U.PORTO
FACULDADE DE CIÊNCIAS DA NUTRIÇÃO E ALIMENTAÇÃO
UNIVERSIDADE DO PORTO

OXIDATIVE STRESS, MUSCLE DAMAGE AND INFLAMMATION IN KAYAKERS AND CANOEISTS: EFFECTS OF ACUTE AND CHRONIC EXERCISE AND ANTIOXIDANTS SUPPLEMENTATION

Vitor Hugo da Costa Gomes Moreira Teixeira

Porto, 2008

Dissertation submitted to the Faculty of Nutrition and Food Sciences of the University of Oporto for the degree of Doctor of Philosophy in Human Nutrition

Supervisor: Prof. Doutor Pedro Moreira

Co-supervisor: Prof. Doutor Franklim Marques

This investigation was partially funded by PRISFAR - Produtos Farmacêuticos, SA

CONTENTS

ACKOWLEGMENTS

This thesis was made possible due to the effort and cooperation of a lot of people to whom I am grateful. I like to express my sincere gratitude to:

My family

Prof. Doutor Pedro Moreira

Prof. Doutor Franklim Marques

Prof.ª Doutora Susana Casal

Professora Doutora Maria Daniel Vaz de Almeida

Prof. Doutor Bruno Oliveira

Prof. Doutor Nuno Borges

Dr.ª Bárbara Duarte

Dr.ª Laura Pereira

Dr. Hugo Valente

Dr. Mário Santos

Dr. José Garcia

Prof. Ryszard Hoppe

Dr. Tiago Lourenço

Sr. Rui Fernandes

Sr. Marcos Oliveira

Sr. Carlos Oliveira

Dr.ª Mariana Andrade

Dr.ª Alexandra Freitas

Dr.ª Ana Bessa

All the sedentary volunteers

All athletes of the Portuguese Canoeing Federation

LIST OF ABBREVIATIONS

$^1O_2$ - singlet oxygen
AAS - atomic absorption spectrometry
$ABTS^{\bullet+}$ - 2,2'-azino-di-(3-ethylbenzothiazoline-6-sulphonate
ACSM - American College of Sports Medicine
AI - adequate intake
AIS - Australian Institute of Sport
AOX - antioxidant
ATPase - adenosine triphosphatase
BMI - body mass index
BMR - basal metabolic rate
CAT – catalase
$CCl_3^{\bullet}$ - trichloromethyl
CK - creatine kinase
EAR - estimated average requirement
EDTA - ethylenediaminetetraacetic acid
em - emission
ex - excitation
FNB - Food and Nutrition Board
GPx - glutathione peroxidase
Gr - glutathione reductase
GSH - glutathione
GSSG - oxidised glutathione
H_2O_2- hydrogen peroxide
HDL - high-density lipoproteins
HOCl - acid hypochlorous acid
HPLC - high-performance liquid chromatography
HSP - heat shock proteins
IL-10 - interleukin-10
IL-1β - interleukin-1 beta
IL-1ra – interleukin-1 receptor antagonist
IL-6 - interleukin-6
Kcal - kilocalories
LDL - low-density lipoproteins
MDA - malondialdehyde
NAD^+ - nicotinamide adenine dinucleotide
NADPH oxidase - nicotinamide adenine dinucleotide phosphate-oxidase
$NO^{\bullet}$ - nitric oxide
$NO_2^{\bullet}$ - nitrogen dioxide
NS - non-significant
$O_2^{\bullet-}$ - superoxide
O_3- ozone

$OH^{\bullet}$ - hydroxyl
$ONOO^{-}$ - peroxynitrite
PAL - physical activity level
PLA - placebo
$RO^{\bullet}$ - alkoxyl
$ROO^{\bullet}$ - peroxyl
$ROOH^{\bullet}$ - hydroperoxyl
ROS - reactive oxygen species
rpm – rotations per minute
RS - thinyl
RSS - perthynil
SDS - sodium dodecyl sulfate
SOD - superoxide dismutase
SPSS - statistical package for social sciences
TAS - total antioxidant status
TBARS - thiobarbituric acid reactive substances
TNF-α - tumor necrosis factor-alpha
VLDL - very low-density lipoproteins
XD - xanthine dehydrogenase
XO - xanthine oxidase

ABSTRACT

It is postulated that exercise generates a surplus production of reactive oxygen species that may even surpass the strengthened antioxidant defences of athletes, causing irreparable cellular oxidative damage, which can harm their health and physical performance. This work aimed to describe antioxidant status, oxidative stress and damage of a group of elite kayakers and canoeists at rest and in response to acute exercise (1000m kayak trial) and chronic exercise (1 year of habitual training and competition), and investigate if a supplementation with a combination of antioxidants could attenuate these effects.

Dietary intake of antioxidants was estimated, and blood superoxide dismutase (SOD), glutathione reductase (Gr), glutathione peroxidase (GPx) and creatine kinase (CK) activities, and total antioxidant status (TAS), uric acid, α-tocopherol, retinol, α-carotene, β-carotene, lycopene, lutein plus zeaxanthin, thiobarbituric acid reactive substances (TBARS), interleukin-6 (IL-6) and cortisol levels were determined in several occasions in 2 seasons, while athletes were training on the Portuguese National Team Campus. The blood samples were collected in resting conditions and 15 min after a 1000m kayak race, both before and after supplementation. In last study, athletes were randomly assigned to receive a placebo or an antioxidant supplement (272 mg α-tocopherol, 400 mg vitamin C, 30 mg β-carotene, 2 mg lutein, 400 μg selenium, 30 mg zinc and 600 mg magnesium) for 4 weeks.

Kayakers/canoeists exhibited significantly elevated markers of lipid peroxidation (TBARS) and muscle damage (CK) compared with sedentary subjects, despite having increased α-tocopherol, α-carotene and β-carotene levels and SOD activity. The activities of GPx and Gr, and the levels of lycopene, lutein + zeaxanthin, retinol and uric acid were similar between groups. Athletes presented reduced TAS values. Vitamin A, selenium, zinc, copper, iron, magnesium and manganese intake was higher in sportsmen, whereas that of vitamin C, α-tocopherol and β-carotene did not differ significantly from non-trained controls.

Enzymatic and non-enzymatic antioxidants remained unchanged following the 1000m kayak race, with the exception of α-carotene in men, which increased, and lycopene in women, which decreased. Uric acid levels incremented significantly after exercise in both sexes. This acute exercise resulted in a statistically significant decline in TAS and increment in CK activity and TBARS and IL-6 levels more markedly and statistical significant in male athletes.

From the competitive period of one season to that of another, TAS and cortisol levels diminished significantly. The increase in plasma α-tocopherol levels from the competitive period of the first season to the non-competitive period of the second season might have attenuated the basal levels of lipid peroxidation (TBARS) in the latter in comparison to the former period. Within the same season, the activity of GPx increased and that of SOD and cortisol and uric acid levels lowered significantly from the pre- to the competitive period. The other variables determined did not exhibited significant alterations throughout the entire study. Antioxidant vitamins levels were within reference values for athletes at all the moments analysed.

Plasmatic α-tocopherol and β-carotene concentrations increased significantly only in the antioxidant supplemented athletes. The levels of other vitamins and the activities of antioxidant enzymes were not different between groups, either before or after supplementation. The exercise-induced increases in IL-6, TBARS and uric acid levels were unaffected by antioxidant treatment. CK increased significantly in response to the exercise in both groups, but decreased to a less extent from pre- to post-supplementation period with the administration of antioxidants. Cortisol levels augmented more during the 4-week period in the placebo group. Antioxidants caused TAS to increase in response to exercise, instead of the decline observed without supplementation. The antioxidant intervention did not influence performance.

Our work suggests that the enhanced antioxidant status of elite kayak paddlers over non-trained individuals is not sufficient to avoid an increase in lipid peroxidation and muscle damage elicited by regular high-load training. A single 1000m kayak race induces oxidative stress and damage, eventually more markedly in male athletes. The greater fluctuations of redox status within the same season than in two consecutive seasons alerts for the need of a frequently monitoring over the course of the habitual training period, instead of off-season and/or pre-competition transversal evaluations. According to our results, antioxidant supplementation does not afford protection against exercise-induced lipid peroxidation and inflammation in already highly-trained kayakers, and further research is needed to exclude the hypothesis of a hindered muscle damage recovery.

RESUMO

Presume-se que o exercício possa elevar a geração de espécies reactivas de oxigénio a um nível que ultrapasse a fortalecida defesa antioxidante dos atletas, causando danos oxidativos irreparáveis a nível celular, com prejuízo da sua saúde e *performance* física. Este trabalho teve como objectivo descrever o estado antioxidante, o stress e dano oxidativos de um grupo de caiaquistas e canoístas em repouso e em resposta ao exercício agudo (prova de caiaque de 1000 m) e ao exercício crónico (1 ano de treino e competição), bem como investigar se a sumplementação com uma combinação de antioxidantes poderia atenuar estes efeitos.

