This technique was adapted on a Hitachi 904 analyzer. Results are expressed as μmoles of nicotinamide adenine dinucleotide phosphate (NADPH, Boehringer-Mannheim) oxidized per minute per gram of hemoglobin for erythrocyte GSH-Px and as unit per liter for plasma GSH-Px.

Erythrocyte Cu-Zn SOD activity was measured after hemoglobin precipitation by monitoring the autoxidation of pyrogallol according to the technique of Marklund and Marklund (1974). This technique was adapted on a Hitachi 904 analyzer.

Total Antioxidant Status.   Total antioxidant status (TAS) was measured in plasma by the chemiluminescent technique using a Hitachi 904 analyzer with a TAS Randox-kit (Randox Laboratories Ltd, Roissy, France).

STATISTICAL ANALYSIS

All data are expressed as means and standard deviations (*SD*). Basal biochemical data (PV1) were analyzed by Student *t*-test for nonpaired values. To evaluate changes in duathlon performance, body mass, resting HR, and perception of training difficulty, we used Student *t*-test for paired values. Biochemical or physiological data were analyzed by ANOVA with repeated measures, first to determine interaction effects between groups (Triathlete group, Sedentary group) and training, and second to determine main effects (training or exercise) and interaction effects between training (NT, OT) and exercise in the Triathlete group. Subjective data (sleep patterns and total POMS score) were also analyzed by repeated-measures ANOVA. When significant changes were observed in ANOVA tests, Fisher's PLSD post hoc test was applied to locate the source of significant differences. Statistical significance level was set at $p < 0.05$.

Results

EFFECTS OF OT IN TRIATHLON, REST CONDITIONS

At the beginning of the study, the biochemical parameters were identical for both groups. The only exception was that the free-testosterone-to-cortisol ratio was higher in the Sedentary group than in the Triathlete group, 0.20 ± 0.06 vs. 0.13 ± 0.04 (10^{-3}), $p < 0.05$. Overloaded training in triathlon had no effect on body mass (67.8 ± 7.5 to 67.3 ± 7.6 kg), resting heart rate (49.9 ± 5.3 to 48.0 ± 3.8 beats.min^{-1}), or sleep patterns (quantity = 7.5 ± 0.8 to 7.5 ± 0.7 hrs a day; quality scale = 2.7 ± 0.2 to 2.6 ± 0.6).

The perception of training difficulty (modified Borg scale) increased significantly in response to OT in swimming, from 10.9 ± 1.3 for NT to 12.8 ± 1.4 for OT, $p < 0.01$; in cycling, from 11.2 ± 1.1 for NT to 13.0 ± 1.4 for OT, $p < 0.05$; and in running, from 11.3 ± 1.0 for NT to 13.3 ± 1.2 for OT, $p < 0.01$. The total POMS score was significantly increased with OT, from 87.2 ± 8.3 for NT to 98.6 ± 6.9 for OT, $p < 0.05$.

$\dot{V}O_2$max and maximal HR did not change during OT. For the Triathlete group ($n = 9$), $\dot{V}O_2$max was 66.6 ± 3.8 to 66.0 ± 4.0 ml·kg^{-1}·min^{-1}; heart rate was 181.2 ± 13.1 to 179.7 ± 12.6 beats·min^{-1}. For the Sedentary group ($n = 6$), $\dot{V}O_2$max was 43.8 ± 6.9 to 42.2 ± 5.6 ml·kg^{-1}·min^{-1}; heart rate was 180.0 ± 7.5 to 180.3 ± 9.2 beats·min^{-1}. Duathlon performance decreased significantly in response to OT (66.0 ± 5.9 to 68.4 ± 6.7 min, $p < 0.05$).

