Original Research

Multivitamin-Mineral Supplementation Prevents Lipid Peroxidation during "The Marathon des Sables"

Guillaume Machefer, PhD, Carole Groussard, PhD, Sophie Vincent, PhD, Hassane Zouhal, PhD, Henri Faure, PhD, Josiane Cillard, PhD, Zsolt Radák, PhD, Arlette Gratas-Delamarche, PhD

Laboratory of Physiology and Biomechanics of Muscular Exercise, UFR-APS, University of Rennes 2 (G.M., C.G., S.V., H.Z., A.G.-D.), G.I.S.«Sciences of Movement» (G.M., C.G., S.V., H.Z., J.C., A.G.-D.), Laboratory of Cellular and Plant Biology, Faculty of Pharmacy, University of Rennes 1 (J.C.), Rennes Cedex, Department of Integrated Biology, CHUG, Grenoble (H.F.), FRANCE, Department of Exercise Physiology, School of Sport Science, Semmelweiss University Budapest, Budapest, HUNGARY (Z.R.)

Key words: exercise, marathon, oxidative stress, supplementation, vitamins, antioxidant

Objective: We investigated the effect of a moderate mutivitamin and mineral supplementation containing mainly vitamin C (150.0 mg.day^{-1}), vitamin E (24.0 mg.day^{-1}) and β-carotene (4.8 mg.day^{-1}) prior to and during an extreme running competition -the Marathon des Sables (MDS)- that consisted of six long races in the desert.

Methods: Seventeen athletes participated in our double blind, placebo-controlled study. Blood samples were collected prior to the supplementation i.e. three weeks before the competition (D-21), two days prior to the MDS (D-2), after the third race (D3) and at the end of the competition (D7). Erythrocyte antioxidant enzyme activity (glutathione peroxidase (GPx), superoxide dismutase (SOD)), erythrocyte glutathione level (GSH), plasma non-enzymatic antioxidant status (uric acid, vitamin C, α-tocopherol, retinol, β-carotene), markers of plasma lipid peroxidation (thiobarbituric reactive substances (TBARS)), reactive carbonyl derivatives (RCD) and membrane damage (creatine kinase and lactate dehydrogenase activities) were measured.

Results: In both groups, GSH levels, uric acid levels and membrane damage significantly increased during the competition while SOD activity significantly decreased. In Supplemented group, plasma α-tocopherol, β-carotene and retinol levels significantly increased after three weeks of supplementing. In contrast to Placebo group, α-tocopherol, vitamin C and retinol levels were significantly affected by the competition in Supplemented group. Moreover, no increase in TBARS was observed in Supplemented group during the competition, whereas TBARS significantly increased at D3 in the placebo group.

Conclusion: The moderate multivitamin-mineral supplementation prevented the transient increase in TBARS levels during this extreme competition.

INTRODUCTION

Acute exercise and repetition of long exercises are now well known to produce reactive oxygen species (ROS) and to induce oxidative damage [1–3]. So, the efficiency of antioxidant protection in athletes is essential.

The exogenous antioxidants that are provided by the diet interact with endogenous antioxidant. Some minerals as copper, zinc and selenium contribute to the antioxidant defense system by acting as co-factors for antioxidant Cu-Zn superoxide dismutase and glutathione peroxidase activities respectively but the main antioxidant diets are the antioxidant vitamins such as vitamin E, vitamin C and β-carotene. These vitamins neutralize several ROS [4–5] and also play a major role in exercise-induced oxidative stress. Their needs are increased in trained athletes and, sometimes, antioxidant vitamin intakes appeared insufficient in athletes [6–8]. In this way, some investigators showed beneficial effects of antioxidant vitamin

Address reprint requests to: Guillaume Machefer, PhD, Laboratory of Physiology and Biomechanics of Muscular Exercise. UFR-APS, University of Rennes 2, av. Charles Tillon, 35044 Rennes Cedex, FRANCE. E-mail: machefer_guillaume@yahoo.fr

This research was supported by NHS Laboratory, Paris, Rungis, France.

supplementation during acute exercise or training period [9–12]. In particular, these studies showed that antioxidant vitamin supplementation prevents exercise induced lipid peroxidation [9–12]. The supplementation generally contains antioxidant vitamin concentrations that largely exceeded the Dietary Recommended Intake (DRI) (for example 1000 mg vitamin C, 400 mg of α-tocopherol equivalent, 32 mg of β-carotene per day [9]).

The competition entitled "Marathon des Sables" (MDS) is an unusual running competition. It consists of 6 very long duration races in the Moroccan desert, during which athletes carried their own food. Previously, we showed that athletes who participated to the 2001 edition of the MDS exhibited an imbalance between oxidant and antioxidant systems 72 hours after this competition [13]. So, we hypothesize that a moderate vitamin and mineral supplementation, near the French DRI of athletes, could improve antioxidant system and limit lipid peroxidation during this extreme competition.

The objective of the study was to investigate the effects of a multivitamin and mineral complex containing mainly antioxidant agents prior to and during the 2002 MDS on 1) plasma markers of oxidative damage and markers of membrane alterations and on 2) the blood antioxidant status.

METHODS

Subject Characteristics

Seventeen healthy long-distance runners (16 men and 1 woman) participating in regular running exercises were involved in the present placebo-controlled, double-blind study. The athletes usually ran 85.0 ± 6.4 km per week (marathon time average 188.3 ± 6.1 min) and trained for ultradistance events for 8.2 ± 1.4 years. Over the three weeks preceding the MDS, training volume was reduced about 30–40%. All subjects were non-smokers and none was taking any medication or vitamin supplementation before and during the study. Oral and written informed consents were obtained after purposes and risks were explained. The protocol of the study was reviewed and approved by the local ethics committee for human subjects.

At the beginning of the study, the athletes were double blind randomly divided into a Supplemented group (S) (10 men) and a Placebo group (P) (6 men and 1 woman). The characteristics of the subjects in S and in P group were described in Table 1. The percentage of body fat was estimated from 4 skinfold thicknesses [14]. Maximal oxygen uptake ($\dot{V}O_{2max}$) was measured using a graded treadmill test performed until exhaustion according to a previously described method [15]. Heart rate was recorded with an electrocardiogram (Personal 120/210 LAPTOP) throughout the test and $\dot{V}O_2$ was measured using a breath-by-breath automated exercise metabolic system (CPX, Medical Graphics, St. Paul, Minnesota, USA). At the beginning of the study, there were no significant differences in subject characteristics between P and S group (Table 1). Physical characteristics, including the measurement of height, weight and body fat, were also monitored and recorded during the competition.