Estimou-se a ingestão alimentar de antioxidantes e determinou-se a actividade da dismutase do superóxido (SOD), reductase da glutationa (Gr), peroxidase da glutationa e cínase da creatina e os níveis do estado antioxidante total (TAS), ácido úrico, α-tocoferol, retinol, α-caroteno, β-caroteno, licopeno, luteína e zeaxantina, substâncias reactivas ao ácido tiobarbitúrico (TBARS), interleucina-6 (IL-6) e cortisol em várias ocasiões em 2 épocas deportivas, quando os atletas se encontravam no Centro de Treinos Nacional. As amostras de sangue foram colhidas em repouso e 15 minutos depois do conclusão da prova de 1000m de caiaque, antes e após do período de suplementação. Neste último estudo, os atletas foram distribuídos aleatoriamente para receber um placebo ou um suplemento antioxidante (272 mg α-tocoferol, 400 mg vitamina C, 30 mg β-caroteno, 2 mg luteína, 400 μg selénio, 30 mg zinco and 600 mg magnésio) durante 4 semanas.

Os caiaquistas/canoístas exibiram níveis significativamente mais elevados dos marcadores de peroxidação lipídica (TBARS) e de dano muscular (CK) em comparação com os indivíduos sedentários, apesar de terem níveis aumentados de α-tocoferol, α-caroteno e β-caroteno e maior actividade da SOD. A actividade da GPx e da Gr e os níveis de licopeno, luteína + zeaxantina, retinol e ácido úrico foram semelhantes entre os grupos. Os atletas apresentaram valores mais reduzidos de TAS. A ingestão de vitamina A, selénio, zinco, cobre, ferro, magnésio e manganês foi maior nos atletas, enquanto que a de vitamina C, α-tocoferol e β-caroteno não diferiu significativamente da dos indivíduos não treinados.

Os antioxidantes enzimáticos e não-enzimáticos permaneceram inalterados após a prova de 1000 m em caiaque, com excepção do nível de α- caroteno nos homens, que aumentou, e do licopeno nas mulheres, que diminuiu. Os níveis de ácido úrico incrementaram significativamente após o exercício em ambos os sexos. Este exercício agudo resultou na

diminuição do TAS e na elevação da actividade da CK e dos níveis de TBARS e IL-6 de forma marcada e estatisticamente significativa em atletas do sexo masculino.

Os níveis de TAS e cortisol diminuíram significativamente do período competitivo de uma época para o correspondente da época seguinte. O aumento dos níves de α-tocoferol do período competitivo da primeira época para o período não-competitivo da época seguinte pode ter contribuído para a atenuação dos níveis basais de peroxidação lipídica (TBARS) neste último. Dentro da mesma época, a actividade da GPx aumentou e a actividade da SOD e os níveis de cortisol e ácido úrico diminuíram significativamente do período pré-competitivo para o pós-competitivo. Não foram observadas alterações significativas nas outras variáveis determinadas ao longo de todo o estudo. Os níveis plasmáticos de vitaminas antioxidantes encontravam-se dentro dos valores de referência para atletas em cada um dos momentos analisados.

As concentrações plasmáticas basais de α-tocoferol e β-caroteno aumentaram de forma significativa nos atletas suplementados com antioxidantes. Os níveis das outras vitaminas e a actividade das enzimas antioxidantes não diferiram entre os grupos, quer antes quer depois da suplementação. O aumento nos níveis de IL-6, TBARS e ácido úrico com o exercício não foi alterado pelo tratamento com antioxidantes. A actividade da CK elevou-se significativamente em resposta ao exercício em ambos os grupos, mas a diminuição da sua actividade do período antes para o período após a suplementação foi menor com a administração de antioxidantes. O aumento dos níveis de cortisol durante o período de 4 semanas de suplementação foi maior no grupo placebo. A suplementação antioxidante resultou num aumento do TAS em resposta ao exercício, ao invés do declínio observado na sua ausência. A intervenção com antioxidantes não influenciou a *performance*.

O nosso trabalho sugere que o estado antioxidante reforçado dos canoístas e caiaquistas de elite em relação aos indivíduos sedentários não é suficiente para abolir o aumento da peroxidação lipídica e dano muscular causados pelo treino intenso regular. Uma prova de caiaque de 1000 m induz stress e dano oxidativos, eventualmente mais marcados em atletas masculinos. As maiores fluctuações do estado redox dentro da mesma época que entre épocas consecutivas alerta para a necessidade da sua monitorização frequente ao longo do curso do treino habitual, ao invés de avaliações tranversais nos períodos não competitivos e/ou pré-competitivos. De acordo com os nossos resultados, a suplementação antioxidante não oferece protecção contra a peroxidação lipídica e inflamação induzidas pelo exercício em caiaquistas e

canoístas altamente treinados, sendo necessária mais investigação para excluir a hipótese de que possa dificultar a recuperação das lesões musculares induzidas pelo exercício.

RÉSUMÉ

Il est postulé que l'exercice peut augmenter la génération d'espèces réactives d'oxygène a un niveau qui dépasse inclusivement la renforcé défense antioxydant des athlètes, provoquant des dommages oxydatifs irréparables au niveau cellulaire, préjudiciant sa santé et performance physique. Ce travail a eu comme objectif décrire l'état antioxydant, le stress et dommage oxydatif d'un groupe de kayakistes e canoéistes en repos et en réponse à un exercice aiguë (essai de 1000m kayak) et au exercice chronique (1 an d'entrainement et de compétition habituel), et investiguer si la supplémentation avec une combinaison d'antioxydants pourrait atténuer ces effets.

L'apport alimentaire d'antioxydants a été estimé, et l'activité de la superoxyde dismutase (SOD), de la glutathione reductase (Gr), de la glutathione peroxidase (GPx) et de la créatine kinase (CK) et les niveaux de statut antioxydant total (TAS), acide urique, α-tocophérol, rétinol, α-carotène, β-carotène, lycopène, lutéine, zéaxanthine, substances réactives à l'acide thiobarbiturique (TBARS), interleukine 6 (IL-6) et cortisol ont été déterminés en diverses occasions en 2 saisons sportifs pendant que les athlètes se trouvaient au Centre National d'Entraînements du Portugal. Les prises de sang ont été réalisées en repos et 15 min après la fin d'un essai de 1000 m de kayak, avant et après la période de supplémentation. Dans ce dernier étude, les athlètes ont été distribués aléatoirement pour recevoir un placebo ou un supplément antioxydant (272 mg α-tocophérol, 400 mg vitamine C, 30 mg β-carotène, 2 mg lutéine, 400 μg sélénium, 30 mg zinc et 600 mg magnésium) pendant 4 semaines.

Les kayakistes/canoéistes ont exhibés des niveaux significativement plus élevé des marqueurs de peroxydation lipidique (TBARS) et de dommages musculaires (CK) en comparaison avec des individus sédentaires, malgré avoir des niveaux augmentés de α-tocophérol, α-carotène et β-carotène et de l'activité de la SOD. Les activités de la GPx et Gr et les niveaux de lycopène + zéaxanthine, rétinol et acide urique ont été semblable entre les groupes. Les athlètes ont présenté des valeurs réduits de TAS. L'apport de vitamine A, sélénium, zinc, cuivre, fer, magnésium et manganèse a été plus élevé dans les athlètes, tant que ce de vitamine C, α-tocophérol et β-carotène n'a pas différé significativement des individus non entraînés.

Les antioxydants enzymatiques et non-enzymatiques ont restés inaltérables après l'essai de 1000m de kayak, avec exception du α-carotène chez les hommes, qui ont augmentés, et du

lycopène chez les femmes, qui ont diminués. Les niveaux d'acide urique ont incrémentés significativement après l'exercice dans les deux sexes. Cet exercice aiguë a résulté en une diminution statistiquement significatif du TAS et en un incrément de l'activité de la CK et des niveaux de TBARS et IL-6 de façon plus accentué et statistiquement significatif dans les athlètes du sexe masculin.

Du période compétitif d'une saison sportif à l'un d'autre saison, le TAS et les niveaux de cortisol ont diminués significativement. L'augmentation des niveaux de α-tocophérol des le période compétitif de la première saison jusqu'à la période non-compétitif de la deuxième saison peut avoir contribué pour l'atténuation des niveaux basales de peroxydation lipidique (TBARS) dans ce dernier. Dans la même saison, l'activité de la GPx a augmentée et celle de la SOD et des niveaux de cortisol et acide urique ont diminué significativement des la pré-compétition jusqu'au période compétitif. Les autres variables déterminées n'ont pas exhibées des altérations significatives pendant l'ensemble de l'étude. Les niveaux des vitamines antioxydants étaient dans les paramètres référenciés pour les athlètes dans tous les moments analysés.

Les concentrations de α-tocophérol et β-carotène plasmatique ont augmentés significativement seulement dans les athlètes supplémentés avec antioxydants. Les niveaux des autres vitamines et l'activité des enzymes antioxydants n'ont pas différés entre groupes, même avant comme après la supplémentation. L'augmentation du IL-6, TBARS et acide urique, induit pas l'exercice, n'ont pas été affectés par le traitement antioxydant. La CK a augmentée significativement en réponse à l'exercice dans les deux groupes, mais a diminuée pour une moindre mesure des avant jusqu'après le période de supplémentation avec l'administration d'antioxydants. Les niveaux de cortisol ont augmentés plus pendant le période de 4 semaines dans le groupe placebo. Les antioxydants ont causés une augmentation du TAS à la suite de l'exercice, au lieu de la baisse observée sans supplémentation. L'intervention avec antioxydants n'a pas eu d'influence sur la performance.