Overloaded training resulted in a significant ($p < 0.05$) increase in urinary epinephrine excretion but did not affect urinary norepinephrine excretion (Table 1). The free-testosterone-to-cortisol ratio did not change significantly in response to OT. For the Triathlete group ($n = 9$) the values were 0.13 ± 0.04 to 0.12 ± 0.05 (10^{-3}). For the Sedentary group ($n = 6$) the values were 0.20 ± 0.06 to 0.17 ± 0.10 (10^{-3}).

Sport practice as well as OT had no effect on GSSG level or on GSH/GSSG ratio (Table 2). The TBARS levels did not differ significantly in rest conditions.

Table 1   Nocturnal Urinary Epinephrine and Norepinephrine Excretions Before and After OT

| | Triathletes ($n = 9$) | | Sedentary ($n = 6$) | | ANOVA |
	Before	After	Before	After	(Group × OT)
U-epinephrine	2.5 ±1.5	5.0* ±2.4	3.7 ± 2.1	2.5 ±1.3	$p < 0.05$
U-norepinephrine	18.8 ±9.0	30.3 ±26.7	24.8 ±10.3	30.2 ±15.2	n.s.

Note: Values are means $\pm$ *SD* in nmol/mmol of creatinine.
*Significant before/after differences, $p < 0.05$

Table 2   Markers of Free Radical Production (mean $\pm$ *SD*) Before and After OT at Rest

| | Triathlete group ($n = 9$) | | Sedentary group ($n = 6$) | |
	Before	After	Before	After
GSSG (µmol·L^{-1})	19.8 ± 4.6	21.0 ± 6.4	18.9 ± 5.0	19.9 ± 4.4
GSH/GSSG ratio	55.7 ± 15.5	59.4 ± 29.3	52.3 ± 18.2	47.1 ± 13.6
TBARS (µmol·L^{-1})	2.16 ± 0.30	2.11 ± 0.31	2.22 ± 0.17	2.17 ± 0.13
Tail moment	10.6 ± 5.3	23.7 ± 12.5	11.9 ± 9.2	13.3 ± 3.8

Note: GSSG = whole blood oxidized glutathione; GSH/GSSG ratio = whole blood reduced glutathione to oxidized glutathione; TBARS = plasma thiobarbituric acid reactive substances.

Table 3 **Indices of Muscle Damage in Plasma (mean ± *SD*) Before and After OT at Rest**

| | Triathletes (*n* = 9) | | Sedentary (*n* = 6) | | ANOVA |
	Before	After	Before	After	(Group × OT)
Total CK activity	161.2	182.37*	156.4	121.0	
(U/l)	±111.9	±115.7	±130.6	±50.5	*p* < 0.05
CK-MB mass	3.8	3.2	1.4	1.3	
	±2.8	±2.0	±0.5	±0.6	n.s.
Myoglobin	35.4	35.4	41.7	43.9	
(ng/ml)	±7.6	±8.9	±13.7	±18.1	n.s.

Note: CK-MB mass = mass level of creatine kinase isoenzyme MB.
*Significant before/after differences, *p* < 0.05

Results of the SCG assay (tail moment) (Table 2) did not show significant change in response to OT. Total CK activity increased significantly, *p* < 0.05, in response to OT (Table 3). On the other hand, OT did not cause any changes in the CK-MB mass and myoglobin levels.

OT resulted in a significant increase in plasma GSH-Px activity and a significant decrease in plasma TAS (both *p* < 0.05). The erythrocyte SOD, GSH-Px activities, and blood GSH levels did not differ significantly between groups after OT (Table 4).

EFFECTS OF OT IN TRIATHLON ON EXERCISE-INDUCED RESPONSE

ANOVA tests showed a significant interaction effect between training and exercise for the blood GSH/GSSG ratio, TBARS level (both *p* < 0.05), CK-MB mass, and TAS level (both *p* < 0.01) in plasma (Figure 2). This effect was mainly due to significant variations after OT, evidenced by post hoc analysis. For the TAS levels, post hoc analysis revealed that the increase in TAS with exercise before OT disappeared afterward in response to OT (Figure 2).

