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Antioxidant supplementation preserves antioxidant response in physical training and low antioxidant intake

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The present controlled-training double-blind study (supplemented (S) group, *n* 7; placebo (P) group, *n* 10) was designed to investigate whether an antioxidant mixture (Se 150 μg, retinyl acetate mg, ascorbic acid 120 mg, α-tocopheryl succinate) would allow overloaded triathletes to avoid adaptation failure in the antioxidant system. Dietary intakes were recorded. The supplement of Se, and vitamins A and E provided 100 % of the French RDA. Four weeks of overloaded training (OT) followed 4 weeks of normal training (NT). After NT and OT, biological studies were conducted at rest and after a duathlon test (run 5 km, cycle 20 km, run 5 km). During the 4-week period of NT, blood levels of GSH levels increased in response to supplementation ($P < 0{\cdot}05$) and remained elevated during OT. Plasma glutathione peroxidase activity was significantly higher in the S group in all situations after NT and OT ($P < 0{\cdot}01$). The S group had increased erythrocyte Cu,Zn-superoxide dismutase activity in response to OT ($P < 0{\cdot}05$). Supplementation significantly reduced ($P < 0{\cdot}05$) the magnitude in duathlon-induced creatine kinase isoenzyme MB mass increase, which tended to be higher with OT ($P = 0{\cdot}09$). We conclude that the antioxidant mixture helped to preserve the antioxidant system during an OT-induced stress in subjects with initially low antioxidant intakes. Effects of supplementation during NT and/or OT are shown mostly by the alleviated muscle damage. The effects of the antioxidant mixture were observed for doses that can be provided by a diversified and well-balanced diet. The maintenance of normal nutritional status with regard to the antioxidant intake (Se, vitamins C and E) plays a key role in antioxidant adaptive effects during NT and OT.

Selenium: Ascorbate: α-Tocopherol: Oxidative stress: Physical training

The increase in reactive oxygen species production during physical exercise may disturb intracellular pro-oxidant–antioxidant homeostasis, inducing oxidative stress that initiates oxidative damage of lipid, protein and nucleic acids (Powers & Hamilton, 1999). Cu,Zn-superoxide dismutase (SOD), Se-dependent glutathione peroxidase (GSH-Px), vitamins C and E, and GSH can prevent exercise-induced oxidative stress (Dekkers *et al.* 1996; Ji, 1999; Powers & Hamilton, 1999). Training-induced upregulation of endogenous antioxidants may reduce the risk of cellular injury during exercise (Powers *et al.* 1999). To interact with endogenous antioxidants, exogenous antioxidants are provided by the diet. As a result of physical training, adaptive effects of the antioxidant system are known to reduce the magnitude of the exercise-induced stress. Paradoxically, as physical training requires repeated bouts of physical aerobic endurance training, an increase in energy requirement increases O_2 utilization, which in turn increases free radical oxygen-derivative generation by the mitochondria of active muscles (Ji, 1999).

We previously showed that overloaded training (OT) in triathletes increases exercise-induced oxidative stress, lipid peroxidation and muscle damage, and that overloaded triathletes fail to adapt to oxidative exercise-induced stress and cell damage response in exercise conditions (Palazzetti *et al.* 2003). These outcomes can be attributed to inefficient adaptation (Hinchcliff *et al.* 2000) or increased antioxidant trace element and vitamin requirements with increased energy expenditure (Clarkson, 1995). Se, and vitamins C and E, play a major and synergistic role as exogenous antioxidants in the regulation of the endogenous antioxidant defence system, and lowers exercise-induced oxidative damage (Goldfarb, 1999).

Abbreviations: GSH-Px, glutathione peroxidase; NT, normal training; OT, overloaded training; P, placebo; POMS, profile of mood states; S, antioxidant-supplemented; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances.

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For overloaded subjects, the efficacy of antioxidant micronutrients, either individually or in combination, has not been established by randomized clinical trial. We hypothesized that the antioxidant system downregulation and its consequences would be avoided, at least in part, by the ingestion of a supplement of antioxidant nutrients. The present study was designed to test the efficacy of a micronutrient daily supplementation with antioxidant vitamins (ascorbic acid, α-tocopherol, retinyl acetate) and Se, at nutritional doses, in reducing the effects of OT-induced oxidative stress and damage.

Methods

Subjects

Twenty well-trained male triathletes (age 32·9 (SD 9·9) years, height 1·754 (SD 0·070) m, body mass 69·4 (SD 5·3) kg, body fat 11·4 (SD 3·7) %, maximal O_2 uptake V_{O2max} 65·0 (SD 6·1) ml/min per kg) participated in this 8-week double-blind study. During the first 4-week period, normal training (NT) was conducted; during the second 4-week period, training was overloaded by qualitative change and quantitative increase in swimming, cycling and running loads (Table 1). Training loads were defined for each subject, quantitatively by collecting personal data about past training, and qualitatively by functional assessments as described by Palazzetti *et al.* (2003). Individual training loads were quantified by a modified version of the method of Morton *et al.* (Palazzetti *et al.* 2003). Total training load was increased by 42 % during OT. The 4-week OT induced an overloaded state shown by a significant decrease in duathlon performance capacity and V_{O2max} during running. Triathletes were randomly assigned in blind fashion to an antioxidant-supplemented (S, *n* 10) or placebo (P, *n* 10) group. After 5 weeks, three triathletes in the S group dropped out because of injuries and for personal reasons. Triathletes were long-distance competitors who managed social, occupational, family and sporting activities. Experimental procedures were approved by the Local Committee for the Protection of Persons in Biomedical Research (no. 99002), and all triathletes gave written informed consent after having been explained the purpose, possible risks and stress associated with the study. All triathletes were non-smokers, had no history of medical disorders and had not taken antioxidant supplements for at least 6 months before the study. They were instructed to refrain from making any drastic changes in diet and to abstain from anti-inflammatory or analgesic drugs throughout the study.