Table 1. Physiological Characteristics and Training Status of the Athletes in Supplemented (S) and Placebo (P) Group

Variable	Supplemented group (n = 10)	Placebo group (n = 7)
Age (year)	41.3 ± 2.6	37.9 ± 1.8
Height (cm)	174.9 ± 2.7	173.1 ± 1.6
Fat (%)	16.8 ± 0.6	16.8 ± 1.2
Body mass (kg) D-21	70.4 ± 3.2	64.9 ± 2.9
Weekly exercise sessions	5.7 ± 0.7	5.2 ± 0.6
Weekly distance of training (km)	88.0 ± 9.0	80.7 ± 9.1
Best performance in marathon (min)	187.8 ± 8.6	189.2 ± 8.6
$\dot{V}O_2$max (mL · min^{-1} · kg^{-1})	58.8 ± 1.1	58.9 ± 0.5

Values for each group are presented as means ± SEM.

Supplementation Protocol

Subjects were instructed to ingest three tablets per day for three weeks before the competition and during the week of competition. Tablets were ingested with breakfast except before the competition when tablets where ingested following the blood draw. Supplemented subjects took tablets of Isoxan-Endurance® (NHS, Paris, Rungis, France). The supplementation tablets contained antioxidant vitamins, vitamins of B group and trace minerals (Table 2). Placebo subjects consumed tablets of lactose and cellulose that had similar form and taste.

Diet

The subjects were all asked to maintain their normal eating habits during the three weeks prior to the MDS. A quantitative assessment of dietary intake was provided by means of a 7-day food record. The food records were analyzed using a computer dietary analysis (Profil v 6.5, C.I.A.M., Saint-Chouldard, France) employing the Ciqual table of food composition. During the competition, it was impossible to evaluate the exact antioxidant intake since the athletes throw away their packaging of food in order to limit their bag weight. Moreover, the

Table 2. Vitamins and Trace Elements Contained in 1 Tablet (Isoxan-Endurance®, NHS, Paris, Rungis, France) of the Supplementation

Vitamins				Minerals	
Vitamin E	8.0 mg	Vitamin B3	5.0 mg	Cu	1.1 mg
Vitamin C	50.0 mg	Vitamin B5	2.0 mg	Mg	43.3 mg
β-carotene	1.6 mg	Vitamin B6	1.3 mg	Zn	4.7 mg
Vitamin B1	1.1 mg	Vitamin B8	33.3 μg	Fe	3.3 mg
Vitamin B2	1.2 mg	Vitamin B9	100.0 μg	Mn	1.16 mg
Vitamin B12	1.0 μg			Se	38.3 μg

precise composition of food (especially in antioxidant nutrients) did not appear on the pre-packaged food concerning specific energetic food, liquid or paste.

Blood Collection

The subjects went to the laboratory twice: at D-21, the day of the beginning of the supplementation i.e. 21 days before the competition, at D-2, two days before the competition i.e. after 19 days of supplementation. Four blood samples were drawn from an antecubital vein, at rest, in the laboratory at D-21 and at D-2 between 09.00 to 10.00 h prior to the ingestion of the 3 tablets, and in Morocco during the MDS at day 3 (D3), approximately 3 hours after the third race and at day 7 (D7), approximately 3 hours after the sixth and last race.

Exercise Protocol

In 2002, the MDS competition took place for 7 days. It consisted of 6 races according to the following protocol. During the first three days, subjects ran 26, 36 and 31 km respectively each day. The distance of the fourth race, at day 4, was 71 km. Day 5 was a resting day. Races 5 and 6 at day 6 and day 7 were 42 km- and 20 km-races respectively. All the races began at 09.00 h. During this competition, all athletes carried their own food and equipment which weighed between 5 and 15 kg at the beginning of the competition. The organization only provided the water, which was restricted to 9.0 to 10.5 L per day according to the distance.

Materials and Reagents

Metaphosphoric acid (MPA), butylated hydroxytoluene (BHT), 1,1,3,3 tetramethoxypropane, phosphotungstic acid, thiobarbituric acid (TBA), H_2SO_4, trichloroacetic acid (TCA), hydrochloride (HCl) and 2,4-dinitrophenylhydrazone (DNPH), were purchased from Sigma (St Quentin Fallavier, France). Acetic acid, ethanol, and n-butanol were from Merck (Darmastadt, Germany) and vacutainers were from Terumo Europe (Leuven, Belgium).

Blood Samples Preparation

Preparations of blood samples were immediately carried out after collection. Blood was drawn into two 7 mL vacutainer tubes (Terumo Europe, Belgique). The first one contained heparin. An aliquot (500 μL) of whole blood was frozen at -80°C for SOD activity measurement and another aliquot (150 μL) was frozen at -20°C for GPx activity determination (with a maximum conservation of 20 days). For reduced GSH analysis, 500 μL of fresh whole blood were centrifuged at 2500 $\times$ *g* (5 min, 4°C). The plasma supernatant was discarded and the erythrocyte pellets were resuspended in 4 volumes of metaphosphoric acid (6% w/v in water). After being shaken vigorously, the solution was centrifuged at 3000 x g (10 min, 4°C). The aciditic protein-free supernatants were stored at -80°C until analysis. The rest of the plasma was aliquoted (500 μL for plasma TBARS estimation, 1 mL for plasma alpha-tocopherol, retinol and βcarotene measurement, 300 μL for the RCD concentration, 300 μL for the CK and LDH activities determination and 200 μL for the uric acid measurement) and was stored at -80°C. For vitamin C determination, an aliquot (500 μL) of plasma was added to 4.5 mL of MPA (5% w/v) prior to the storage at -80°C.

The second 7 mL vacutainer containing EDTA was used to determine haematocrit (Hct) and blood concentration of hemoglobin (Hb). For the Hct measurements, whole blood samples were collected into micro Hct tubes, which were then centrifuged (Hema-C, Jouan, France) for 3 min. Hct was calculated by measuring the ratio between the blood cell volume and the total volume of the sample. Hct measurements were done in triplicate. For hemoglobin determination, the vacutainer was analyzed with an automate (Hemocue B-haemoglobin, Hemocue®, UK).