The lack of changes in erythrocyte GSH-Px and SOD activities was not modified by OT (Table 5). Independent of training phase, duathlon induced an increase in blood GSSG level, GSH-Px activity (both *p* < 0.01), total CK activity, and myoglobin (both *p* < 0.001) in plasma, and induced a decrease in blood GSH level (*p* < 0.001) (Table 5).

Discussion

Chronic stress induced by OT impairs physiological and/or biochemical adaptations, with or without associated clinical signs (Fry et al., 1991). We hypothesized that OT would lead to a lack of adaptation referring to redox status, and consequently that it would increase the oxidative damage.

**Table 4   Parameters of Antioxidant Potential (mean ± *SD*) Before and After OT at Rest**

| | Triathletes (*n* = 9) | | Sedentary (*n* = 6) | | ANOVA |
	Before	After	Before	After	(Group × OT)
Cu-Zn-SOD	1.33	1.32	1.32	1.32	
(U/mgHb)	±0.12	±0.11	±0.12	±0.14	n.s.
GSH-Px	41.9	40.9	41.0	40.3	
(U/gHb)	±5.2	±6.0	±6.2	±7.5	n.s.
Plasma GSH-Px	390.7	425.9*	378.6	381.8	
(U/l)	±23.2	±36.4	±45.4	±41.1	$p < 0.05$
GSH	1048.0	1093.6	923.2	904.2	
(μmol·L^{-1})	±144.6	±185.6	±129.9	±191.9	n.s.
TAS	1.51	1.45*	1.52	1.54	
(mmol·L^{-1})	±0.07	±0.06	±0.12	±0.10	$p < 0.05$

Note: Cu-Zn-SOD = erythrocyte superoxide dismutase activity; GSH-Px = erythrocyte glutathione peroxidase activity; plasma GSH-Px = plasma glutathione peroxidase activity; GSH = whole blood reduced glutathione; TAS = plasma total antioxidant status. *Significant before/after differences, $p < 0.05$

The increased urinary epinephrine excretion suggests that the 4-wk OT leads to an overloaded state. This state was confirmed by the decreased duathlon performance and the increased total POMS score and perception of training difficulty. We would expect changes in anabolic and catabolic activity balance with OT. In our study, the stressful state occurred without any changes in plasma levels of free testosterone and cortisol, as already shown (Fry et al., 1991; Kirwan et al., 1990).