Table 1. Calculated total training loads and training volumes per week for swimming, cycling and running in triathletes
(Mean values and standard deviations for seventeen subjects)

	NT		OT	
	Mean	SD	Mean	SD
Total training load (AU)	648	172	906***	229
Swimming (km per week)	5·9	1·7	7·6*	2·4
Cycling (km per week)	174	41	266***	57
Running (km per week)	28	10	42***	12

NT, normal training; OT, overloaded training; AU, arbitrary units.
Mean values were significantly different from those of NT: *$P<0·05$; ***$P<0·001$.

Experimental procedures

Referring to the competition program of long-distance triathletes, training was overloaded for 4 weeks following a 4-week NT phase. Maximal functional assessments in swimming, cycling and running were performed by each triathlete before NT (Palazzetti *et al.* 2003). After NT and OT, the triathletes completed a duathlon test (run 5 km, cycle 20 km, run 5 km) at 84 (SD 2) % V_{O2max} preceded 2 d before by a maximal treadmill test. Each functional assessment before NT or duathlon was separated by 1 day off and performed at the same time of day. Before each functional assessment, body mass was measured with a calibrated balance. The % body fat was determined from the skinfold thicknesses measured at four sites (biceps, triceps, subscapular, suprailiac), as described by Durnin & Rahaman (1967). Venous blood samples were collected in basal conditions at the start of the study and after NT and OT, and in post-exercise conditions after NT and OT.

Supplementation

The S group consumed two tablets of an antioxidant complex preparation per d: Selenium A-C-E® (Richelet Laboratories, Paris, France; each containing 75 µg organic Se, mg retinyl acetate, 60 mg ascorbic acid and mg D-α-tocopheryl succinate). The P group received a placebo preparation that was identical in form and taste, and the same amount of tablets as the S group. Before the study, triathletes were told to take one tablet before breakfast and one tablet before lunch during NT and OT phases.

Nutritional status

Dietary records were kept each day of the study for each meal. All triathletes were instructed on proper nutritional recording, including estimating portion sizes. Data acquisition was made in grams and daily nutritional consumption was quantified by the CIQUAL database (Regal micro Windows 9x, NT version 1.2; Max Feinberg, Paris, France).

Nutritional status was assessed by reference to the French RDA for sportsmen, based on additional needs for daily energy expenditure exceeding 9·207 MJ, recently been established by the French Food Agency of Sanitary Security (Guilland *et al.* 2001).

Energy expenditure estimation

Each day, triathletes kept a record of the total duration of sleep, professional activity, meals, washing, dressing, travelling, training and others specific activities. This allowed assessment of the triathletes' daily energy expenditures on a 24 h basis. For each activity recorded, a multiplying coefficient was assigned from a corresponding physical activity level (Vermorel *et al.* 2001). The relationship between activity duration and physical activity level enabled calculation of a mean energy expenditure (kJ) for each activity. During training sessions, the heart rate was recorded by a telemetric system (Polar Accurex Plus; Polar Electro Oy, Kempele, Finland) and the energy expenditure was calculated from the relationship between O_2 consumption and heart rate established individually for swimming, cycling and running. The total energy expenditure was the sum of energy expenditure calculated for each activity.

Profile of mood states

Every week, each triathlete completed the profile of mood states questionnaire (POMS) (McNair *et al.* 1992). The relationship between physiological, biochemical disturbances, and behaviour and/or psychological indices is well documented. Originally, POMS was devised to indicate the occurrence of these disturbances in pathological situations. POMS is now widely used as one of the tools for identifying overloaded state. In our present study, POMS was administered to quantify the influence of training loads on mood state.

Functional assessments

Identical series of measurements were repeated by all triathletes after NT and after OT. Each test was performed at the same time of day.

Maximal treadmill test. Triathletes performed 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/h (2 % slope) for 5 min; running speed was then increased by 2 km/h every 2 min up to 14 km/h and by 1 km/h to exhaustion. During treadmill tests, ventilatory and gas exchange responses were measured on a breath-by-breath basis using an automatic spiroergometric system (Vmax 29; Sensor Medics, Rungis, France). Heart rate was monitored continuously and recorded using an electrocardiograph monitor (SMS 182; HELLIGE, Freibourg in Breisgau, Germany) and a telemetric system (Polar Accurex Plus; Polar Electro Oy). The criteria used for determining V_{O2max} were a plateau in V_{O2max} despite an increase in running speed, a respiratory exchange ratio $>1{\cdot}1$ and an heart rate $>90\,\%$ of the predicted maximal heart rate.