Notre travaille suggère que l'état antioxydant renforcé d'athlètes d'élite de kayak sur individus non-entrainés n'est pas suffisant pour éviter une augmentation de peroxydation lipidique et de dommages musculaires suscité par l'entrainement régulier de haute-charge. Une simple course de 1000m de kayak induit stress oxydative et dommage, éventuellement plus marqué en athlètes masculins. La plus grande fluctuation du statut redox dans la même saison de que en deux consécutive saisons nous alerte pour la nécessité d'un suivit fréquent au cours du période habituel d'entrainement, au lieu de hors-saison et/ou évaluations en pré-

compétition. Selon nos résultats, la supplémentation d'antioxydants ne protège pas contre la peroxydation lipidique el inflammation induite par l'exercice en kayakistes de haut niveau d'entrainement, et d'autres recherches sont besoin pour exclure l'hypothèse d'une difficulté de réparations des dommages musculaires.

1. INTRODUCTION

Reactive oxygen species (ROS) are continuously generated in all cells of aerobic organisms as part of their normal metabolism [1, 2]. The term ROS encompasses free radicals (e.g. superoxide, $O_2^{\bullet -}$; hydroxyl, $OH^{\bullet}$; alkoxyl, $RO^{\bullet}$; peroxyl, $ROO^{\bullet}$; and hydroperoxyl, $ROOH^{\bullet}$) and non-radical compounds (e.g. hydrogen peroxide, H_2O_2; singlet oxygen, $^1O_2$; hypochlorous acid, HOCl; and ozone, O_3), that can be converted into free radicals by transition metals, both having the ability to oxidative damage cellular macromolecules [3, 4]. There also exist other non oxygen-based oxidants, namely reactive sulphur species, including thinyl (RS) and perthynil (RSS), reactive nitrogen species, comprising nitric oxide ($NO^{\bullet}$), nitrogen dioxide ($NO_2^{\bullet}$) and peroxynitrite ($ONOO^-$), as well as reactive carbon species, such as trichloromethyl ($CCl_3^{\bullet}$) [5, 6]. Free radicals are molecules or ions capable of independent existence containing one or more unpaired electrons in their outer orbital or valence shell [7], which make them extremely unstable and highly reactive [8].

There is evidence that ROS, at low physiologically relevant levels, are involved in the regulation of a wide range of biological processes, such as signal transduction, gene transcription, cell differentiation, enzyme activation, prostaglandin synthesis, xenobiotics detoxification, glycogen repletion, apoptosis, cell survival and tissue repairing, immune response and muscular contraction [2, 4, 9-20].

The production of these oxidants may be elevated by both psychological and physical stress [21], and is influenced by personal characteristics (e.g. age and genetic predisposition) and exogenous stimulus (e.g. infection, toxin, cold, radiation exposure, air pollutants, intoxication by oxygen, smoke, alcohol, diet and physical activity) [5, 9, 22]. Davies *et al*. [23] were the first to show an increase in free radical production during muscle contractile activity. Since that pioneer work, there has been accumulating consistent evidence supporting the generation of ROS after a single bout of aerobic [24-28], anaerobic [29-32] or mixed (i.e. involving a balanced contribution of both aerobic and anaerobic metabolism) exercises [33, 34]. Exercising muscles produces ROS in an intensity-dependent manner [5, 35], and the magnitude of the augment was estimated to be between 2-10% [3]. Recently, it was demonstrated in humans that ROS outflow from the active muscle bed, by determining venoarterial differences in spin adducts using electron paramagnetic resonance spectroscopy [35, 36].

Although the available evidence strongly supports the exercise-incremented ROS generation, the mechanisms involved and their relative contribution are not yet perfectly understood. In order to meet elevated energy demands during exercise, the oxygen uptake by the whole body can increase up to 15-fold and the oxygen flux through active skeletal muscles fibres by 100-fold above resting values [1, 2, 4, 14]. It is estimated that some 2–4% of the molecular oxygen is not completely reduced to water, instead forming $O_2^{\bullet-}$, via side-reactions with flavin or ubisemiquinone [4], that can "leak out" from the mitochondrial electron transport chain [1, 37]. However, when mitochondria are in State 3, that is with a high electron flow into oxygen during exercise, this proportion can fall to 10% of the value at rest (State 4) [38]. Thus, there are some data that suggest that mitochondrial leakage could have a minor role than was initially thought [4, 14]. Another important source of ROS may be the ischemia-reperfusion phenomena induced by exhaustive or anaerobic exercise [1, 4, 5]. The temporary ischemia or hypoxia in several organs, as blood is shunted away to the active muscles and the skin [38], elicit the proteolytic conversion of xanthine dehydrogenase (XD) to the oxidase form, xanthine oxidase (XO) [14]. XO, instead of using NAD^+ as an electron acceptor, reduces molecular O_2 (after reperfusion) directly to $O_2^{\bullet-}$ and H_2O_2, as a by-product of the degradation of hypoxanthine into xanthine and, subsequently, to urate [3, 37]. After the initial muscle injury caused by mechanical forces during intense and traumatizing exercise, the body triggers an extensive immune response, by mobilizing neutrophils and other phagocytic cells from the blood to inflammatory sites, in order to remove the debris and repair tissues [39]. Activated phagocytes attracted to the myocytes may release ROS ($O_2^{\bullet-}$, H_2O_2 and other) on non-injured tissue, leading to further muscle damage and a delayed-onset muscle soreness [3, 40]. The mobilization and activity of ROS producing immune cells is dependent upon the exercise intensity and mediated by a number of hormones, like cortisol, and cytokines, such as interleukin-6 (IL-6) [37, 41]. Thus, ROS seems to play an important role as mediators of skeletal muscle damage and inflammation after strenuous exercise [41]. Other poorly characterized sources of ROS that accompanies physical exercise include the release of transition metals, such as iron and copper, from their transporters, auto-oxidation of haem proteins (oxyhaemoglobin and oxymyoglobin), hydroquinones, catecholamines and thiols, altered calcium homeostasis, prostanoid metabolism, increased central temperature and enhanced activity of myeloperoxidase, cytochrome P450 enzymes, NADPH oxidase, nitric oxide synthase and mitochondrial membrane-bound glycerol 3-phosphate dehydrogenase [3, 5-7, 13, 21, 37, 42, 43]. These several pathways of ROS production may act synergistically and is possible that different types of exercises involve different mechanisms. While the increase during aerobic exercise seems to relate mainly to electron leakage from mitochondrial respiratory chain and to

xanthine oxidase activity, phagocytic respiratory burst activity is implicated in their generation during and after anaerobic exercise [44]. A XO-mediated pathway was thought to be the responsible mechanism for the ROS increase 20 minutes after an anaerobic test (Wingate) [30].

Under normal physiological circumstances, these species are neutralized by an elaborate antioxidant network that evolved to counteract their deleterious effects. This system consists of antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and non-enzymatic antioxidants, encompassing α-tocopherol, vitamin C, β-carotene and other carotenoids, coenzyme Q10, lipoic acid, glutathione (GSH), cysteine, uric acid and flavonoids [1, 5, 43]. We can also consider the role of some indirect antioxidants, as heat shock proteins (HSP), bilirubin, ferritin, albumin, caeruloplasmin, and iron, copper, zinc, selenium and manganese, as cofactors for antioxidant enzymes [5]. Some antioxidants are highly specific (e.g. SOD) while other are less specific (e.g. vitamins C, α-tocopherol and glutathione) [13]. Antioxidants act in concert, complementing each other functionally [1], and are strategically compartmentalized in order to provide an optimized protection [43]. Lipid soluble antioxidants (α-tocopherol, carotenoids, coenzyme Q, flavonoids and polyphenols) are more effective in the cells' lipid environment, such as mitochondria, sarcoplasmic reticulum and the plasma membrane [9, 43], while water-soluble antioxidants (vitamin C, glutathione, uric acid and lipoic acid) function better in aqueous environments, such as plasma, cytosol, mitochondrial matrix and interstitial fluids [3, 5, 9, 45]. The efficiency of the defence machinery depends on the orchestrated synergism between endogenous antioxidants [7, 9], influenced by genetic factors, ageing, exercise and nutrition, and exogenous antioxidants, modulated by dietary intake [2, 46]. A low intake and/or availability of dietary antioxidants can weaken the antioxidant system [47]. The Food and Nutrition Board (FNB) [45] defined dietary antioxidant has "a substance in foods that significantly decreases the adverse effects of reactive species, such as reactive oxygen and nitrogen species, on normal physiological function in humans". Vitamin C, α-tocopherol and selenium meet this definition [45].

Oxidative stress is classically defined as "a condition in which cellular production of ROS overwhelms the physiological antioxidant capacity to render them inactive" [21, 48]. A more recent definition ("a disruption of redox signalling and control") [48] recognizes the contingency that cellular redox imbalance may be compartmentalized and not global. An increased oxidative stress has been associated with the ageing process [49] and numerous diseases, such as atherosclerosis, diabetes, muscular dystrophy, rheumatic arthritis, neurodegenerative diseases (Parkinson and Alzheimer), cataracts, pulmonary diseases, hypertension, and cancer

[7, 19, 38, 50]. Dietary antioxidants were epidemiologically linked to the prevention of oxidative stress-related diseases [9], which might have contributed to the benefits that have been proposed for antioxidant supplementation for a long time [50].