Biochemical Analysis

Oxidative Stress Markers. Lipid peroxidation was estimated by analyzing plasma TBARS levels [16]. The method was adapted taking into account the recommendations of Halliwell and Chirico [17]. Tetramethoxypropane was used to establish a standard curve of MDA-TBA complex. TBARS levels were estimated by spectrophotometric absorbance at 532 nm. The measurement of RCD was carried out by spectrophotometric method at 360 nm as described earlier [18]. In summary, proteins of 100 μL of plasma were precipitated with trichloroacetic acid (TCA). After centrifugation (10 min, 20000 g, 4 °C), the pellets were suspended and incubated in a solution containing 10 mM DNPH or 2 N HCl for 60 min at 24°C. TCA (500 μL) was added and resulting proteins hydrazones were centrifuged at 16000 x g for 10 min. The pellets were washed twice with absolute ethanol (1:1) and once with acetone. The final precipitates were dissolved in 1 mL buffer containing 8 M urea. After this, we measured the protein content of all samples [19] in order to bring the RCD into mg of protein.

Muscle Damage. Membrane damage was evaluated by the measurement of plasma CK (EC 2.7.3.2) and LDH (EC 1.1.1.27) activities. Both of the assays used a Sigma test (47-10 and 228-20 respectively) (St Quentin Fallavier, France).

Antioxidant Status. Plasma vitamin C level was determined by high performance liquid chromatography (HPLC) [20]. Plasma α-tocopherol, retinol, and β-carotene levels in plasma were measured using a reversed HPLC procedure [21]. Plasma uric acid level was measured by spectrophotometric method using a Sigma kit (686-A) (St Quentin Fallavier, France). Erythrocyte reduced GSH was determined spectrophotometrically using the GSH-400 kit (Bioxytech, Oxis international Inc., Portland, USA). Erythrocyte antioxidant enzymes were evaluated by measuring erythrocyte GPx (EC 1.11.1.9) and SOD (EC 1.15.1.1) activities. Both assays used a Randox test combination (Randox, Montpellier, France). The Ransel

test (RS506) was used for GPx activity. This method uses an enzyme-coupled reaction and measures the oxidation of NADPH by cumen hydroperoxide as substrate. The Ransod test (SD125) for SOD activity determination employs xanthine and xanthine oxidase to generate superoxide radicals which react with 2-(4-iodophényl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. SOD activity is then measured by the degree of inhibition of this reaction.

Expression of the Results and Statistical Analysis

Repetitive exercise and heat exposure induce plasma volume changes (ΔPV) which necessarily modify all measured plasma concentrations. Therefore, the plasma chemical values (TBARS and antioxidant vitamins concentrations, CK and LDH activities) measured in this study were all corrected taking into account ΔPV using the equation that takes into account the Hct and Hb variation [22]:

$$V_C = V_M \times \left[\frac{Hb_1}{Hb_2} \times \frac{[1 - (Hct_2 \times 10^{-2})]}{[1 - (Hct_1 \times 10^{-2})]}\right]$$

V_C: value corrected

V_M: value measured

Hb_1 and Hct_1: heamoglobin (g/100mL) and hematocrit (%) at D-21

Hb_2 and Hct_2: heamoglobin (g/100mL) and hematocrit (%) at D-2, D3 or D7

Moreover, as possible changes in erythrocyte number may occur following exercise, the antioxidant enzyme activities were corrected taking into account Hb variations.

Results are expressed as means ± SEM, as absolute values (basal values), or in percentage where 100 % value represents value at D-21. An unpaired t test was used to analyze differences between P and S group. One-way ANOVA with repeated measures was used to test for significant differences of oxidant and antioxidant markers between D-21, D-2, D3 and D7. Two-way ANOVA with repeated measures was used to test for significant differences between P and S group. The limit of significance was set at $p < 0.05$.

RESULTS

Physiological Characteristics

All of the athletes that participated to our study completed the competition. As expected, the one-week race session induced a significant decrease in body mass in P and S group (−4.2 ± 0.6 kg and −3.3 ± 1.0 kg respectively) between D-21 and D7 without any significant changes in body fat.

Diet

No significant differences were found between groups concerning daily energy intake (9 ± 1.6 MJ.day^{-1} vs 9 ± 0,9 MJ.day^{-1}, P vs S respectively), and caloric profile prior to the competition. Without the supplementation, antioxidant vitamin intakes were not statistically different between groups (Retinol: 0.5 ± 0.2 mg.day^{-1} vs 0.5 ± 0.1 mg.day^{-1}; Vitamin C: 224.5 ± 71.1 mg.day^{-1} vs 118.3 ± 27.8 mg.day^{-1}; Vitamin E: 6.8 ± 0.8 mg.day^{-1} vs 6.6 ± 2.6 mg.day^{-1}; β-carotene: 3,4 ± 2.4 mg.day^{-1} vs 3.5 ± 1.1 mg.day^{-1} in P and S respectively).

Markers of Oxidative Stress and Muscle Damage

Plasma TBARS Levels. Basal values of TBARS concentrations were not statistically different at the beginning of the study between the two groups: 2.47 ± 0.14 μmol.l^{-1} and 2.49 ± 0.10 μmol.l^{-1} in P and S group respectively. TBARS levels remained stable throughout the MDS in S group (Fig. 1A) whereas in P group, a transient and significant increase (about 80%) in TBARS was observed at D3. In addition, at D3,

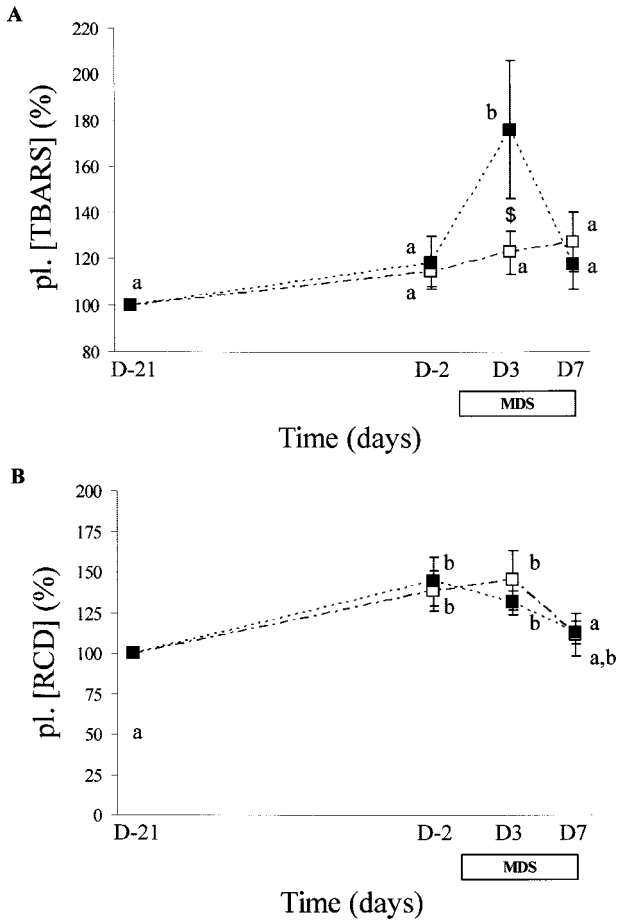

Fig 1. Plasma Thiobarbituric Reactive Substances (TBARS) (A) and Reactive Carbonyl Derivatives (RCD) (B) levels in Supplemented (□) and Placebo (■) group at the beginning of the study (D-21), after 19 days of supplementation (D-2), during (D3) and at the end (D7) of the competition. Results are expressed as means as ± SEM where 100% value represents value at D-21. In each group, the values not sharing the same letter were significantly different at $p < 0.05$. $ indicated significant differences between P and S group at $p < 0.05$.