Duathlon test. All duathlon tests took place outdoors between March and April in Nice, France. Outside temperature ranged from 17 to 22 °C. Triathletes performed all tests under the same equipment conditions and drank the same energy-providing beverage. Before each duathlon test (run 5 km, cycle 20 km, run 5 km), all triathletes warmed-up for 30 min by alternate jogging and stretching. Running trials were performed on a flat circuit alternating lawn and asphalt. Cycling was performed on an exercise bike (EliteTravel, Fontaniva, Italy), over which was positioned the personal bike of triathletes. Duathlon tests were performed at 84 (SD 2) % V_{O2max}.

Blood sampling

Venous blood samples were collected at the start of the study (baseline), after NT and after OT in resting and post-duathlon conditions. 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 06.00 and 08.00 hours. Post-exercise venous blood samples were obtained immediately after the duathlon tests, which took place the same day at the end of afternoon. Blood samples were collected by venepuncture from an antecubital vein of each subject. Whole blood (400 μl) for glutathione analysis was immediately treated as described later. The blood samples were centrifuged (4000 rpm, 4 °C, 10 min), and plasma or serum were divided into portions and frozen in liquid N_2 before storage at −80 °C until assay.

Biochemical analysis

Oxidized and reduced glutathione. Immediately after venepuncture, 400 μl whole blood was transferred into a tube containing 3600 μl metaphosphoric acid (60 ml/l water). The contents was mixed and centrifuged for 10 min at 4 °C. Acidic protein-free supernatant fractions were stored at −80 °C until analysis. Glutathione level was determined using enzymatic cycling of GSH by means of NADPH and glutathione reductase coupled with 5,5′-dithiobis(2-nitrobenzoic acid). We estimated GSSG level according to the method of Akerboom & Sies (1981), slightly modified by Emonet *et al.* (1997). For this, we masked GSH by adding 10 μl 2-vinyl-pyridine to 500 μl deproteinized extract adjusted to pH 6 with triethanolamine. The mixture was allowed to stand for 60 min. The fraction of GSH was calculated as:

$$\text{GSH} = \text{total glutathione} - (2 \times \text{GSSG}).$$

Mass of creatine kinase isoenzyme MB. The mass of creatine kinase isoenzyme MB, determined in plasma by immunoassay using the ELISA sandwich principle with fluorogenic marker, was used to evaluate exercise-induced muscle damage.

Single-cell gel electrophoresis assay. The single-cell electophoresis assay (or comet assay), a sensitive technique for the measurement of DNA breakage in individual cells, was performed as described by Singh *et al.* (1988) with minor modifications by Emonet *et al.* (1998). One hundred and fifty μl agarose (5 g/l) 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. Next, the coverslips were removed and 5 μl whole blood mixed with 60 μl low-melting-point agarose (6 g/l; Biozym, Hessisch Oldendorf, Germany) diluted in Ca- and Mg-free PBS buffer (60 μl) was added. The slides were covered again with a coverslip and kept in the refrigerator for another 10 min to solidify the low-melting-point agarose. After removal of the coverslips, the slides were immersed in a jar containing cold lysing solution

(2·5 mM-NaCl, 100 mM-EDTA, 10 mM-TRIS, sodium sarcosinate (10 g/l), Triton X-100 (10 g/l) and dimethyl sulfoxide (100 g/l) were added fresh) and kept at 4 °C for at least 16 h. Electrophoresis was conducted using a freshly made alkaline buffer (10 M-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 (μm x % DNA). After electrophoresis (25 V, 300 mA, 30 min), the slides were placed horizontally and Tris buffer (0·4 M Tris, pH 7·5) was added to neutralize the excess alkali. The slides were allowed to sit for 5 min and this neutralization step was repeated three times. Finally, 50 μl ethidium bromide (20 μg/ml) was added 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 Division, Oberkochen, Germany) equipped with a short-arc Hg lamp HBO® (50 W, 516–560 nm; Carl Zeiss), and filters 5 and 15 (Carl Zeiss) at × 20 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 tail distance (the distance between the centre position of the head and the centre of gravity of the tail) × % DNA in the tail (relative to the total amount of DNA in the entire comet (head + tail)) (Hellman *et al.* 1995).

Index of lipid peroxidation. Thiobarbituric acid-reactive substances (TBARS) were evaluated in plasma by a fluorometer (model LS 50; Perkin-Elmer Ltd, Bucks., UK) with a malondialdehyde kit (Sobioda, Grenoble, France) as previously described (Richard *et al.* 1992).

Metalloenzymes. Plasma and erythrocyte Se-dependent GSH-Px activities were evaluated according to Günzler *et al.* (1974) using *tert*-butylhydroperoxide (Sigma Chemical Co., Paris, France) as substrate instead of H_2O_2. This technique was adapted on a Hitachi 904 analyser (Boerhinger-Mannheim, Mannheim, Germany). Results were expressed as μmol NADPH (Boehringer-Mannheim) oxidized/min per litre plasma for plasma GSH-Px. Erythrocyte Cu,Zn-SOD activity was measured after Hb precipitation by monitoring the autoxidation of pyrogallol according to the technique of Marklund & Marklund (1974). This technique was adapted for the Hitachi 904 analyser.