The elevation in oxidative stress stemming from increased physical activity was related [3] with the unexpected outcome of some epidemiologic studies [51, 52] that encountered an increased (albeit non-significant) risk of morbidity and mortality for those individuals engaged in very high amounts of exercise (> ~3,510 kcal/week). Paradoxically, physical activity is a well-documented health promoter and deterrent of several chronic disease [2]. Actually, there is irrefutable evidence of the effectiveness of regular physical activity in the primary and secondary prevention of cardiovascular disease, diabetes, cancer (colon and breast), hypertension, obesity, depression, bone and joint diseases and premature death [53]. The recommendations state that healthy adults aged 18 to 65 years should include a minimum of 30 minutes of moderate intensity aerobic physical activity on five days each week or a minimum of 20 minutes of vigorous intensity aerobic physical activity on three days each week, in addition to resistance exercises at least twice a week, for promoting and maintaining health [54].

The apparent contradiction between the oxidative stress caused by acute exercise and the health benefits associated with regular exercise may be explained, at least in part, by the oxidative stress-induced adaptations when exercising chronically [19]. Actually, chronic exercise (training) not only causes an improvement in exercise performance, but also induce adaptations that strengthen antioxidant defences and/or reduce basal production of oxidants [55], giving an additional protection against oxidative stress [3]. Therefore, the magnitude of oxidative stress in response to acute exercise is influenced by the training status of the subjects [3]. The adaptations result from the cumulative effects of repeated exercise bouts, providing they are of sufficient intensity and length [56, 57]. Very light intensity exercise fails to induce adaptations because the ROS load is adequately eliminated by the antioxidant defense network [55]. The adaptive mechanisms might include an up-regulation of antioxidant enzymes activities, a decline of ROS leakage during oxidative phosphorylation, due to an augmented mitochondrial volume, a decrease in the loosely bound iron in muscles and a diminished capacity of neutrophils to produce ROS [3, 21, 58]. Engage in an aerobic training program demonstrated to increase antioxidant enzymes activities and decrease ROS production and oxidative stress at rest or after exercise in previously sedentary subjects [56, 59]. It were observed increases in the activities of antioxidant enzymes after endurance [56, 59-61], anaerobic [57, 60, 62] and mixed training programs [33, 63-66], that may be due to altered gene expression [67].

Published data describe a greater increase on GPx and SOD activities than on CAT activity following a training period [5]. Athletes generally present augmented antioxidant enzymes activities than age- and sex-matched sedentary individuals [60, 62, 63, 65, 66, 68]. Regarding non-enzymatic antioxidants, the impact of training appears somewhat more controversial [5].

The antioxidant system seems adequate to deal with the physiologic production of ROS at rest or the slightly increased generation during low-intensity exercise [1, 40]. Nevertheless, high intensity or long duration exercises cause a large increase in ROS production that may overwhelm the antioxidant capacity [69-72]. Some studies [73-78] demonstrate that periods of intensive training and competing load can compromise antioxidant status and result in oxidative damage, particularly in overload-training athletes [79, 80]. This suggests that even the training-strengthened antioxidant system of athletes is unable to absolutely cope with the augmented oxidant insults they experienced in some conditions [14, 55]. Trained athletes may, indeed, exhibit higher resting oxidative stress markers than sedentary controls [60, 81]. The effects that training has on oxidative stress in dependent on the individuals' training status and the load and particularities of the training program [5]. Redox status evolve throughout the season and it is not predictable *a priori* which athletes will present an increased oxidative damage [82]. Therefore, the determination of antioxidant status and oxidative damage parameters should be included in the biological longitudinal follow-up of elite sportsmen [5, 83]. Nonetheless, few studies [73, 75, 82, 84] have longitudinally monitored the redox status of elite athletes. Further investigation is needed to better understand the effects of large volumes of training performed by elite athletes on antioxidant status and oxidative damage.

Low levels of ROS are required for optimal muscle contraction, by increasing myofibrillar calcium sensitivity and calcium release from the sarcoplasmic reticulum [38]. On the other hand, massive amounts of ROS, as those produced during strenuous exercise, may cause muscle force production to fall, and fatigue may occur in a dose-dependent manner [7, 15, 38, 43, 85]. The mechanisms involved in the putative detrimental effect of ROS in exercise performance are the oxidative damage to adenosine triphosphatase (ATPase) pumps, muscle contractile proteins or mitochondrial enzymes [14]. The repetitive muscular ROS-induced muscle fatigue without an adequate recovery was implicated in the overtraining syndrome [5]. If muscle fatigue is caused in part by oxidative stress, then lowering the muscular basal redox status with a previous treatment with antioxidants would allow muscle to withstand more effectively the ROS generated during exercise, lengthening the time to muscle effort termination [9]. However, antioxidant supplementation did not enhance exercise performance in the large majority of studies in non-deficient athletes [42, 86, 87], with the exception of exercise done at a high-altitude

[88], a condition that exacerbates oxidative stress [89, 90]. Some animal experiments data have, indeed, suggested an impaired skeletal muscle contractile function and exercise performance following antioxidant supplementation [43]. We must also consider that performance is not a reasonable end-point to evaluate the efficacy of antioxidant supplementation, since it is an outcome with multi-factor determinants [2, 9].

Despite there is little experimental evidence to support an ergogenic role for antioxidant supplements [2, 4], they are widely used among the athletic population [7]. Nonetheless, we have to bear in mind that the indiscriminate absolute elimination of ROS by excessive antioxidant intake may not be physiologically favourable [2], affecting body's normal functioning due to their participation in a variety of molecular mechanisms [21]. In this context, the ROS-mediated decrease in force generation could function as a mechanism to protect muscle cells from overstimulation and further injury, and antioxidant supplementation can override this protection [5, 38]. On the other side, prolonged and high intensity exercise can generate ROS to a level that causes tissue damage, that must be repaired for adaptation to recommence [37], and may accelerate the normal age-related loss of skeletal muscle mass [91]. However, the administration of antioxidants may attenuate the inflammatory response, limiting the rate of muscle recovery from muscle damaging exercise [92, 93]. The investigations concerning the role of antioxidants supplements to attenuate signs and symptoms of muscle injury generated mixed findings [94]. More work is needed to clarify this dual role of ROS.

ROS regulate several physiologic functions, such as redox-regulation of vascular tone, substrate metabolism and muscle contractility [14], and their generation might be a desired, or even required, consequence of exercise. A moderate rise of ROS may, in fact, be a stimulus to initiate training-associated benefit adaptations, such as mitochondrial biogenesis, increased muscle blood supply and altered fuel consumption patterns [37]. These molecules act by modulating redox-sensitive signal transduction pathways and the activation of key transcription factors leading to an altered pattern of the expression of a variety of different genes [7, 67, 91]. The administration of allopurinol, an inhibitor of XO, prevented muscle damage after exercise but also blunted the activation of signaling pathways responsible for relevant adaptations to training, such as the upregulation of SOD and nitric oxide synthetase [38]. If ROS is involved in the systemic adaptive response to training [19], then the strategy of pre-treatment with high levels of antioxidants may blunt some of the induced cellular adaptations by exercise [43].

Although a basal level of ROS is needed for several physiologic processes, excessive ROS production when performing strenuous exercise may exceed antioxidant defence capabilities and trigger an oxidative attack of biological structures [2]. As highly reactive substances, ROS can damage all major cellular constituents, such as lipids, proteins, carbohydrates, and nucleic acids, leading to impaired cellular function [3, 7, 21, 43]. Dillard *et al*. [95] were the first to establish that physical exercise induces lipid peroxidation, by detecting a near 2-fold heightened exhaled pentane concentration above the resting value. Lipid peroxidation causes substantial damage to the integrity, stability and fluidity of cell, mitochondria, endoplasmic reticulum and nuclear membranes [3, 37].

As free radicals are hard to detect *in vivo*, due to their high instability and extremely short-life (from milliseconds to nanoseconds) [37], the majority of the studies focused on indirect markers, measuring an array of by-products or end-products of ROS-associated oxidative damage in body fluids or tissues and expired air (as pentane in Dillard's work) [3]. The only direct method to detect and measure free radicals in biological samples is by electron paramagnetic resonance spectroscopy, particularly in conjunction with spin traps that increase their life-span [4], firstly used by Ashton and colleagues [96]. The most frequently studied method for estimating the extent of peroxidation is the thiobarbituric acid reactive substances (TBARS) assay [3]. The assessment of blood and/or tissues' antioxidant enzymes activity (GPx, SOD, CAT and glutathione reductase), non-enzymatic antioxidant concentrations (antioxidant vitamins, GSH and uric acid) or a parameter of the overall antioxidant capacity (e.g. Total Antioxidant Status, TAS) are also used as indicators of the effects of exercise on redox status [37], but their significance is harder to determine [4]. Lipid peroxidation damage to cellular membranes augments its permeability, compromising the integrity of the barrier [9], eliciting the release of cytosolic proteins, as creatine kinase (CK) [21]. Therefore, CK determination can be considered a marker of muscular cellular damage and, indirectly, of oxidative stress [5]. It is recommended that several assays should be used for a more accurate and reliable assessment of redox status in biological systems [42].