Blood GSH/GSSG rest ratio is a sensitive index of cellular redox status (Sen, 1995), for which no change was detected after 4 weeks OT in triathletes. Increased blood GSH/GSSG ratio was reported only after a 10-wk endurance training program in previously sedentary subjects (Tessier et al., 1995). Furthermore, in that case the increased GSH/GSSG ratio was associated with lower blood GSH and GSSG levels. In our study, the magnitude of exercise-induced decrease in blood GSH/GSSG ratio was increased in the OT condition and manifested as an increase in exercise-induced oxidative stress. Such a decrease in skeletal muscle and blood GSH/GSSG ratio has been observed in response to acute exercise in elite marathon runners (Corbucci et al., 1984), national level runners (Sastre et al., 1992), and sedentary subjects (Laaksonen et al., 1999). In well-adapted athletes, a long-distance triathlon race did not induce oxidative stress (Margaritis et al., 1997). The exercise-induced decrease in blood GSH/GSSG ratio seems to occur in high physiological stress or sedentary states. In these cases it may be due to a lack of ability to reduce glutathione oxidized in exercise conditions.
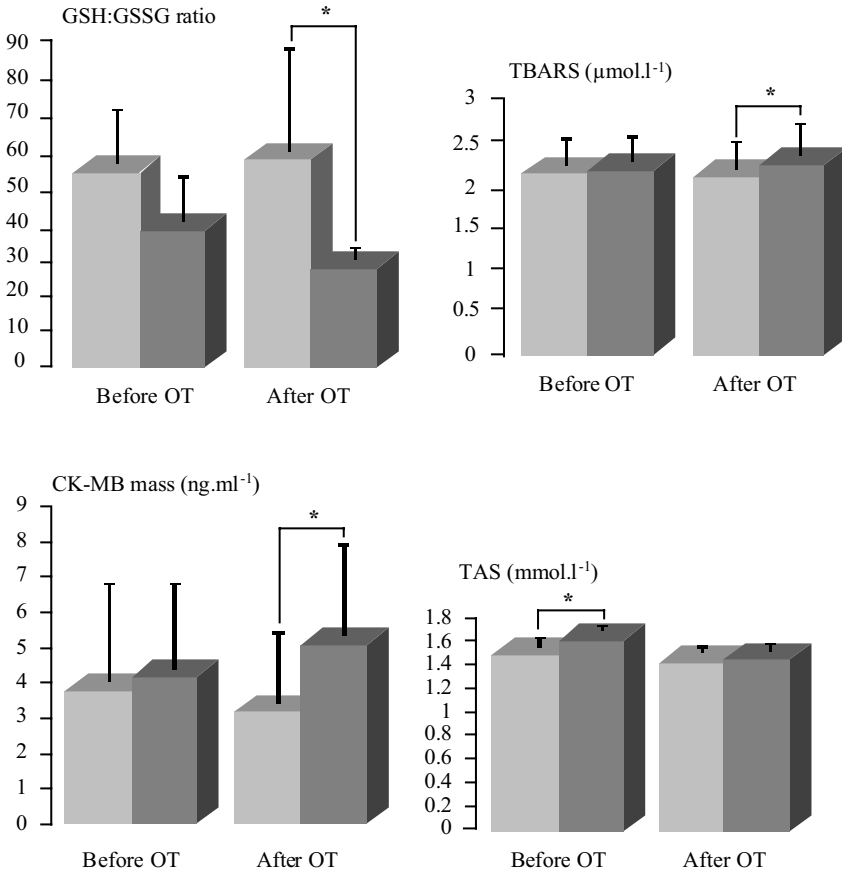
Figure 2. Indices of oxidative stress (whole blood GSH/GSSG ratio), lipid peroxidation (plasma TBARS), muscle damage (plasma CK-MB mass), and antioxidant potential (plasma TAS) in Triathlete group in pre- (light bars) and post- (darker bars) duathlon test before and after OT. *$p < 0.05$ relative to preexercise.

It is known that training reduces the likelihood of damage to skeletal muscle during subsequent bouts of the same form of exercise (Armstrong, 1986), but overloaded training has the opposite effect (Kirwan et al., 1990; Okamura et al., 1997). The increased resting total CK activity confirms the state of stress and shows that repeated days of extensive and/or intensive training cause muscle cells to lose membrane integrity in already-trained triathletes. In addition, CK-MB mass was enhanced after intense acute exercise ($84 \pm 2\%$ of $\dot{V}O_2max$) in response to OT without any variation in rest conditions.

Heavy endurance exercise induces DNA damage in leukocytes (Hartmann et al., 1998; Niess et al., 1996; 1998). It is likely that ROS generated during neu-

Table 5 Markers of Free Radical Production, and Indices of Muscle Damage and Antioxidant Potential (mean $\pm$ SD) in Triathletes ($n = 9$) Before and After OT