Selenium determination. Serum Se concentrations were determined with an atomic absorption spectrometer (Perkin-Elmer 5100; Perkin-Elmer Ltd, Norwalk, CT, USA) equipped with an HGA 600 furnace, an electron discharge lamp and Zeeman background correction (Arnaud *et al.* 1993).

Vitamin determination. Vitamin C concentration was evaluated by fluorimetry using an automated method in serum after stabilization and extraction with metaphosphoric acid solution (50 g/l) according to Speek *et al.* (1984). Retinol (0·6 mg) and α-tocopherol (20 mg) concentrations were determined by HPLC as described by Arnaud *et al.* (1991).

Statistical analysis

All values are expressed as means and standard deviations. To determine training main effect, one-way ANOVA test (supplementation) with repeated measures (NT, OT) was used to estimate daily energy intake, energy expenditure and macronutrient intake and to evaluate body mass, body fat and BMI. Differences between energy intake and energy expenditure were analysed by two-way ANOVA (supplementation, training) with repeated measures (energy intake, energy expenditure). To compare estimated daily micronutrient intakes and French RDA in NT and OT, we used Student's t test for paired values. A one-way ANOVA test (supplementation) with repeated measures (NT, OT) was applied to determine supplementation main effect on estimated dietary intake. Physiological and psychological data were pooled and Student's t test for paired values was applied. Biochemical data were analysed by two-way ANOVA test (supplementation, training) with repeated measures (pre-duathlon, post-duathlon) to determine: (1) interaction effects between supplementation, training and duathlon (supplementation × training × duathlon); supplementation and training (supplementation × training); supplementation and duathlon (supplementation × duathlon); (2) main effects (supplementation, training or duathlon). When significant changes were observed in ANOVA tests, Fisher's protected least significant difference *post hoc* test was applied to locate the source of significant differences. Statistical significance level was set at $P<0·05$.

Results

A decrease in duathlon performance capacity (68·3 (SD 7·9) *v.* 71·2 (SD 10·4) min; n 17, $P<0·05$) and in running V_{O_2max} (4·516 (SD 0·433) *v.* 4·386 (SD 0·370) l/min; n 17, $P<0·05$), and an increase in total POMS score (100·1 (SD 21·4) *v.* 108·3 (SD 21·8); n 17, $P<0·05$) showed that the 4-week OT induced an overloaded state in the triathletes.

Nutritional analysis in both groups show that energy intake, energy expenditure and macronutrient intake were increased in response to OT, and that energy intake was significantly lower ($P<0·01$) than energy expenditure in NT and OT (Table 2). On the other hand, the S and P groups did not differ in food antioxidant intakes (Table 2), but the antioxidant supplement intakes of the two groups were significantly different ($P<0·05$; Fig. 1). Supplementation during OT allowed compensation for the deficit in dietary vitamin C and E and Se (Fig. 1, Table 2). In response to OT, body mass, body fat and BMI decreased in both groups (Table 2).The observance of the supplementation (supplementation provided:effective intake ratio) was 97·9 %.

Supplementation

The supplementation induced changes in circulating concentrations in Se after the first 4 weeks (Table 3). The changes in serum α-tocopherol were significant ($P<0·05$)

Table 2. Estimation of energy intake, energy expenditure, macronutrient and micronutrient intakes/d, and evaluation of body mass, body fat, and BMI in normal and overloaded training for supplemented and placebo group triathletes ||

(Mean values and standard deviations)

	S group (*n* 5)				P group (*n* 7)			
	NT		OT		NT		OT	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Energy intake (MJ/d)	13·8††	3·1	15·0***§§	3·2	12·5††	2·5	13·8***§§	3·0
Energy expenditure (MJ/d)	15·1	1·4	17·0***	1·9	15·2	2·3	16·8***	2·4
Carbohydrate (g/d)	425	80	486***	92	367	78	404***	91
Lipid (g/d)	119	39	122*	40	115	38	127*	40
Protein (g/d)	125	32	130*	31	113	21	122*	27
Se (μg/d)¶	95	13	98	17	85	22	82	21
β-Carotene (μg RE/d)	625	244	600	192	497	275	496	348
Vitamin C (mg/d)¶	168	82	187	76	144	67	138	83
Vitamin E (mg TE/d)¶	11·0	3·6	11·3	3·6	10·2	1·7	11·2	5·3
EDI Se (μg/d)	245	13	248	17	85‡	22	82‡	21
FRDA Se (μg/d)	102†††	10	116†††	13	103†	16	114†	17
EDI β-carotene (μg RE/d)	625	244	600	192	497	275	496	348
FRDA β-carotene (μg RE/d)	633	55	711	74	637	90	702	94
EDI Vitamin C (mg/d)	288	82	307	76	144‡	67	138‡	83
FRDA Vitamin C (mg/d)	250	33	296	45	252†††	54	291†††	57
EDI Vitamin E (mg TE/d)	31·0	3·6	31·3	3·6	10·2‡	1·7	11·2‡	5·3
FRDA Vitamin E (mg TE/d)	28·8	4·0	34·3	5·4	29·1†††	6·5	33·7†††	6·8
Body mass (kg)	69·1	4·1	68·4**	3·9	69·7	4·7	68·9**	4·5
Body fat (%)	12·4	3·9	11·4**	3·7	12·5	1·6	11·5**	1·7
BMI (kg/m^2)	22·4	1·5	22·0***	1·5	22·5	1·6	22·2***	1·5

S, antioxidant supplemented; P, placebo; NT, normal training; OT, overloaded training; EDI, estimated dietary intake (including antioxidant supplementation (selenium A–C–E®; Richelet Laboratories, Paris, France)); FDRA, French recommended dietary allowance (Guilland *et al.* 2001); RE, retinol equivalents; TE, tocopherol equivalents.