TBARS level was significantly lower in S group than in P group. In P group, TBARS returned to their initial level at D7.

Plasma RCD Levels. Basal values of RCD concentrations were 0.35 ± 0.03 nmol RCD per mg of protein in P group and 0.34 ± 0.04 nmol RCD per mg of protein in S group. No significant different was observed between S and P group during all the study (Fig. 1B). However, P group exhibited a significant decrease in RCD at D7 compared with D-2 and D3.

Plasma CK and LDH Activities. CK (Fig. 2A) and LDH (Fig. 2B) activities had a similar pattern in S and P group. In both groups, a significant increase (fivefold) in plasma CK and LDH activities was observed at D3 and remained higher at D7 compared with their pre-race values at D-21 and D-2.

Antioxidant Systems

Erythrocyte Antioxidants. Erythrocyte GPx activity (Fig. 3A) did not show any significant change. SOD activity in-

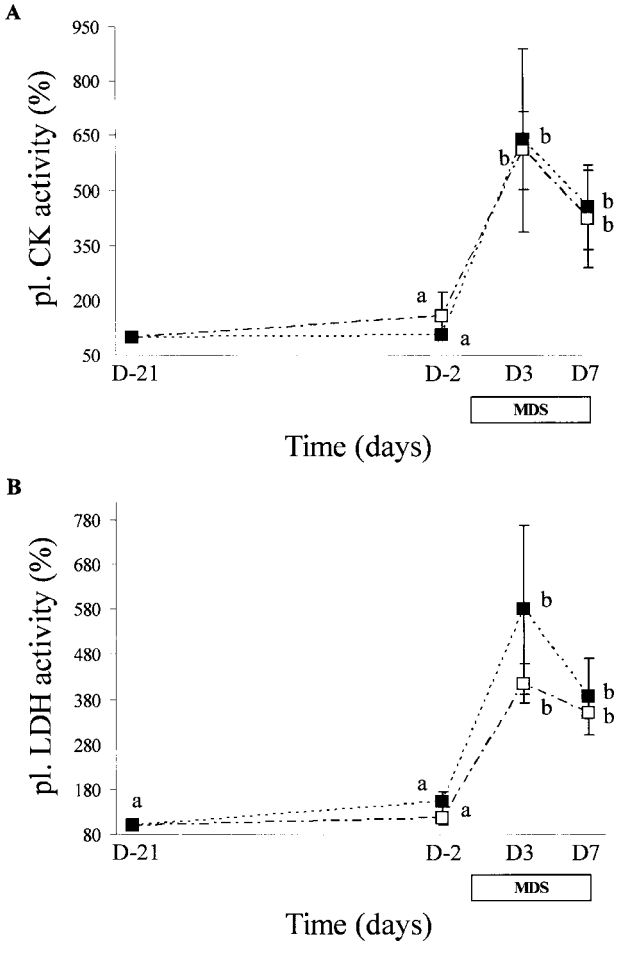

Fig 2. Plasma creatine kinase (CK) (A) and lactate dehydrogenase (LDH) (B) activities in Supplemented (□) and Placebo (■) group at the beginning of the study (D-21), after 19 days of supplementation (D-2), during (D3) and at the end (D7) of the competition. Results are expressed as means as ± SEM where 100 % value represents value at D-21. In each group, the values not sharing the same letter were significantly different at $p < 0.05$.

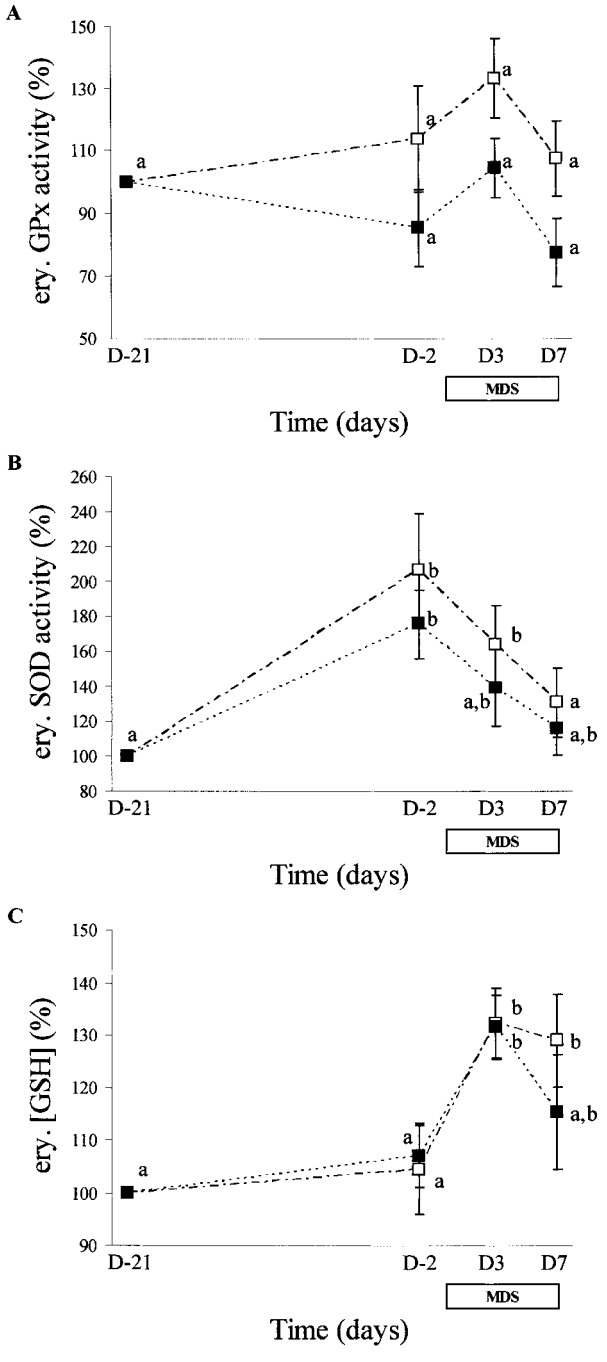

Fig 3. Erythrocyte glutathione peroxidase (GPx) (A), superoxide dismutase (SOD) (B) activities and reduced glutathione (GSH) level (C) in Supplemented (□) and Placebo (■) group at the beginning of the study (D-21), after 19 days of supplementation (D-2), during (D3) and at the end (D7) of the competition. Results are expressed as means as ± SEM where 100 % value represents value at D-21. In each group, the values not sharing the same letter were significantly different at $p < 0.05$.

creased in both groups at D-2 compared with D-21 (Fig. 3B) whereas SOD activity significantly decreased at D7 in both groups compared with D-2. A significant increase in GSH level compared with D-21 and D-2 was observed at D3 and at D7 in S group but only at D3 in P group.