	Pre-duathlon	Post-duathlon	ANOVA (Trng)	ANOVA (Duathlon)	ANOVA (Tr $\times$ Du)
GSSG (μmol·L^{-1})					
Before	19.8 $\pm$ 4.6	25.6 $\pm$ 9.1*	n.s.		
After	21.0 $\pm$ 6.4	30.8 $\pm$ 4.1*	n.s.	$p < 0.01$	n.s.
Total CK (U/l)					
Before	161.2 $\pm$ 111.9	212.4 $\pm$ 114.6*			
After	182.3 $\pm$ 115.7	237.4 $\pm$ 119.8*	n.s.	$p < 0.001$	n.s.
Myoglobin (ng/ml)					
Before	35.4 $\pm$ 7.6	90.4 $\pm$ 44.6*			
After	35.4 $\pm$ 8.9	94.4 $\pm$ 37.4*	n.s.	$p < 0.001$	n.s.
Cu-Zn-SOD (U/mgHb)					
Before	1.33 $\pm$ 0.12	1.36 $\pm$ 0.10			
After	1.32 $\pm$ 0.11	1.32 $\pm$ 0.11	n.s.	n.s.	n.s.
GSH-Px (U/gHb)					
Before	41.9 $\pm$ 5.2	42.4 $\pm$ 5.7			
After	40.9 $\pm$ 6.0	40.7 $\pm$ 4.8	n.s.	n.s.	n.s.
Plasma GSH-Px (U/l)					
Before	390.7 $\pm$ 23.2	414.1 $\pm$ 25.7*			
After	425.9 $\pm$ 36.4[†]	451.2 $\pm$ 49.2*[†]	$p < 0.05$	$p < 0.01$	n.s.
GSH (μmol·L^{-1})					
Before	1048.0 $\pm$ 144.6	908.2 $\pm$ 86.4*			
After	1093.6 $\pm$ 185.6	874.0 $\pm$ 151.2*	n.s.	$p < 0.001$	n.s.

Note: GSSG = whole blood oxidized glutathione; Total CK = total CK activity; Cu-Zn-SOD = erythrocyte superoxide dismutase activity; GSH-Px = erythrocyte glutathione peroxidase activity; plasma GSH-Px = plasma glutathione peroxidase activity; GSH = whole blood reduced glutathione .
*Significant pre/post differences, $p < 0.05$; [†]Significant before/after OT differences, $p < 0.05$

trophil activation would be the main cause of nuclear damage. However, 4 weeks of OT in triathlon did not induce significant DNA damage in leukocytes. Thus, on that point the effects of OT are not comparable to the acute effects of exercise. These results may be related to a great efficiency of the DNA repair enzymes. To our knowledge, the effects of training procedure on DNA damage have not been studied. Moreover, it remains unclear how strenuous exercise affects the leukocyte nucleus and what the biological significance of these DNA lesions is (Hartmann et al., 1998).

The 4-week OT was shown to induce an overloaded state in amateur triathletes of good level performance, without significant modifications of TBARS levels in rest conditions. A few studies have compared only the basal lipid peroxidation levels between trained and sedentary subjects and these have led to conflicting results. Kretzschmar et al. (1991) and Robertson et al. (1991) observed no difference. Marzatico et al. (1997) described two lipid peroxidative indices (conjugated dienes and malondialdehyde) which were higher in endurance athletes than in sedentary subjects, indicating that daily training sessions may cause basal oxidative stress. On the other hand, Niess et al. (1996) reported that plasma levels of malondialdehyde were lower in trained runners than in untrained subjects.

Mena et al. (1991) and Okamura et al. (1997) evaluated the effects of repeated exercise on oxidative damage in elite cyclists and runners, respectively. For both groups, chronic exercise increased lipid peroxidation. These two studies led to conditions similar to those of our OT procedures. The fact that the results of these studies differed from ours may be due, first, to the different practice level of the subjects, and second, to their level of adaptation at the end of each study. The high level performance (Mena et al., 1991) may be closely related to a high level of training and therefore suggests a potential basal oxidative stress associated with an adaptation failure in some functions. Despite the lack of information on physiological and/or psychological states at the end of those studies, it may be supposed that cyclists and runners are in an advanced state of overtraining. This suggests that a threshold of chronic oxidative stress may be reached in conditions of chronic high level physiological demands.