Mean values were significantly different from those of NT: *$P<0·05$, **$P<0·01$, ***$P<0·001$.

Mean values were significantly different from EDI: †$P<0·05$, ††$P<0·01$, †††$P<0·001$.

Mean values were significantly different from those of the S group: ‡$P<0·05$.

Mean values were significantly different from energy expenditure: §§$P<0·01$.

|| For details of subjects, training loads, supplementation and procedures, see Table 1 and pp. 92–94.

¶ Values do not include antioxidant supplementation.

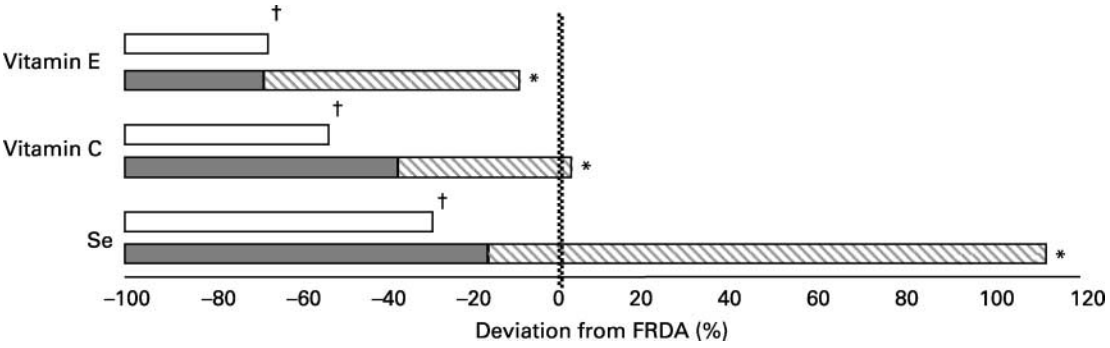

Fig. 1. Food and supplement vitamin C and E and selenium intakes/d relative to the French RDA (FRDA; Guilland *et al.* 2001) in supplemented (*n* 5) and placebo (*n* 7) group triathletes during overloaded training. □, Placebo group; ■, supplemented group; ▨, supplement contribution. For details of subjects and procedures, see p. 92. Mean values were significantly different from those of the P group:*$P<0·05$. Mean values were significantly different from those of the FRDA: †$P<0·05$.

when the complete duration of the study was taken into account (Table 3), and concentrations of retinol did not change with supplementation. An interaction effect of supplementation × training was observed for ascorbic acid ($P<0·05$; Table 3). From the beginning of the study during NT, blood GSH levels increased in response to supplementation (Fig. 2) and remained high during OT. During OT the levels tended to be still higher with supplementation ($P=0·09$). In all situations after NT and OT, plasma GSH-Px activity was significantly higher ($P<0·05$) in the S group than in the P group (Table 4).

Supplementation × training effect

There were no effects of antioxidant supplementation on plasma TBAR levels or on GSH:GSSG ratio at rest

Table 3. Antioxidant status in supplemented and placebo group triathletes at baseline after NT and OT‡
(Mean values and standard deviations)

		S group (n 7)		P group (n 10)		Statistical significance of effect (ANOVA)	
		Mean	SD	Mean	SD	Supplemented	Supplemented × Training
Serum Se (μmol/l)	Baseline	0·98	0·13	1·06	0·13		
	After NT	1·37†	0·13	1·03***	0·15	$P<0·001$	NS
	After OT	1·35†	0·16	1·08***	0·12		
Serum ascorbic acid (μmol/l)	Baseline	57·6	16·4	70·0	19·4		
	After NT	65·5	10·9	61·1	18·0	NS	$P<0·05$
	After OT	69·6	8·6	56·9	18·1		
Serum α-tocopherol (μmol/l)	Baseline	27·0	5·8	24·1	4·1		
	After NT	30·1	5·6	24·6*	6·1	$P<0·05$	NS
	After OT	28·1	4·8	23·7*	4·9		

S, antioxidant supplemented; P, placebo; NT, normal training; OT, overloaded training.
Mean values were significantly different from those of the s group; *$P<0·05$, *** $P<0·001$.
Mean values were significantly different from those at baseline: † $P<0·05$.
‡ For details of subjects, training loads, supplements and procedures, see Table 1 and pp. 92–94.