Plasma Antioxidants. The plasma uric acid level (Fig. 4A) increased significantly during the race at D3 and at D7 compared with D-21 and D-2 in both groups without any significant difference between the two groups.

Changes in plasma retinol, vitamin C, α-tocopherol and β-carotene concentrations were described in Fig. 4B, Fig. 5A, Fig. 5B and Fig. 5C, respectively. At baseline, there were no statistically significant differences in antioxidant vitamin concentrations between P and S groups (retinol: 1.7 ± 0.2 μmol.L^{-1} vs 1.9 ± 0.1 μmol.L^{-1}; $^{-}$vitamin C: 59.7 ± 4.3 μmol.L^{-1} vs 61.1 ± 6.7 μmol.L^{-1}; α-tocopherol: 27.4 ± 2.4 μmol.L^{-1} vs 31.6 ± 2.2 μmol.L^{-1}; β-carotene: 0.7 ± 0.1 μmol.L^{-1} vs 0.5 ± 0.1 μmol.L^{-1} in P vs S respectively). In P group, no significant change were observed concerning the whole plasma antioxidant vitamin concentrations throughout the study period. By contrast, in S group, plasma α-tocopherol, retinol and β-carotene levels

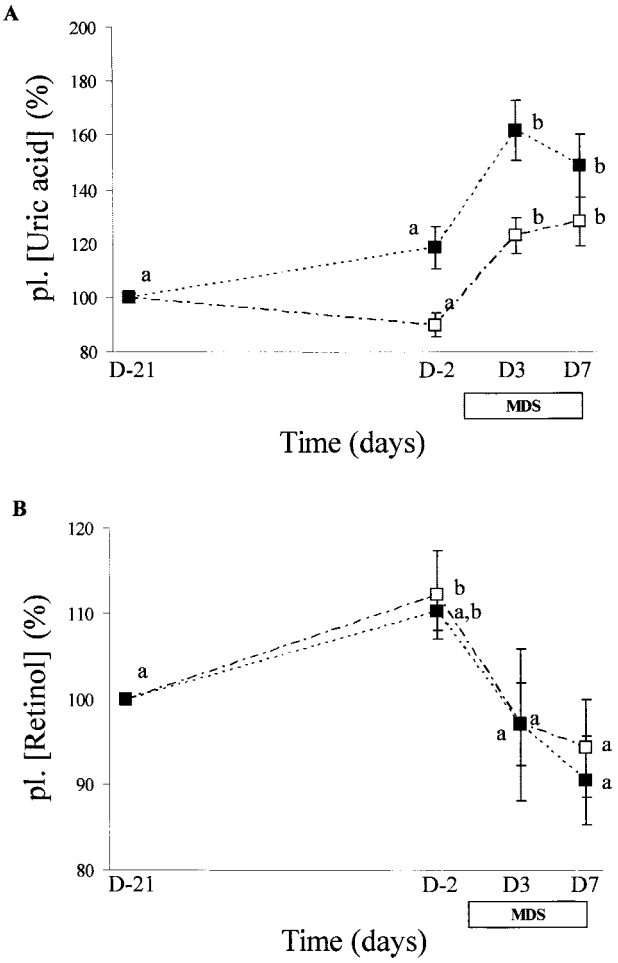

Fig 4. Plasma acid uric (A) and retinol (B) levels in Supplemented (□) and Placebo (■) group at the beginning of the study (D-21), after 19 days of supplementation (D-2), during (D3) and at the end (D7) of the competition. Results are expressed as means as ± SEM where 100 % value represents value at D-21. In each group, the values not sharing the same letter were significantly different at $p < 0.05$.