Our study demonstrates that 4 weeks of OT led to increased exercise-induced lipid peroxidation. The literature on acute exercise-induced lipid peroxidation has shown that the higher the performance and training levels, the higher the increase in exercise-induced lipid peroxidation (Marzatico et al., 1997; Mena et al., 1991; Vasankari et al., 1997). Furthermore, acute exercise also leads to an increase in lipid peroxidation in sedentary subjects (Kretzschmar et al., 1991; Laaksonen et al., 1999), who are typically considered nonadapted. It therefore seems that the lipid peroxidation increase in response to exercise is similar for both highly trained and sedentary subjects. Moreover, the high performance level associated with a high training demands may lead to a lack of biochemical adaptation. In tapered and adapted subjects, a sporting event does not always induce an increase in lipid peroxidation (Duthie et al., 1990; Margaritis et al., 1997; Vasankari et al., 1997). As a consequence of the imbalance between oxidants and antioxidants, an increase in exercise-induced lipid peroxidation with OT may result from an inability of antioxidants to deal with increased free-radical production.

Plasma GSH-Px is antigenically distinct from its cytosolic counterpart and has a renal origin. So the kidney gene of GSH-Px reacts by an increased expression to the overproduction of lipid peroxides, which results in an increased secretion of GSH-Px in plasma, when logically erythrocyte GSH-Px has no possibility of adapting. Exercise-induced damage indicates that upregulation of plasma GSH-Px activity fails to prevent oxidative damage in overloaded-training triathletes. Plasma GSH-Px activity, not extensively studied during training until now, is sensitive to repeated stimulations, as once observed (Tessier et al., 1995). We report a higher plasma GSH-Px activity during OT, which may indicate an increased peroxide production in the blood.

In overloaded triathletes, erythrocyte SOD and GSH-Px activities were unchanged after the duathlon test, as reported for well-adapted subjects in response to a long-distance triathlon race (Margaritis et al., 1997), a half-marathon race (Duthie et al., 1990), or the first stage of a bicycle race (Mena et al., 1991).

Total antioxidant capacity is positively correlated with peak $\dot{V}O_2$ in trained runners (Child et al., 1998), which may be considered a beneficial response to exercise. Inversely, in overloaded triathletes, total antioxidant status (TAS) is significantly decreased. This decrease suggests that nonenzymatic antioxidants are strongly consumed during OT, which could explain why reserves are progressively depleted in spite of a satisfactory micronutrient status.

Some of the conflicting results on acute or chronic exercise-induced lipid peroxidation and/or antioxidant responses seem to be due to a lack of identification of the subjects' training state. Therefore, physical performance and/or maximal oxygen uptake may be considered insufficient criteria for identifying an adaptive level, as trained subjects may exhibit the same performance even though they may be in a different adaptive state. Circulating biochemical parameters, rather than spiroergometric data, may be more representative of the physiological state. Biochemical parameters, however, respond in an anachronistic way, because they do not respond concomitantly to training stimulation. Indeed the types of stimulation and differences among individuals vary widely. Most studies reporting exercise-increased lipid peroxidation evaluated the subjects at a certain stage of the training season without always taking into account the adaptation state or competition program. Consequently, the lack of information about training phase in most of the studies makes it impossible to draw conclusions about the relationship between training level and exercise-induced lipid peroxidation in overloaded subjects.

In the case of overloaded training, antioxidant parameters did not vary in the same way: erythrocyte SOD, GSH-Px activities, and blood GSH were unchanged, and plasma TAS was decreased, but plasma GSH-Px activity upregulation failed to prevent oxidative exercise-induced damage in overloaded triathletes. This failure suggests, first, the absence of concomitant responses in antioxidant parameters during OT, and second, even if some parameters eventually adapt, it may be insufficient to prevent oxidative damage. Thus we may suppose that antioxidant supplementation in the case of OT would protect against exercise-induced oxidative damage, knowing that antioxidants such as selenium and vitamins A, C, and E play a major and synergistic role in regulating the endogenous antioxidant defense system (Goldfarb et al., 1999).