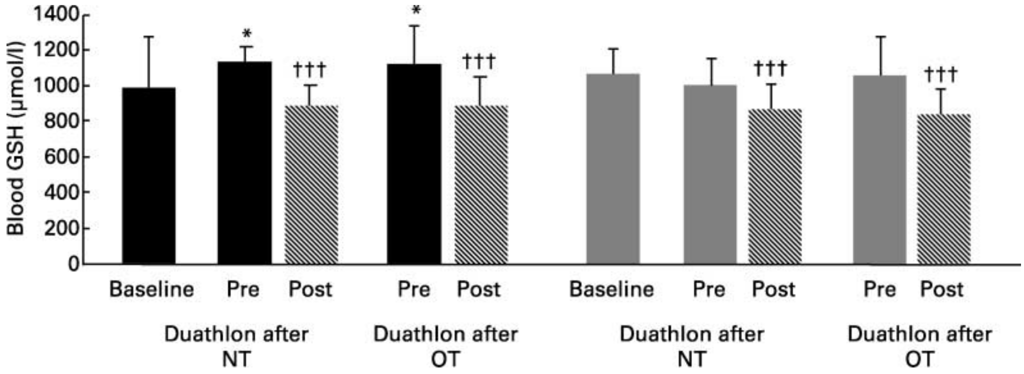

Fig. 2. Blood GSH levels in antioxidant-supplemented (n 7; ■) and placebo (n 10; ■) group triathletes at baseline, pre-duathion (■ and ▧) and post-duathion (■ and ▧), after normal training (NT) and overloaded training (OT) respectively. For details of subjects, supplements, training and procedures, see Table 1 and pp. 92–94. Values are means with their standard deviations shown by vertical bars. Mean values were significantly different from those at baseline within the same group: *$P<0·05$ (Fisher's protected least significant difference *post hoc* analysis). Mean values were significantly different from those pre-duathlon within the same group: $^{†††}P<0·001$ (Fisher's protected least significant difference *post hoc* analysis).

(Table 4). No effect of OT on creatine kinase isoenzyme MB mass was observed at rest (Fig. 3). The S group had an increase in erythrocyte Cu,Zn-SOD activity in response to OT (F 5·1, $P<0·05$) (Fig. 4). Supplementation did not modify a decrease in erythrocyte GSH-Px activity at rest in response to OT (Table 4). In both groups, OT induced an increase in leucocyte-DNA damage (S group 12·0 (SD 13·6) *v*. 20·1 (SD 6·9), $P<0·05$; P group 10·4 (SD 5·9) *v*. 23·0 (SD 8·4), $P<0·05$) and in plasma GSH-Px activity (Table 4). After NT, plasma GSH-Px activity decreased in the P group (412·9 (SD 65·9) *v*. 358·1 (SD 63·0) U/l, $P<0·05$), and there was smaller change in the S group (460·8 (SD 78·0) *v*. 444·0 (SD 72·9) U/l, $P<0·05$).

Supplementation × duathlon effect

Antioxidant supplementation significantly reduced ($P<0·05$) the magnitude of duathlon-induced creatine kinase isoenzyme MB mass increase, an effect that tended to be greater with OT ($P=0·09$, Fig. 3). The increase in plasma GSH-Px activity pre- and post-duathlon was not modified by supplementation (Table 4). There was no interaction effect of OT × duathlon or supplementation × duathlone on the increase in erythrocyte GSH-Px activity (Table 4). Supplementation did not prevent a decrease in blood GSH in response to the duathlon (Fig. 2).

Supplementation × training × duathlon

No interaction effect of supplementation × training × duathlon was observed for the all biochemical variables analysed.

Discussion

We hypothesized that an antioxidant supplementation at physiological doses would partially avoid antioxidant system downregulation and consequently lower chronic and/or acute exercise-induced oxidative damage to lipids or DNA in overloaded triathletes. Based on French RDA for energy expenditure, the increase in energy expenditure in the triathletes with OT induced an increase in theoretical micronutrient need. Spontaneous nutritional intakes did

Table 4. Markers of free radical production and variables of endogenous antioxidant potential in supplemented and placebo group triathletes at baseline, and pre- and post-duathlon after normal training and overloaded training||

(Mean values and standard deviations)

		S group (*n* 7)				P group (*n* 10)			
		Pre-duathlon		Post-duathlon		Pre-duathlon		Post-duathlon	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
GSSG (μmol/l)	Baseline	17·4	5·9	na	na	15·0	5·5	na	na
	After NT	17·5	5·9	27·0***	7·5	17·6	7·4	26·4***	8·0
	After OT	17·8	4·0	29·4***	6·1	16·6	5·8	26·8***	5·9
GSH:GSSG	Baseline	59·1	14·9	na	na	75·7	20·5	na	na
	After NT	71·0	20·8	35·3***	10·2	61·5	19·5	34·3***	10·6
	After OT	67·2	21·2	31·1***	4·5	70·3	29·8	31·0***	4·3
TBARS (μmol/l)	Baseline	2·70	0·45	na	na	2·48	0·27	na	na
	After NT	2·36	0·54	2·46*	0·34	1·95	0·30	2·13*	0·29
	After OT	2·25	0·30	2·38*	0·39	1·95	0·30	2·05*	0·32
Erythrocyte GSH-Px (U/g Hb)	Baseline	44·1	11·7	na	na	41·8	9·7	na	na
	After NT	44·9	11·6	45·9*	11·2	42·0	10·1	43·1*	9·9
	After OT	42·4‡§	9·8	44·2*	11·4	39·6‡§	8·6	40·6*	9·3
Plasma GSH-Px (U/l)	Baseline	460·8	78·0	na	na	412·9††	65·9	na	na
	After NT	444·0‡	72·9	475·3***	63·9	358·1††‡	63·0	408·6***††	60·7
	After OT	479·8§	65·1	492·5***	72·8	400·0††§	40·7	416·8***††	55·0

S, antioxidant supplemented; *P*, placebo; TBARS, thiobarbituric acid-reactive substances; GSH-Px, glutathione peroxidase; NT, normal training; OT, overloaded training; na, not available.