Whether or not regular vigorous exercise does increase athletes' antioxidants needs from the diet is currently an unsolved question [42, 43]. Due to the considerable inter-individual difference in susceptibility to oxidative stress, it is suggested that the specific antioxidant requirements of athletes engaged in strenuous training loads should be addressed on an individual-basis [2, 7, 9]. Sportsmen may have increased antioxidant requirements during periods of intensive training and/or competition [79, 80, 97] and, under these circumstances, the normal

dietary intake may be insufficient even when nutritional intakes are within recommendations [74].

However, there are not broadly established dietary reference intakes specific for athletes [98]. The Australian Institute of Sport (AIS) suggests that athletes should be supplemented with vitamin C (500 mg) and vitamin E (500 UI) daily for 2 weeks when undertaking a sudden increase in training stress or a shift to a stressful environment (e.g. heat, altitude) [99]. The American College of Sports Medicine (ACSM) [100] stated that physically active individuals may have increased requirements of micronutrients, but they can generally be attained by following the guidelines for the general population regarding fruit and vegetable intake [101], which, however, do not consider their augmented energy expenditure [7]. Regarding this issue, the French authorities [102] recommend that 12 mg of vitamin E, 110 mg of vitamin C and 1 mg of β-carotene must be added daily for each 1000 kcal expended above 2200 kcal, for men.

Antioxidant nutrient deficiencies are not widely reported among athletes [43]. Sportsmen generally consume antioxidant vitamins within or above the recommendations for the general population [103]. However, micronutrient inadequate intakes are also often described [5], particularly in some groups of athletes, mainly due to voluntary restriction of energy intake or high-fat diets for aesthetic or competitive purposes [9, 103]. The fine balance between oxidants and antioxidants can be repeatedly disrupted by an insufficient intake of antioxidants and a heavy oxidant load elicited by high-intensity exercise during routine training. Thus, from a theoretical standpoint, there appears to be a plausible rationale to consider the supplementation of some athletes with antioxidants.

The more investigated single supplemented antioxidants were vitamin E [29, 95, 104-111] and vitamin C [34, 112-119], and to a far less extent coenzyme Q10 [120], *N*-acetylcysteine [85, 121], polyphenols [122-124] and selenium [125-127]. Several works have also determined the effects of a combination of antioxidant compounds, for example: vitamin C and vitamin E [128-133]; vitamin C, vitamin E and β-carotene [74, 134-136] ; vitamin C, vitamin E and coenzyme Q10 [137, 138] ; vitamin E and coenzyme Q10 [139]; vitamin C, vitamin E and selenium [31, 79, 97] ; vitamin C, vitamin E, β-carotene, zinc and selenium [89, 140]; vitamin C, vitamin E and zinc [141]; vitamin C, vitamin E, magnesium and copper [142]; vitamin C, vitamin E, β-carotene, selenium , lipoic acid, *N*-acetylcysteine, catechin, lutein and licopene [143] ; and vitamin C, vitamin E, β-carotene and selenium [144]. The administration of a supplement with a broad range of antioxidants may be a more reasonable approach due to the known interaction between these compounds [2]. Indeed, some studies data indicate that the combination of several antioxidants appears to be

more effective than a single-compound supplementation [79, 80, 97, 128]. The length of supplementation may reach 6 months [145], but administrations during [121] or after exercise [93] were also investigated, and the timing of supplementation does not seem to be a despicable issue [94].

Generally, works investigating the effects of antioxidants supplements on exercise-induced oxidative stress, muscle injury or inflammatory responses have met equivocal findings [2]. A recent meta-analysis [7] reported that the number of studies demonstrating an attenuation in oxidative stress with antioxidant supplementation was almost identical to those that did not found any significant effect. Some few studies even illustrated that antioxidant interventions are capable of further increasing exercise-related oxidative stress [29, 93, 104, 128]. Although, vitamin C, α-tocopherol and β-carotene are generally well tolerated at doses several-fold higher than the recommended by the FNB [2, 9], caution is needed against the use of antioxidants in megadoses [2, 146], because they may have hazardous effects [20, 42, 93]. As redox agents, antioxidants can protect against ROS generation in some circumstances, whereas in other conditions can promote its formation [147]. The discrepancies in results could be explained by the diversity concerning the antioxidant (form, type, dose and supplementation length), the exercise protocol (type, intensity and duration), the subjects' characteristics (age, sex, nutritional status and fitness) and methodological procedures (redox biomarkers, time of sampling...) used in different studies [2, 3, 41]. Most of the published studies were laboratory-based, used untrained individuals or applied exercise protocols that were very different from those practiced by the athletic population. Despite the academic value of these works, the applicability of their findings to the athletes' "real-world" is arguable [148]. There is paucity of research on elite athletes performing exercises that are used to during their normal training programme.

At the present time, it is not known whether exercise-induced oxidative stress is needed to the adaptive processes or whether it is hazardous, by causing additional muscle damage and/or hindering the recovery, thus compromising the chance to train and compete at a level of excellence. This poses a significant challenge that deserves a careful analysis of the significance of antioxidants supplements. Some questions should be addressed meanwhile: are athletes at an increased risk of oxidative stress in comparison with sedentary individuals? Is the risk modified within the training season? What is the impact of the chronic high-volume of training in already-trained athletes? Are antioxidant supplements necessary for athletes? What is the optimal type, dose and duration of antioxidants to use? It should be demonstrated that oxidative stress has more disadvantages that advantages to athletes' health and performance,

before antioxidant supplements be included in their nutritional plan. There is, currently, insufficient evidence to recommending or discouraging antioxidant supplementation.

The comprehensive understating of effect of acute and chronic exercise on redox status and its modification by antioxidants supplementation would be very useful not only in a sports context, but also for some oxidative stress-related medical conditions that exercise may mirror.

REFERENCES

1. Banerjee AK, Mandal A, Chanda D, Chakraborti S. Oxidant, antioxidant and physical exercise. Mol Cell Biochem. 2003; 253(1-2):307-12
2. Atalay M, Lappalainen J, Sen CK. Dietary antioxidants for the athlete. Curr Sports Med Rep. 2006; 5(4):182-6
3. Knez WL, Coombes JS, Jenkins DG. Ultra-endurance exercise and oxidative damage: implications for cardiovascular health. Sports Med. 2006; 36(5):429-41
4. Cooper CE, Vollaard NB, Choueiri T, Wilson MT. Exercise, free radicals and oxidative stress. Biochem Soc Trans. 2002; 30(2):280-85
5. Finaud J, Lac G, Filaire E. Oxidative stress: Relationship with exercise and training. Sports Med. 2006; 36(4):327-58
6. Madger S. Reactive oxygen species: toxic molecules or spark of life? Crit Care. 2006; 10(1):208
7. Williams SL, Strobel NA, Lexis LA, Coombes JS. Antioxidant requirements of endurance athletes: implications for health. Nutr Rev. 2006; 64(3):93-108
8. Packer L. Oxidants, antioxidant nutrients and the athlete. J Sports Sci. 1997; 15(3):353-63
9. Sen CK. Antioxidants in exercise nutrition. Sports Med. 2001; 31(13):891-908
10. Stone JR, Yang S. Hydrogen peroxide: a signaling messenger. Antioxid Redox Signal. 2006; 8(3-4):243-70
11. Knight JA. Review: Free radicals, antioxidants, and the immune system. Ann Clin Lab Sci. 2000; 30(2):145-58
12. Roy S, Khanna S, Nallu K, Hunt TK, Sen CK. Dermal wound healing is subject to redox control. Molecular Ther. 2006; 13(1):211-20

13. Packer L, Cadenas E, Davies KJA. Free radicals and exercise: An introduction. Free Radical Biology and Medicine. 2008; 44(2):123-25

14. Vollaard NB, Shearman JP, Cooper CE. Exercise-induced oxidative stress: myths, realities and physiological relevance [review]. Sports Med. 2005; 35(12):1045-62

15. Reid MB. Invited Review: redox modulation of skeletal muscle contraction: what we know and what we don't. J Appl Physiol. 2001; 90(2):724-31

16. Lamprecht M, Greilberger J, Oettl K. Analytical aspects of oxidatively modified substances in sports and exercises. Nutrition. 2004; 20(7-8):728-30

17. Sen CK. Antioxidant and redox regulation of cellular signaling: introduction. Med Sci Sports Exerc. 2001; 33(3):368-70

18. Sen CK, Packer L. Antioxidant and redox regulation of gene transcription. Faseb J. 1996; 10(7):709-20

19. Radak Z, Chung HY, Goto S. Systemic adaptation to oxidative challenge induced by regular exercise. Free Radic Biol Med. 2008; 44(2):153-59

20. Duarte T, Lunec J. Review: When is an antioxidant not an antioxidant? A review of novel actions and reactions of vitamin C. Free Radic Res. 2005 39(7):671-86

21. Bloomer RJ, Goldfarb AH. Anaerobic exercise and oxidative stress: a review. Can J Appl Physiol. 2004; 29(3):245-63

22. Ji LL. Antioxidant signaling in skeletal muscle: A brief review. Exp Gerontol. 2007; 42(7):582-93

23. Davies KJA, Quintanilha AT, Brooks GA, Packer L. Free radicals and tissue damage produced by exercise. Biochem Biophys Res Commun. 1982; 107(4):1198-205