We conclude that OT could compromise the antioxidant defense mechanism with respect to exercise-induced response. The resulting increase in exercise-induced oxidative stress and further cellular susceptibility to damage needs more study.

Acknowledgments

References

Armstrong, R.B. (1986). Muscle damage and endurance events. **Sports Med.** 3: 370-381.

Camus, G., Deby-Dupont, G., Delby, C., Juchmès-Ferir, A., Pincemail, J., and Lamy, M. (1993). Inflammatory response to strenuous exercise in man. **Mediat. Inflamm.** 2: 335-342.

Child, R.B., Wilkinson, D.M., Fallowfield, J.L., and Donnelly, A.E. (1998). Elevated serum antioxidant capacity and plasma malondialdehyde concentration in response to a simulated half-marathon run. **Med. Sci. Sports Exerc.** 30: 1603-1607.

Corbucci, G.G., Montanari, G., Cooper, M.B., Jones, D.A., and Edwards, R.H.T. (1984). The effect of exertion on mitochondrial oxidative capacity and on some antioxidant mechanisms in muscle from marathon runners. **Int. J. Sports Med.** 5: S135.

Duthie, G.G., Robertson, J.D., Maughan, R.J., and Morrice, P.C. (1990). Blood antioxidant status and erythrocyte lipid peroxidation following distance running. **Arch. Biochem. Biophys.** 282: 78-83.

Emonet, N., Leccia, M.T., Favier, A., Beani, J.C., and Richard, M.J. (1997). Thiols and selenium: Protective effect on human skin fibroblasts exposed to UVA radiation. **J. Photochem. Photobiol. B** 40: 84-90.

Emonet, N., Richard, M.J., Ravanat, J.L., Signorini, N., Cadet, J., and Beani, J.C. (1998). Protective effects of antioxidants against UVA-induced DNA damage in human skin fibroblasts in culture. **Free Radic. Res.** 29: 307-313.

Fry, R.W., Morton, A.R., and Keast, D. (1991) Overtraining in athletes: An update. **Sports Med.** 12: 32-65.

Goldfarb, A.H. (1999). Nutritional antioxidants as therapeutic and preventive modalities in exercise-induced muscle damage. **Can. J. Appl. Physiol.** 24: 249-266.

Hartmann, A., Pfuhler, S., Dennog, C., Germadnik, D., Pilger, A., and Speit, G. (1998). Exercise-induced DNA effects in human leukocytes are not accompanied by increased formation of 8-hydroxy-2'-deoxyguanosine or induction of micronuclei. **Free Radic. Biol. Med.** 24: 245-251.

Hellman, B., Vaghef, H., and Boström, B. (1995). The concepts of the tail moment and tail inertia in the single cell gel electrophoresis assay. **Mut. Res.** 336: 123-131.

Kirwan, J.P., Costill, D.L., Houmard, J.A., Mitchell, J.B., Flynn, M.G., and Fink, W.J. (1990). Changes in selected blood measures during repeated days of intense training and carbohydrate control. **Int. J. Sports Med.** 11: 362-366.

Kretzschmar, M., Müller, D., Hübscher, J., Marin, E., and Klinger, W. (1991). Influence of aging, training and acute physical exercise on plasma glutathione and lipid peroxides in man. **Int. J. Sports Med.** 12: 218-222.

Laaksonen, D.E., Atalay, M., Niskanen, L., Uusitupa, M., Hänninen, O., and Sen, C.K. (1999). Blood glutathione homeostasis as a determinant of resting and exercise-induced oxidative stress in young men. **Redox Report** 4: 53-59.

Lavoie, J.M., Leger, L.A., Leone, M., and Provencher, P.J. (1985). A maximal multistage swim test to determine the functional and maximal aerobic power of competitive swimmers. **J. Swimming Res.** 1: 17-22.