Mean values were significantly different from those pre-duathlon: *$P < 0·05$, ***$P < 0·001$.

Mean values were significantly different from those of the S group: ††$P < 0·01$.

Mean values were significantly different from those at baseline: ‡$P < 0·05$.

Mean values were significantly different from those after NT: §$P < 0·05$.

|| For details of subjects, training loads, supplementation and procedures, see Table 1 and pp. 92–94.

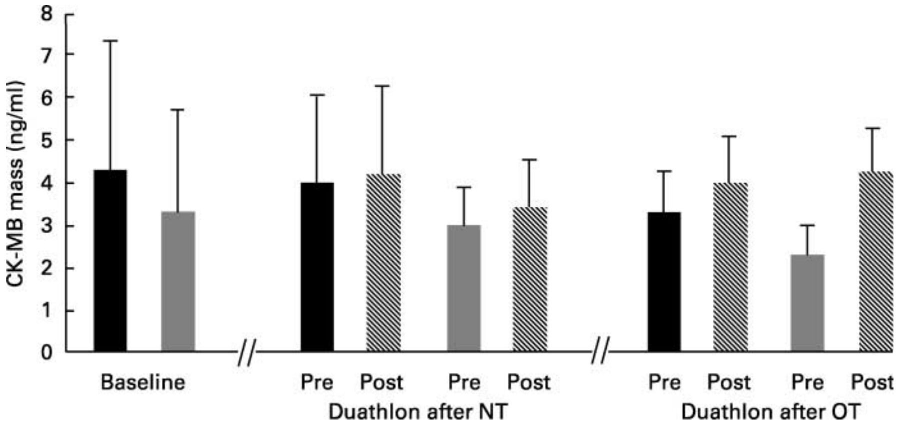

Fig. 3. Plasma creatine kinase (CK) isoenzyme MB mass in antioxidant-supplemented (*n* 7; ■) and placebo (*n* 10; ■) group triathletes at baseline, pre-duathion (■ and ▨) and post-duathion (■ and ▨), after normal training (NT) and overloaded training (OT) respectively. For details of subjects, supplements, training and procedures, see Table 1 and pp. 92–94. Values are means with their standard deviations shown by vertical bars. There was a significant supplementation × duathlon interaction effect after NT and OT: *F* 4·9, $P < 0·05$.

not enable any of the athletes to reach the French recommendations for intakes of Se, and vitamins C and E. With regard to energy expenditure, it has to be emphasized that for Se, and vitamins C and E, the supplement was administered at physiological doses and allowed the subjects to reach 100 % of the French recommendations without reaching the non-observable adverse effect level. The 97·9 % observance of the supplement intake had an effect on Se, α-tocopherol and ascorbic acid plasma concentrations.

As lipoperoxidation (and muscle damage) may be greater in trained than in sedentary subjects (Kanter *et al.* 1993; Marzatico *et al.* 1997), it was possible that poorly conducted training or OT led to a lack of the expected protective adaptations to training, especially if combined with a decreased density of antioxidant intakes provided by food. From the beginning of the study and during NT, blood GSH levels increased with supplementation and remained elevated during OT. During OT, the levels tended to be still higher with supplementation ($P=0·09$). GSH is a component of the antioxidant system and its efficiency depends on the synergic effects of the components in the system. Some of them are reinforced, for example, by exogenous supplementation. In our present study,

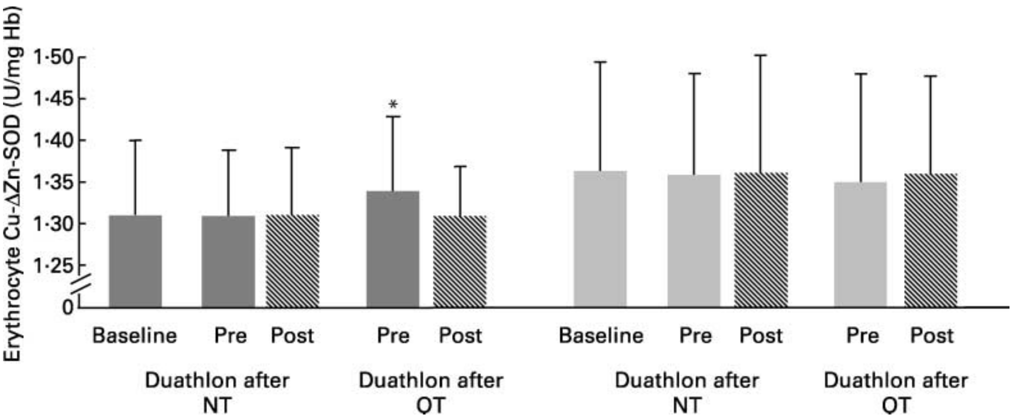

Fig. 4. Erythrocyte Cu,Zn-superoxide dismutase (SOD) activity in antioxidant-supplemented (*n* 7; ■) and placebo (*n* 10; ▨) group triathletes on baseline, pre-duathlon (□) and post-duathlon (▨), after normal training (NT) and overloaded training (OT). For details of subjects, supplements, training and procedures, see Table 1 and pp. 92–94. Values are means with their standard deviations shown by vertical bars. Mean value was significantly different from that pre-duathlon after NT: *$P < 0{\cdot}05$ (Fisher's protected least significant difference *post hoc* analysis).