24. Vasankari TJ, Kujala UM, Vasankari TM, Vuorimaa T, Ahotupa M. Effects of acute prolonged exercise on serum and LDL oxidation and antioxidant defences. Free Radic Biol Med. 1997; 22(3):509-13

25. Mastaloudis A, Leonard SW, Traber MG. Oxidative stress in athletes during extreme endurance exercise. Free Radic Biol Med. 2001; 31(7):911-22

26. Child RB, Wilkinson DM, Fallowfield JL, Donnelly AE. Elevated serum antioxidant capacity and plasma malondialdehyde concentration in response to a simulated half-marathon run. Med Sci Sports Exerc. 1998; 30(11):1603-7

27. Aguiló A, Tauler P, Fuentespina E, Tur JA, Córdova A, Pons A. Antioxidant response to oxidative stress induced by exhaustive exercise. Physiol Behav. 2005; 84(1):1-7

28. Vider J, Lehtmaa J, Kullisaar T, Vihalemm T, Zilmer K, Kairane C, et al. Acute immune response in respect to exercise-induced oxidative stress. Pathophysiology. 2001; 7(4):263-70

29. McBride JM, Kraemer WJ, Triplett-McBride T, Sebastianelli W. Effect of resistance exercise on free radical production. Med Sci Sports Exerc. 1998; 30(1):67-72

30. Groussard C, Rannou-Bekono F, Machefer G, Chevanne M, Vincent S, Sergent O, et al. Changes in blood lipid peroxidation markers and antioxidants after a single sprint anaerobic exercise. Eur J Appl Physiol. 2003; 89(1):14-20

31. Goldfarb AH, Bloomer RJ, McKenzie MJ. Combined antioxidant treatment effects on blood oxidative stress after eccentric exercise. Med Sci Sports Exerc. 2005; 37(2):234-39

32. Ramel A, Wagner KH, Elmadfa I. Plasma antioxidants and lipid oxidation after submaximal resistance exercise in men. Eur J Nutr. 2004; 43(1):2-6

33. Chang CK, Tseng HF, Hsuuw YD, Chan WH, Shieh LC. Higher LDL oxidation at rest and after a rugby game in weekend warriors. Ann Nutr Metab. 2002; 46(3-4):103-07

34. 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. 2003; 89(3-4):393-400

35. Bailey DM, Young IS, McEneny J, Lawrenson L, Kim J, Barden J, et al. Regulation of free radical outflow from an isolated muscle bed in exercising humans. Am J Physiol Heart Circ Physiol. 2004; 287(4 56-4):H1689-H99

36. Bailey DM, Davies B, Young IS, Jackson MJ, Davison GW, Isaacson R, et al. EPR spectroscopic detection of free radical outflow from an isolated muscle bed in exercising humans. J Appl Physiol. 2003; 94(5):1714-18

37. Sachdev S, Davies KJA. Production, detection, and adaptive responses to free radicals in exercise. Free Radic Biol Med. 2008; 44(2):215-23

38. Gomez-Cabrera M, Domenech E, Viña J. Moderate exercise is an antioxidant: Upregulation of antioxidant genes by training. Free Radic Biol Med. 2008; 44(2):126-31

39. Malm C. Exercise-induced muscle damage and inflammation: fact or fiction? Acta Physiol Scand. 2001; 171(3):233-9

40. Urso ML, Clarkson PM. Oxidative stress, exercise, and antioxidant supplementation. Toxicology. 2003; 189(1-2):41-54

41. Sacheck JM, Blumberg JB. Role of vitamin E and oxidative stress in exercise. Nutrition. 2001; 17(10):809-14

42. Clarkson PM, Thompson HS. Antioxidants: what role do they play in physical activity and health? Am J Clin Nutr. 2000; 72(2 Suppl):637S-46S

43. Powers SK, DeRuisseau KC, Quindry J, Hamilton KL. Dietary antioxidants and exercise. J Sports Sci. 2004; 22(1):81-94

44. Sahlin K, Cizinsky S, Warholm M, Hoberg J. Repetitive static muscle contractions in humans - a trigger of metabolic and oxidative stress? Eur J Appl Physiol Occup Physiol. 1992; 64(3):228-36

45. Monsen ER. Dietary reference intakes for the antioxidant nutrients: vitamin C, vitamin E, selenium, and carotenoids. J Am Diet Assoc. 2000; 100(6):637-40

46. Dekkers JC, van Doornen LJ, Kemper HC. The role of antioxidant vitamins and enzymes in the prevention of exercise-induced muscle damage. Sports Med. 1996; 21(3):213-38

47. Wiswedel I, Hirsch D, Kropf S, Gruening M, Pfister E, Schewe T, et al. Flavanol-rich cocoa drink lowers plasma F(2)-isoprostane concentrations in humans. Free Radic Biol Med. 2004; 37(3):411-21

48. Jones DP. Redefining oxidative stress. Antioxid Redox Signal. 2006; 8(9-10):1865-79

49. Golden T, Hinerfeld D, Melov S. Oxidative stress and aging: beyond correlation. Aging Cell 2002; 1(2):117-23

50. Viña J, Gomez-Cabrera MC, Borras C. Fostering antioxidant defences: Up-regulation of antioxidant genes or antioxidant supplementation? Br J Nutr. 2007; 98(SUPPL. 1):S36-S40

51. Lee IM, Hsieh CC, Paffenbarger Jr RS. Exercise intensity and longevity in men: The Harvard Alumni Health Study. JAMA. 1995; 273(15):1179-84

52. Paffenbarger Jr RS, Hyde RT, Wing AL, Hsieh CC. Physical activity, all-cause mortality, and longevity of college alumni. N Engl J Med. 1986; 314(10):605-13

53. Warburton DER, Nicol CW, Bredin SSD. Health benefits of physical activity: The evidence. CMAJ. 2006; 174(6):801-09

54. Haskell WL, Lee IM, Pate RR, Powell KE, Blair SN, Franklin BA, et al. Physical activity and public health: Updated recommendation for adults from the American College of Sports Medicine and the American Heart Association. Medicine and Science in Sports and Exercise. 2007; 39(8):1423-34

55. Radak Z, Taylor AW, Ohno H, Goto S. Adaptation to exercise-induced oxidative stress: From muscle to brain. Exerc Immunol Rev. 2001; 7:90-107

56. Miyazaki H, Oh-ishi S, Ookawara T, Kizaki T, Toshinai K, Ha S, et al. Strenuous endurance training in humans reduces oxidative stress following exhausting exercise. Eur J Appl Physiol. 2001; 84(1-2):1-6

57. Hellsten Y, Apple FS, Sjodin B. Effect of sprint cycle training on activities of antioxidant enzymes in human skeletal muscle. J Appl Physiol. 1996; 81(4):1484-7

58. Leeuwenburgh C, Heinecke JW. Oxidative stress and antioxidants in exercise. Curr Med Chem. 2001; 8(7):829-38

59. Elosua R, Molina L, Fito M, Arquer A, Sanchez-Quesada JL, Covas MI, et al. Response of oxidative stress biomarkers to a 16-week aerobic physical activity program, and to acute physical activity, in healthy young men and women. Atherosclerosis. 2003; 167(2):327-34

60. Marzatico F, Pansarasa O, Bertorelli L, Somenzini L, Della Valle G. 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. 1997; 37(4):235-9

61. Selamoglu S, Turgay F, Kayatekin BM, Gönenc S, Yslegen C. Aerobic and anaerobic training effects on the antioxidant enzymes of the blood. Acta Physiol Hung. 2000; 87(3):267-73

62. Ortenblad N, Madsen K, Djurhuus MS. Antioxidant status and lipid peroxidation after short-term maximal exercise in trained and untrained humans. Am J Physiol. 1997; 272(4 Pt 2):R1258-63

63. Cazzola R, Russo-Volpe S, Cervato G, Cestaro B. Biochemical assessments of oxidative stress, erythrocyte membrane fluidity and antioxidant status in professional soccer players and sedentary controls. Eur J Clin Invest. 2003; 33(10):924-30

64. Metin G, Atukeren P, Alturfan AA, Gulyasar T, Kaya M, Gumustas MK. Lipid peroxidation, erythrocyte superoxide-dismutase activity and trace metals in young male footballers. Yonsei Med J. 2003; 44(6):979-86

65. Brites FD, Evelson PA, Christiansen MG, Nicol MF, Basilico MJ, Wikinski RW, et al. Soccer players under regular training show oxidative stress but an improved plasma antioxidant status. Clin Sci (Lond). 1999; 96(4):381-5

66. Evelson P, Gambino G, Travacio M, Jaita G, Verona J, Maroncelli C, et al. Higher antioxidant defences in plasma and low density lipoproteins from rugby players. Eur J Clin Invest. 2002; 32(11):818-25

67. Ji LL. Modulation of skeletal muscle antioxidant defense by exercise: Role of redox signaling. Free Radic Biol Med. 2008; 44(2):142-52

68. Robertson JD, Maughan RJ, Duthie GG, Morrice PC. Increased blood antioxidant systems of runners in response to training load. Clin Sci (Lond). 1991; 80(6):611-18

69. Lovlin R, Cottle W, Pyke I, Kavanagh M, Belcastro AN. Are indices of free radical damage related to exercise intensity. Eur J Appl Physiol Occup Physiol. 1987; 56(3):313-6