Margaritis, I., Tessier, F., Richard, M.J., and Marconnet, P. (1997). No evidence of oxidative stress after a triathlon race in highly trained competitors. **Int. J. Sports Med.** 18: 186-190.

Marklund, S., and Marklund, G. (1974). Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. **Eur. J. Biochem.** 47: 469-474.

Marzatico, F., Pansarasa, O., Bertorelli, L., Somenzini, L., and Della Valle, G. (1997). Blood free radical antioxidant enzymes and lipid peroxides following long-distance and lactacidemic performances in highly trained aerobic and sprint athletes. **J. Sports Med. Phys. Fitness** 37: 235-239.

McNair, D.M., Lorr, M., and Droppleman, L.F. (1992). **EdITS Manual for the Profile of Mood States.** San Diego, CA: Educational and Industrial Testing Services.

Mena, P., Maynar, M., Guttierrez, J.M., Maynar, J., Timon, J., and Campillo, J.E. (1991). Erythrocyte free radical scavenger enzymes in bicycle professional racers. Adaptation to training. **Int. J. Sports Med.** 12: 563-566.

Morton, R.H., Fitz-Clarke, J.R., and Banister, E.W. (1990). Modeling human performance in running. **J. Appl. Physiol.** 69: 1171-1177.

Niess, A.M., Hartmann, A., Grünert-Fuchs, M., Poch, B., and Speit, G. (1996). DNA damage after exhaustive treadmill running in trained and untrained men. **Int. J. Sports Med.** 17: 397-403.

Niess, A.M., Baumann, M., Roecker, K., Horstmann, T., Mayer, F., and Dickhuth, H.H. (1998). Effects of intensive endurance exercise on DNA damage in leucocytes. **J. Sports Med. Phys. Fitness** 38: 111-115.

Okamura, K., Doi, T., Hamada, K., Sakurai, M., Yoshioka, Y., Mitsuzono, R., Migita, T., Sumida, S., and Sugawa-Katayama, Y. (1997). Effect of repeated exercise on urinary 8-hydroxydeoxyguanosine excretion in humans. **Free Radic. Res.** 26: 507-514.

Richard, M.J., Portal, B., Meo, J., Coudray, C., Hadjian, A., and Favier, A. (1992). Malondialdehyde kit evaluated for determining plasma and lipoprotein fractions that react with thiobarbituric acid. **Clin. Chem.** 38: 704-709.

Riggin, R.M., and Kissinger, P.T. (1977). Determination of catecholamines in urine by R.P. liquid chromatography with electrochemical detection. **Anal. Chem.** 49: 2109-2111.

Robertson, J.D., Maughan, R.J., Duthie, G.G., and Morrice, P.C. (1991). Increased blood antioxidant systems of runners in response to training load. **Clin. Sci.** 80: 611-618.

Sastre, J., Asensi, M., Gascó, M., Pallardó, F.V., Ferrerro, J.A., Furukawa, T., and Viña, J. (1992). Exhaustive physical exercise causes oxidation of glutathione status in blood: Prevention by antioxidant administration. **Am. J. Physiol.** 263: R992-R995.

Sen, C.K. (1995). Oxidants and antioxidants in exercise. **J. Appl. Physiol.** 79: 675-686.

Singh, N.P., McCoy, M.T., Tice, R.R., and Schneider, E.L. (1988). A simple technique for the quantification of low levels of DNA damage in individual cells. **Exp. Cell. Res.** 175: 184-191.

Tessier, F., Margaritis, I., Richard, M.J., Moynot, C., and Marconnet, P. (1995). Selenium and training effects on the glutathione system and aerobic performance. **Med. Sci. Sports Exerc.** 27: 390-396.

Vasankari, T.J., Kujala, U.M., Vasankari, T.M., Vuorimaa, T., and Ahotupa, M. (1997). Effects of acute exercise on serum and LDL oxidation and antioxidant defenses. **Free Radic. Biol. Med.** 22: 509-513.