GSSG could have been spared from oxidation because of the increase in ascorbic acid and α-tocopherol. This effect could have been reinforced by training, which is known to increase GSSG concentrations (Margaritis *et al.* 1997). Moreover, upregulation of the endogenous antioxidant system by supplementation was shown by the increase in erythrocyte Cu,Zn-SOD activity in the S group.

The rapid exercise-induced increase in activity of plasma GSH-Px after the duathlon can be seen as an acute response to exercise-induced oxidative stress; indeed, acute stress is observed under other conditions. Acute exposure to such a situation could increase antioxidant activity during and/or after exercise to anticipate and/or respond to the free radical overproduction that occurs during reperfusion of kidney. As plasma GSH-Px is quickly released from the kidney in the case of oxidative stress (Nadif *et al.* 1998), its release may be the anticipated response to the oxidative stress. OT induced an increase plasma GSH-Px activity at rest in both groups. This increase appears to be an adaptive effect over longer periods to repetitive stress induced by exercise. Plasma GSH-Px activity was significantly higher in the S group in all situations after NT and OT. Greater tissue GSH-Px activity due to the increase in circulating Se levels (as a consequence of supplementation) caused an increased release by the kidneys in response to repetitive stress.

Despite this apparent upregulation of endogenous circulating antioxidant response by the antioxidant mixture, there was no effect on plasma TBARS, a finding which is consistent with a previous and independent epidemiological study in sedentary subjects (Preziosi *et al.* 1998). Lipid peroxidation and muscle damage were reduced with supplementation with mg vitamin E/d for 5 weeks in subject previously moderately trained before an OT period (Itoh *et al.* 1999). The same effects were observed with lower doses (330 mg/d) after intensive aerobic training in cyclists (Rokitzki *et al.* 1994*a*). The question of the level of dose administered seems to be important. Administration of 68·5mg vitamin E/d and 200 mg ascorbic acid/d for 4·5 weeks to marathon runners had no effect on lipid peroxidation (Rokitzki *et al.* 1994*b*). In our present study, the doses of α-tocopheryl succinate and ascorbic acid were lower (20 mg/d and 120 mg/d respectively). The 150 μg Se added did not cause a synergistic effect of the mixture components. Moreover, we cannot dismiss the possibility that the increase in clearance of TBARS in well-trained subjects before the study could have blinded the increase in lipid peroxidation products.

DNA is probably the most biologically significant target of oxidative attack (Halliwell, 2000). Some studies dealing with exercise and oxidative stress have shown an increase in leucocyte-DNA damage due to intensive aerobic exercise (Hartmann *et al.* 1994; Tsai *et al.* 2001). Training status (Niess *et al.* 1996; Radàk *et al.* 1999; Sato *et al.* 2003) or vitamin E supplementation (Hartmann *et al.* 1995) seemed to protect against this damage. There was no effect of the antioxidant mixture on the extent of leucocyte-DNA damage after OT in our present study. The mechanisms of oxidative leucocyte-DNA damage induced either by acute exercise, and of reinforcement of endogenous antioxidant by the supplement and the expected protective effect on leucocyte-DNA, are not necessarily identical. Independent of an exercise effect, there have been mixed results on the effect of supplement complexes containing vitamins C and E on leucocyte-DNA damage (Duthie *et al.* 1996; Prieme *et al.* 1997; Huang *et al.* 2000). The lack of carotenoids or closely associated substances available from fruits and vegetables (Collins, 1999) in our administered antioxidant complex may also explain the lack of expected protective effects.

Myocellular enzyme release due to increased sarcolemmal and lysosomal membrane permeability is easily estimated by circulating activities and/or concentrations. Even if muscle damage has several origins, in our present study this effect was largely attributable to membrane peroxidation. The magnitude of exercise-induced muscle damage increase, which tended moreover to be higher with OT, was significantly decreased with supplementation. This decrease suggests that supplementation has a protective effect despite the lack of effect on exercise-induced increase in lipid peroxidation markers. The same decrease in oxidative damage after exercise has already been shown, but at higher supplementation with vitamin E (Meydani *et al.* 1993) or vitamin C (Alessio *et al.* 1997). Our present results show that even at a lower dose of supplementation and at higher training level, antioxidants provide a protective effect against exercise-induced muscle damage.

Conclusion

The effects of antioxidant supplementation during NT and/or OT are shown mostly through alleviation of acute exercise-induced muscle damage. Whatever the mechanisms involved, the antioxidant mixture helped to preserve the antioxidant system during OT-induced stress in subjects with initially low antioxidant intakes. Most of the studies did not attempt to define an optimal dose for protection (McCall & Frei, 1999). The current results are still inadequate to define the optimal dose for protection of OT subjects. In the present study the effects of the antioxidant mixture were observed for doses that can be provided by a diverse and well-balanced diet. Hence, maintaining a normal nutritional status with regard to antioxidant intake (Se, and vitamins C and E) plays a key role in antioxidant adaptive effects during NT and OT.

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