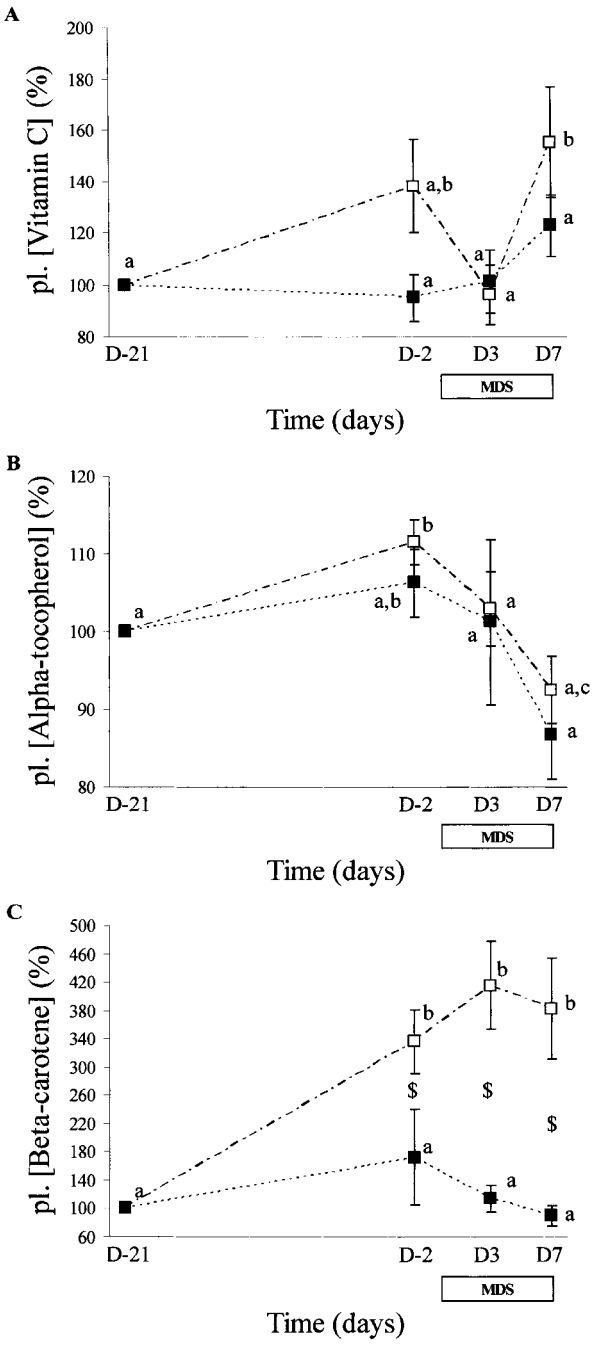

Fig 5. Plasma vitamin C (A) α-tocopherol (B) and β-carotene (C) levels in Supplemented (□) and Placebo (■) group at the beginning of the study (D-21), after 19 days of supplementation (D-2), during (D3) and at the end (D7) of the competition. Results are expressed as means as ± SEM where 100 % value represents value at D-21. In each group, the values not sharing the same letter were significantly different at $p < 0.05$. $ indicated significant differences between P and S group at $p < 0.05$.

increased significantly at D-2 compared with D-21. In addition, α-tocopherol and retinol levels significantly decreased at D7 compared with D-2, whereas vitamin C levels increased significantly at D7 compared to D-21.

Performances during the MDS

No statistically significant differences were found in the total run time (1749.6 ± 109.5 min in S group vs 1850.1 ± 96,7 min in P group) and in the average running speed (8.0 ± 0.5 $km.h^{-1}$ in S group vs 7.4 ± 0.4 $km.h^{-1}$ in P group) between P and S groups.

DISCUSSION

The present study reported on the effects of a moderate vitamin/mineral supplementation on plasma lipid peroxidation and protein oxidation markers, plasma muscular damage and blood antioxidant systems in response to a repetition of long exercises. The main results showed that the combination of multivitamin and mineral antioxidant complex prevented lipid peroxidation induced by repetition of exercises an may improve mobilization and/or utilization of antioxidant vitamins.

In the present study, the results were obtained in 17 athletes; the limited number could be explained by the voluntary participation and mainly by the extreme difficulties of the competition. As indicated by their training habits and by their laboratory performances ($\dot{V}O_{2max}$), all the subjects were well-trained endurance athletes. Their performances during the MDS, between the 6th and 351th place of 700 competitors, confirmed their good physical capacities. The supplementation proposed in this study was only 2 to 3 times the French DRI and, as expected, had no effect on physical performances during the one-week competition.

Thiobarbituric acid reactive substances (TBARS) are usually used a marker for lipid peroxidation during exercise-induced oxidative stress [2,11,13]. In this study, we adapted the protocol by Yagi [16] by adding the chain-breaking antioxidant butylated hydroxy toluene (BHT) in order to prevent the non-specific amplification of peroxidation during the assay as it was recommended by Halliwell and Chirico [17]. We observed a significant increase in plasma TBARS levels in P group at D3 whereas TBARS levels were not significantly changed in S group at the same time. In addition, TBARS levels were significantly higher in P group than in S group at D3. Thus, the moderate multivitamins and mineral supplementation limited lipid peroxidation induced by repetition of 3 races (D3). While this result has never been shown during a week of running exercises with physiological dose of antioxidant supplementation, other studies concerning acute exercise and training period support our finding [9–12]. On D7, in P group, plasma TBARS value returned to its basal value. It is difficult to explain the lack of TBARS response in P group by the 3 hour-delay in sample collection on D7 since samples were collected in the same delay on D3. So this result might suggest that an adaptive response occurred. Radák et al., [18] put forward similar conclusions. They observed a transient increase in urinary 8-hydroxydeoguanosine in athletes during a four-day race (93 km, 120 km, 56 km and 59 km respectively).

Concerning protein oxidation, no effect of supplementation was demonstrated since no increase was observed in both groups during and after the competition. It has been already found that acute exercise did not significantly increase the oxidation of protein [23]. By contrast, a slight but significant decrease in P group was observed at D7 compared with D3. The mechanisms remained to be clarified. A possible protective effect of heat shock proteins (HSP) could not be excluded since prolonged exercise and heat exposure are well known to be important factors of HSP production [24–25].

The supplementation did not prevent the increase in plasma CK and LDH activities since a similar increase was observed in both groups. The increase in plasma CK and LDH activities has been attributed to membrane disruption. Such lesions are mainly due to mechanical damage and/or oxidative stress during exercise [26]. In this study, the lack of any protective effect of the supplementation argues in favor of a major mechanical lesion. Indeed, it is well accepted that repetition of exhaustive eccentric contractions, as observed during long running exercise, leads to cell damage [27].

The beneficial effect of the moderate supplementation on TBARS levels could not be explained by the improvement of antioxidant enzyme activities. Indeed, no significant differences were observed between the two groups concerning the erythrocyte GPx and SOD activities. The absence of significant change in erythrocyte GPx activity and the decrease in erythrocyte SOD activity has been already described after exercise [23,28–30]. The decrease in erythrocyte SOD activity may be attributed either to insufficient intakes in Cu or to the inactivation or protein degradation of this enzyme by ROS such as hydrogen peroxide (H_2O_2) [31] and hypochlorous acid (HOCl) [32]. The hypothesis involving either Cu or H_2O_2 is unlikely since the supplementation brought Cu in sufficient amount and a concomitant decrease in GPx activity is often observed with H_2O_2 production. As a consequence, inhibition of SOD activity by HOCl seemed to be an explanation since HOCl is released by the neutrophils during inflammatory process. Indeed, it is now well demonstrated that running exercise induced inflammatory responses and increased neutrophils levels [33–35]. As these athletes obviously decreased their training volume before the MDS, the inflammatory process may be limited during this period of low training. This is probably the main reason for the initial increase in SOD activity in both groups at D-2.

Moreover, no significant difference was found between the two groups concerning both GSH and uric acid levels. As it has been already found in humans during a three-day recovery period following 90 min of exercise at 65 % $\dot{V}O_{2max}$ [36], we

found significantly higher values in GSH after 3 days of competition. Such a change is often described during oxidant stress and may reflect the production and release of GSH by organs such as the liver [37]. An inter-organ transport could then be established so as to help the muscles to react against the ROS [38]. At D7, the levels of GSH were still higher compared with D-21 and D-2 only in S group. The GSH has a synergistic role with vitamin E and vitamin C and is also able to interact with these vitamins [39]. Thus, the supplementation in vitamin E and vitamin C possibly limited the use in GSH in the S group as suggested by Palazzetti et al. [8]. The increase in uric acid level has been already described after an acute exercise [40–41] and resulted from an increase in purine metabolism during exercise [29,40]. This increase is associated with a limitation of oxidative stress [42].