70. Sastre J, Asensi M, Gasco E, Pallardo FV, Ferrero JA, Furukawa T, et al. Exhaustive physical exercise causes oxidation of glutathione status in blood: Prevention by antioxidant administration. Am J Physiol Regul Integr Comp Physiol. 1992; 263(5 32-5):R992-R95

71. Niess AM, Hartmann A, Grünert-Fuchs M, Poch B, Speit G. DNA damage after exhaustive treadmill running in trained and untrained men. Int J Sports Med. 1996; 17(6):397-403

72. Quindry JC, Stone WL, King J, Broeder CE. The effects of acute exercise on neutrophils and plasma oxidative stress. Med Sci Sport Exerc. 2003; 35(7):1139-45

73. Accominotti M, Dutey P, Lahet C, Vallon J. Evolution des taux de sélénium et de glutathion peroxydase sanguins de sportifs de haut niveau. Sci Sports. 1991; 6(3):165-17

74. 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. 2000; 21(2):146-50

75. Pincemail J, Lecomte J, Castiau JP, Collard E, Vasankari T, Cheramy-Bien JP, et al. Evaluation of autoantibodies against oxidized LDL and antioxidant status in top soccer and basketball players after 4 months of competition. Free Radic Biol Med. 2000; 28(4):559-65

76. Bergholm R, Mäkimattila S, Valkonen M, Liu ML, Lahdenperä S, Taskinen MR, et al. Intense physical training decreases circulating antioxidants and endothelium-dependent vasodilatation in vivo. Atherosclerosis. 1999; 145(2):341-9

77. Balakrishnan SD, Anuradha CV. Exercise, depletion of antioxidants and antioxidant manipulation. Cell Biochem Funct. 1998; 16(4):269-75

78. Subudhi AW, Davis SL, Kipp RW, Askew EW. Antioxidant status and oxidative stress in elite alpine ski racers. Int J Sport Nutr Exerc Metab. 2001; 11(1):32-41

79. Margaritis I, Palazzetti S, Rousseau AS, Richard MJ, Favier A. Antioxidant supplementation and tapering exercise improve exercise-induced antioxidant response. J Am Coll Nutr. 2003; 22(2):147-56

80. Palazzetti S, Richard MJ, Favier A, Margaritis I. Overloaded training increases exercise-induced oxidative stress and damage. Can J Appl Physiol. 2003; 28(4):588-604

81. Santos-Silva A, Rebelo MI, Castro EMB, Belo L, Guerra A, Rego C, et al. Leukocyte activation, erythrocyte damage, lipid profile and oxidative stress imposed by high competition physical exercise in adolescents. Clin Chim Acta. 2001; 306(1-2):119-26

82. Schippinger G, Wonisch W, Abuja PM, Fankhauser F, Winklhofer-Roob BM, Halwachs G. Lipid peroxidation and antioxidant status in professional American football players during competition. Eur J Clin Invest. 2002; 32(9):686-92

83. Lac G, Maso F. Biological markers for the follow-up of athletes throughout the training season. Pathol Biol (Paris). 2004; 52(1):43-49

84. Finaud J, Scislowski V, Lac G, Durand D, Vidalin H, Robert A, et al. Antioxidant status and oxidative stress in professional rugby players: evolution throughout a season. Int J Sports Med. 2006; 27(2):87-93

85. Medved I, Brown MJ, Bjorksten AR, McKenna MJ. Effects of intravenous N-acetylcysteine infusion on time to fatigue and potassium regulation during prolonged cycling exercise. J Appl Physiol. 2004; 96(1):211-17

86. Evans WJ. Vitamin E, vitamin C, and exercise. Am J Clin Nutr. 2000; 72(2 Suppl):647S-52S

87. Singh A, Moses FM, Deuster PA. Chronic multivitamin-mineral supplementation does not enhance physical performance. Med Sci Sports Exerc. 1992; 24(6):726-32

88. Simon-Schnass I, Pabst H. Influence of vitamin E on physical performance. Int J Vitam Nutr Res. 1988; 58(1):49-54

89. Pfeiffer JM, Askew EW, Roberts DE, Wood SM, Benson JE, Johnson SC, et al. Effect of antioxidant supplementation on urine and blood markers of oxidative stress during extended moderate-altitude training. Wilderness Environ Med. 1999; 10(2):66-74

90. Pialoux V, Mounier R, Ponsot E, Rock E, Mazur A, Dufour S, et al. Effects of exercise and training in hypoxia on antioxidant/pro-oxidant balance. Eur J Clin Nutr. 2006; 60(12):1345-54

91. Jackson MJ. Free radicals generated by contracting muscle: By-products of metabolism or key regulators of muscle function? Free Radic Biol Med. 2008; 44(2):132-41

92. Tiidus PM. Radical species in inflammation and overtraining. Can J Physiol Pharmacol. 1998; 76:533-38

93. Childs A, Jacobs C, Kaminski T, Halliwell B, Leeuwenburgh C. Supplementation with vitamin C and N-acetyl-cysteine increases oxidative stress in humans after an acute muscle injury induced by eccentric exercise. Free Radic Biol Med. 2001; 31(6):745-53

94. Bloomer RJ. The role of nutritional supplements in the prevention and treatment of resistance exercise-induced skeletal muscle injury. Sports Med. 2007; 37(6):519-32

95. Dillard CJ, Litov RE, Savin WM. Effects of exercise, vitamin E, and ozone on pulmonary function and lipid peroxidation. J Appl Physiol. 1978; 45(6):927-32

96. Ashton T, Rowlands CC, Jones E, Young IS, Jackson SK, Davies B, et al. Electron spin resonance spectroscopic detection of oxygen-centred radicals in human serum following exhaustive exercise. Eur J Appl Physiol Occup Physiol. 1998; 77(6):498-502

97. 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. 2004; 91(1):91-100

98. Food and Nutrition Board, Institute of Medicine. Dietary reference intakes for vitamin C, vitamin E, selenium, and carotenoids. Washington, DC: National Academy Press; 2000.

99. Australian Institute of Sport. Antioxidant Vitamins C and E. 2008. Available at: http://www.ausport.gov.au/ais/nutrition/supplements/resources/supplement_fact_sheets/antioxidant_vitamins_c_and_e. Accessed April 30, 2008

100. American College of Sports Medicine. Current Comment on Vitamin and Mineral Supplements and Exercise. Available at: http://www.acsm.org/AM/Template.cfm?Section=Search&section=19991&template=/CM/ContentDisplay.cfm&ContentFileID=410. Accessed April 30, 2008

101. United States Department of Health and Human Services and United States Department of Agriculture. Dietary Guidelines for Americans 2005. Available at: http://www.health.gov/dietaryguidelines/dga2005/document/default.htm. Accessed April 30, 2008

102. Martin A. Apports nutritionnels conseillés pour la population française. 3ème ed. Paris: Ed Tec & Doc; 2001.

103. Lukaski HC. Vitamin and mineral status: Effects on physical performance. Nutrition. 2004; 20(7-8):632-44

104. Nieman DC, Henson DA, McAnulty SR, McAnulty LS, Morrow JD, Ahmed A, et al. Vitamin E and immunity after the Kona Triathlon World Championship. Med Sci Sports Exerc. 2004; 36(8):1328-35

105. Zembron-Lacny A, Szyszka K, Sobanska B, Pakula R. Prooxidant-antioxidant equilibrium in rowers: Effect of a single dose of vitamin E. J Sports Med Phys Fitness. 2006; 46(2):257-64

106. Beaton LJ, Allan DA, Tarnopolsky MA, Tiidus PM, Phillips SM. Contraction-induced muscle damage is unaffected by vitamin E supplementation. Med Sci Sports Exerc. 2002; 34(5):798-805

107. Viitala PE, Newhouse IJ, La Voie N, Gottardo C. The effects of antioxidant vitamin supplementation on resistance exercise induced lipid peroxidation in trained and untrained participants. Lipids Health Dis. 2004; 3:14

108. Rokitzki L, Logemann E, Huber G, Keck E, Keul J. alpha-Tocopherol supplementation in racing cyclists during extreme endurance training. Int J Sport Nutr. 1994; 4(3):253-64

109. Itoh H, Ohkuwa T, Yamazaki Y, Shimoda T, Wakayama A, Tamura S, et al. Vitamin E supplementation attenuates leakage of enzymes following 6 successive days of running training. Int J Sports Med. 2000; 21(5):369-74

110. Avery NG, Kaiser JL, Sharman MJ, Scheett TP, Barnes DM, Gomez AL, et al. Effects of vitamin E supplementation on recovery from repeated bouts of resistance exercise. J Strength Cond Res. 2003; 17(4):801-9

111. McAnulty SR, McAnulty LS, Nieman DC, Morrow JD, Shooter LA, Holmes S, et al. Effect of alpha-tocopherol supplementation on plasma homocysteine and oxidative stress in highly trained athletes before and after exhaustive exercise. J Nutr Biochem. 2005; 16(9):530-7

112. Ashton T, Young IS, Peters JR, Jones E, Jackson SK, Davies B, et al. Electron spin resonance spectroscopy, exercise, and oxidative stress: an ascorbic acid intervention study. J Appl Physiol. 1999; 87(6):2032-6