Thus, the beneficial effect of the supplementation on TBARS levels could be attributed to the antioxidant vitamins that were significantly affected by the supplementation during the competition. Before the competition, the antioxidant vitamin intakes were not significantly different between both groups. We could also notice that the vitamin E intake seemed to be insufficient. As other investigations [9,29,43], vitamin supplementation significantly increased the concentration of α-tocopherol, retinol and β-carotene levels in S group but a significant difference between P and S groups at D-2 was only observed for β-carotene levels. In the present study, no significant effect of extreme exercise on β-carotene level was observed in both groups. In S group, β-carotene levels always remained higher than those in P group at D3 and at D7. Alpha-tocopherol and retinol concentrations significantly decrease after 6 races in S group compared with D-2. No significant change was observed in P group. The decrease in α-tocopherol concentrations after exercise might reflect entrance in the muscular compartment or its use to scavenge free radicals [30]. The significant decrease in retinol levels in S group, which potentially react with peroxyl radical [44], could also hypothetically reflect its use against lipid peroxidation during exercise. However, it is difficult to compare this result with other studies since the changes of retinol concentrations during exercise have not been studied much in humans [13,29]. In contrast, we observed an increase in vitamin C concentrations compared with D-21 and D3 only in S group. The significant increase in plasma level vitamin C after exercise is well documented [10,41,45] and has been attributed to adrenal gland release [49] or to the mobilisation of vitamin C from the neutrophils [47]. As vitamin C and vitamin E act together [39], this increase may improve the regeneration of α-tocopherol. In its turn, oxidised vitamin C could be reduced by GSH. As the changes in antioxidant vitamin concentrations were significant only in S group, these results suggested that the supplementation in multivitamins and mineral might improve the utilization and/or mobilization of antioxidant vitamins during the MDS. This could explain the beneficial effect of moderate supplementation on TBARS levels.

In conclusion, the present study showed that an extreme competition that consists of repetitions of 6 long races altered enzymatic and non-enzymatic antioxidant systems, increased membrane damage and induced lipid peroxidation. The moderate dose of the multivitamin and mineral supplementation was sufficient to increase plasma antioxidant vitamin concentrations in health endurance athletes and to prevent the lipid peroxidation, probably by improving the utilization and/or mobilization of the antioxidant vitamins, during the present extreme competition.

ACKNOWLEDGMENTS

The authors thank Martine Chevanne, Maria Sasvari, Dominique Paul, Marie-Thérèse Gougeon, Martine Godard and Michelle Jester for their technical assistance.

The authors thank the Hemocue® company for its hiring of equipment.

The authors thank the Doc Trotter association and especially Patrick Germanèze for their help during the "Marathon des Sables."

REFERENCES

1. Ashton T, Young IS, Peters JR, Jones E, Jackson SK, Davies B, Rowlands CC: Electron spin resonance spectroscopy, exercise, and oxidative stress: an ascorbic acid intervention study. J Appl Physiol 87:2032–2036, 1999.
2. Groussard C, Rannou-Bekono F, Machefer G, Chevanne M, Vincent S, Sergent O, Cillard J, Gratas-Delamarche A: Changes in blood lipid peroxidation markers and antioxidants after a single sprint exercise. Eur J Appl Physiol 89:14–20, 2003.
3. Okamura K, Doi T, Hamada K, Sakurai M, Yoshioka Y, Mitsuzono R, Migita T, Sumida S, Sugawa-Kaayama Y: Effect of repeated exercise on urinary 8-hydroxy-deoxyguanosine excretion in humans. Free Radic Res 26:507–514, 1997.
4. Powers SK, DeRuisseau KC, Quindry J, Hamilton KL: Dietary antioxidants and exercise. J Sports Sci 22:81–94, 2004.
5. Yu BP: Cellular defences against damage from reactive oxygen species. Physiol Rev 74:139–162, 1994.
6. Ziegler PJ, Nelson JA, Jonnalagadda SS: Nutritional and physiological status of U.S. national figure skaters. Int J Sport Nutr 9:345–360, 1999.
7. Groussard C, Machefer G, Rannou F, Gratas-Delamarche A: Evaluation of antioxidant vitamin intakes in sportsmen. Sci Sport 19:193–195, 2004.
8. Palazzetti S, Rousseau AS, Richard MJ, Favier A, Margaritis I: Antioxidant supplementation preserves antioxidant response in physical training and low antioxidant intake. Br J Nutr. 91:91–100, 2004. Erratum in: Br J Nutr 91:655–656, 2004.
9. Schröder H, Navarro E, Tramullas A, Mora J, Galiano D: Nutrition antioxidant status and oxidative stress in professional basketball players: effects of a three compound antioxidative supplement. Int J Sports Med 21:146–150, 2000.

10. Mastaloudis A, Morrow JD, Hopkins DW, Devaraj S, Traber MG: Antioxidant supplementation prevents exercise-induced lipid peroxidation, but not inflammation, in ultramarathon runners. Free Radic Biol Med 36:1329–1341, 2004.
11. Cavas L, Tarhan L: Effects of vitamin-mineral supplementation on cardiac marker and radical scavenging enzymes, and MDA levels in young swimmers. Int J Sport Nutr Exerc Metab 14:133–146, 2004.
12. Sacheck JM, Milbury P, Cannon JG, Roubenoff R, Blumberg JB: Effect of vitamin E and eccentric exercise on selected biomarkers of oxidative stress in young and elderly men. Free Rad Biol Med 34:1575–1588, 2003.
13. Machefer G, Groussard C, Rannou-Bekono F, Zouhal H, Faure H, Vincent S, Sergent O, Cillard J, Gratas-Delamarche A: Extreme running competition decreases blood antioxidant defense capacity. J Am Coll Nutr 23:358–364, 2004.
14. Durnin J, Rahaman M: The assessment of the amount of fat in human body from measurements of skinfold thickness. Brit J Nutr 21:681–689, 1967.
15. Flandrois R, Grandmontagne M, Mayet MH, Favier R, Frutoso G: Maximal oxygen consumption in young French subject, its variation with age, sex, and training. J Physiol (Paris) 78:186–194, 1982.
16. Yagi K: Assay for serum lipid peroxide level and its clinical significance. In Yagi K (ed): "Lipid Peroxides in Biology and Medicine." New York: Academic Press, p 223, 1982.
17. Halliwell B, Chirico S: Lipid peroxidation: its mechanism, measurement, and significance. Am J Clin Nutr 57(Suppl):715S–725S, 1993.
18. Radák Z., Pucsuk J., Boros S., Josfai L., Taylor A.W: Changes in urine 8-hydroxydeoguanosine level of super-marathon runners during a four-day race period. Life Sci 66:1763–1767, 2000.
19. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the folin. J Biol Chem 193:265–275, 1951.
20. Speek SJ, Schriver J, and Schreurs WHP: Fluorometric determination of total vitamin C in whole blood by high-performance liquid chromatography with precolumn derivatization. J Chromatrogr 13:53–60, 1984.
21. Steghens JP, Van Kappel AL, Riboli E, and Collombel C: Simultaneous measurement of seven carotenoids, retinal and α-tocopherol in serum by high-performance liquid chromatography. J Chromatrogr 694:71–81, 1997.
22. Dill DB, Costill DL: Calculation of the percentage changes in volume of blood, plasma, and red cells in dehydratation J Appl Physiol 37:247–248, 1974.
23. Miyasaki H, Oh-ishi S, Ookawa T, Kizaki T, Toshinai K, Ha S, Haga S, Ji LL, Ohno H: Strenuous endurance training in humans reduces oxidative stress following exhausting exercise. Eur J Appl Physiol 84:1–6, 2001.
24. Ryan AJ, Cisolfi, CV, Moseley PL: Synthesis of 70K stress protein by human leucocytes: effect of exercise in the heat. J Appl Physiol 70:466–571, 1991.
25. Csermely P: Stress of life from molecules to man. In Csermely P (ed): "Stress of Life: from Molecules to Man." New York: New York Academy of Sciences, 1998.
26. Davies KJA, Quintanilhat AT, Brooks GA, Packer L: Free radicals and tissue damage by exercise. Biochem Biophys Res Com 107: 1198–1205, 1982.
27. McCully KK, Faulkner JA: Injury to skeletal muscle fibers of mice following lengthening contractions. J Appl Physiol 59:119–126, 1986.
28. Ohno H, Sato Y, Yamashita K, Doi R, Arai K, Kondo T, Taniguchi N: The effect of brief exercise on free radical scavenging enzyme systems in human red blood cells. Can J Physiol Pharmacol 64: 1263–1265, 1986.
29. Rokitzki L, Logemann E, Sagredos AN, Murphy M, Wetzel-Roth W, Keul J: Lipid peroxidation and antioxidant vitamins under extreme endurance stress. Acta Physiol Scand 151:149–158, 1994.
30. Sürmen-Gür E, Ozturk E, Gür H, Punduk Z, Tnucel P: Effect of vitamin E supplementation on post-exercise plasma lipid peroxidation and blood antioxidant status in smokers: with special reference to heamoconcentration effect. Eur J Appl Physiol Occup Physiol 79:472–478, 1999.
31. Salo DC, Pacifici RE, Lin SW, Giulivi C, Davies KJA: Superoxide dismutase undergoes proteolysis and fragmentation following oxidative modification and inactivation. J Biol Chem 265:11919–11927, 1990.
32. Auchère F, Capeillère-Blandin C: Oxidation of Cu, Zn-superoxide dismutase by the myeloperoxidase/hydrogen peroxide/chloride system: functional and structural effects. Free Rad Res 36:1185–1198, 2002.
33. Suzuki K, Totsuka M, Nakaji S, Yamada M, Kudoh S, Liu Q, Sugawara K, Yamada K, Sato K: Endurance exercise causes interaction among stress hormones, cytokines, neutrophils dynamics, and muscle damage. J Appl Physiol 87:1360–1367, 1999.
34. Starkie RL, Rolland J, Angus DJ, Anderson MJ, Febbraio MA: Circulating monocytes are not the source of elevations in plasma IL-6 and TNF-α levels after prolonged running. Am J Physiol 280:C769–C774, 2001.
35. McIntyre DL, Reid WD, Lyster DM, McKenzie DC: Different effects of strenuous eccentric exercise on the accumulation of neutrophils in muscle in women and men. Eur J Appl Physiol 81:47–53, 2000.
36. Gohil K, Viguie C, Stanley WC, Brooks GA, Packer L: Blood glutathione oxidation during human exercise. J Appl Physiol 64: 115–119, 1988.
37. Deneke SM, Fanburg B: Regulation of cellular glutathione. Am J Physiol 257:L163–L173, 1989.
38. Ji LL, Fu R: Responses of glutathione system and antioxidant enzymes to exhaustive exercise and hydroperxide. J Appl Physiol 72:549–554, 1992.
39. Packer JE, Slater TF, Willson RL: Direct observation of a free radical interaction between vitamin E and vitamin C. Nature 278: 737–738, 1979.
40. Thompson D, Williams C, Garcia-Roves P, McGregor SJ, McArdle F, Jackson MJ: Post-exercise vitamin C supplementation and recovery from demanding exercise. Eur J Appl Physiol 89: 393–400, 2003.
41. Mastaloudis A, Leonard SW, Traber MG: Oxidative stress in athletes during extreme endurance exercise. Free Rad Biol Med 31:911–922, 2001.
42. Mikami T, Yoshino Y, Ito A: Does a relationship exist between the urate pool in the body and lipid peroxidation during exercise? Free Rad Res 32:31–39, 2000.
43. Morinobu T, Yoshikawa S, Hamamura K, Tamai H: Measurement

of vitamin E metabolites by high-performance liquid chromatography during high dose administration of α-tocopherol. Eur J Clin Nutr 57:410–414, 2003.
44. Samokyszyn VM, Marnett LJ: Hydroperoxide-dependent cooxidation of 13-cis-retinoic acid by prostaglandin H synthase. J Biol Chem 262:14119–14133, 1987.
45. Petersen EW, Ostrowski K, Ibfelt T, Richelle M, Offord E, Halkjær-Kristensen J, Pedersen B: K: Effect of vitamin supplementation on cytokine response and on muscle damage after strenuous exercise. Am J Physiol 280:C1570–C1575, 2001.
46. Gleeson M, Robertson JD, Maughan RJ: Influence of exercise on ascorbic acid status in man. Clin Sci (Lond) 73:501–505, 1987.
47. Tauler P, Aguiló A, Cases N, Sureda A, Gimenez F, Villa G, Códova A, Pons Biescas A: Acute phase immune response to exercise coexists with decreased neutrophil antioxidant enzyme defences. Free Radic Res 36:1101–1107, 2002.

Received December 4, 2004; Accepted July 25, 2006