113. Khassaf M, McArdle A, Esanu C, Vasilaki A, McArdle F, Griffiths RD, et al. Effect of vitamin C supplements on antioxidant defence and stress proteins in human lymphocytes and skeletal muscle. J Physiol. 2003; 549(Pt 2):645-52

114. Peters EM, Anderson R, Nieman DC, Fickl H, Jogessar V. Vitamin C supplementation attenuates the increases in circulating cortisol, adrenaline and anti-inflammatory polypeptides following ultramarathon running. Int J Sports Med. 2001; 22(7):537-43

115. Bryer SC, Goldfarb AH. Effect of high dose vitamin C supplementation on muscle soreness, damage, function, and oxidative stress to eccentric exercise. Int J Sport Nutr Exerc Metab. 2006; 16(3):270-80

116. Goldfarb AH, Patrick SW, Bryer S, You T. Vitamin C supplementation affects oxidative-stress blood markers in response to a 30-minute run at 75% VO2max. Int J Sport Nutr Exerc Metab. 2005; 15(3):279-90

117. Nieman DC, Henson DA, McAnulty SR, McAnulty L, Swick NS, Utter AC, et al. Influence of vitamin C supplementation on oxidative and immune changes after an ultramarathon. J Appl Physiol. 2002; 92(5):1970-7

118. Thompson D, Bailey DM, Hill J, Hurst T, Powell JR, Williams C. Prolonged vitamin C supplementation and recovery from eccentric exercise. Eur J Appl Physiol. 2004; 92(1-2):133-8

119. Close GL, Ashton T, Cable T, Doran D, Holloway C, McArdle F, et al. Ascorbic acid supplementation does not attenuate post-exercise muscle soreness following muscle-damaging exercise but may delay the recovery process. Br J Nutr. 2006; 95(5):976-81

120. Faff J, Tutak T, Satora P, Sienkiewicz D. The influence of ubiquinone on the intense work capacity and on serum activities of creatine kinase and aspartate aminotransferease. Biol Sport. 1997; 14:37-44

121. Medved I, Brown MJ, Bjorksten AR, Murphy KT, Petersen AC, Sostaric S, et al. N-acetylcysteine enhances muscle cysteine and glutathione availability and attenuates fatigue

during prolonged exercise in endurance-trained individuals. J Appl Physiol. 2004; 97(4):1477-85

122. Morillas-Ruiz JM, Villegas Garcia JA, López FJ, Vidal-Guevara ML, Zafrilla P. Effects of polyphenolic antioxidants on exercise-induced oxidative stress. Clin Nutr. 2006; 25:444-53

123. Morillas-Ruiz J, Zafrilla P, Almar M, Cuevas MJ, Lopez FJ, Abellan P, et al. The effects of an antioxidant-supplemented beverage on exercise-induced oxidative stress: results from a placebo-controlled double-blind study in cyclists. Eur J Appl Physiol. 2005; 95(5-6):543-9

124. Pilaczynska-Szczesniak L, Skarpanska-Steinborn A, Deskur E, Basta P, Horoszkiewicz-Hassan M. The influence of chokeberry juice supplementation on the reduction of oxidative stress resulting from an incremental rowing ergometer exercise. Int J Sport Nutr Exerc Metab. 2005; 15(1):48-58

125. Tessier F, Margaritis I, Richard MJ, Moynot C, Marconnet P. Selenium and training effects on the glutathione system and aerobic performance. Med Sci Sports Exerc. 1995; 27(3):390-6

126. Dragan I, Dinu V, Mohora M, Christea E, Ploesteanu E, Stroescu V. Studies regarding the antioxidant effects of selenium on top swimmers. Rev Roum Physiol. 1990; 27(1):15-20

127. Tessier F, Hida H, Favier A, Marconnet P. Muscle GSH-Px activity after prolonged exercise, training, and selenium supplementation. Biol Trace Elem Res. 1995; 47(1-3):279-85

128. Bryant RJ, Ryder J, Martino P, Kim J, Craig BW. Effects of vitamin E and C supplementation either alone or in combination on exercise-induced lipid peroxidation in trained cyclists. J Strength Cond Res. 2003; 17(4):792-800

129. Fischer CP, Hiscock NJ, Penkowa M, Basu S, Vessby B, Kallner A, et al. Supplementation with vitamins C and E inhibits the release of interleukin-6 from contracting human skeletal muscle. J Physiol. 2004; 558(Pt 2):633-45

130. 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. 2004; 36(10):1329-41

131. Shafat A, Butler P, Jensen RL, Donnelly AE. Effects of dietary supplementation with vitamins C and E on muscle function during and after eccentric contractions in humans. Eur J Appl Physiol. 2004; 93(1-2):196-202

132. Dawson B, Henry GJ, Goodman C, Gillam I, Beilby JR, Ching S, et al. Effect of Vitamin C and E supplementation on biochemical and ultrastructural indices of muscle damage after a 21 km run. Int J Sports Med. 2002; 23(1):10-5

133. Mastaloudis A, Traber MG, Carstensen K, Widrick J. Antioxidants did not prevent muscle damage in response to an ultramarathon run. Med Sci Sports Exerc. 2006; 38(1):72-80

134. Tauler P, Aguilo A, Fuentespina E, Tur JA, Pons A. Diet supplementation with vitamin E, vitamin C and beta-carotene cocktail enhances basal neutrophil antioxidant enzymes in athletes. Pflugers Arch. 2002; 443(5-6):791-7

135. Tauler P, Aguiló A, Gimeno I, Fuentespina E, Tur JA, Pons A. Response of blood cell antioxidant enzyme defences to antioxidant diet supplementation and to intense exercise. Eur J Nutr. 2006; 45(4):187-95

136. Schröder H, Navarro E, Mora J, Galiano D, Tramullas A. Effects of alpha-tocopherol, beta-carotene and ascorbic acid on oxidative, hormonal and enzymatic exercise stress markers in habitual training activity of professional basketball players. Eur J Nutr. 2001; 40(4):178-84

137. Vasankari TJ, Kujala UM, Vasankari TM, Vuorimaa T, Ahotupa M. Increased serum and low-density-lipoprotein antioxidant potential after antioxidant supplementation in endurance athletes. Am J Clin Nutr. 1997; 65(4):1052-6

138. Nielsen AN, Mizuno M, Ratkevicius A, Mohr T, Rohde M, Mortensen SA, et al. No effect of antioxidant supplementation in triathletes on maximal oxygen uptake, 31P-NMRS detected muscle energy metabolism and muscle fatigue. Int J Sports Med. 1999; 20(3):154-8

139. Kaikkonen J, Kosonen L, Nyyssonen K, Porkkala-Sarataho E, Salonen R, Korpela H, et al. Effect of combined coenzyme Q10 and d-alpha-tocopheryl acetate supplementation on exercise-induced lipid peroxidation and muscular damage: a placebo-controlled double-blind study in marathon runners. Free Radic Res. 1998; 29(1):85-92

140. Hagobian TA, Jacobs KA, Subudhi AW, Fattor JA, Rock PB, Muza SR, et al. Cytokine response at high altitude: Effects of exercise and antioxidants at 4300 m. Med Sci Sports Exerc. 2006; 38(2):276-85

141. Bailey D. Effect of antioxidant supplementation in indices of muscle damage following prolonged intermittent shuttle running. Med Sci Sports Exerc. 2003;

142. 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. 2004; 14(2):133-46

143. Schmidt MC, Askew EW, Roberts DE, Prior RL, Ensign WY, Jr., Hesslink RE, Jr. Oxidative stress in humans training in a cold, moderate altitude environment and their response to a phytochemical antioxidant supplement. Wilderness Environ Med. 2002; 13(2):94-105

144. Kaczmarski M, Wójcicki J, Samochowiec L, Dutkiewicz T, Sych Z. The influence of exogenous antioxidants and physical exercise on some parameters associated with production and removal of free radicals. Pharmazie. 1999; 54(4):303-6

145. Lawrence JD, Bower RC, Riehl WP, Smith JL. Effects of alpha-tocopherol acetate on the swimming endurance of trained swimmers. Am J Clin Nutr. 1975; 28(3):205-8

146. Willett WC, Stampfer MJ. What vitamins should I be taking, doctor? New Engl J Med. 2001; 345(25):1819-24

147. Herbert V. Prooxidant effects of antioxidant vitamins. Introduction. J Nutrition. 1996; 126(4 Suppl):1197S-200S

148. Falvo MJ, Bloomer RJ. Review of exercise-induced muscle injury: Relevance for athletic populations. Res Sports Med. 2006; 14(1):65-82

2. HYPOTHESIS AND AIMS

Hypothesis

Antioxidant supplementation attenuates oxidative stress, lipid peroxidation, muscle damage and inflammation induced by exercise in athletes.

Aims

The specific aims of this thesis were:

1. Compare dietary intake of antioxidants, antioxidant status, and oxidative stress, lipid peroxidation and muscle damage markers between athletes and age-matched sedentary individuals
2. Determine the effect of acute exercise (1000m kayak race) on redox status, and lipid peroxidation, muscle damage and inflammation markers
3. Describe the antioxidant status and oxidative stress, lipid peroxidation, muscle damage and inflammation markers evolution with regular training and competition
4. Evaluate the efficacy of antioxidant supplementation in reducing oxidative stress, lipid peroxidation, muscle damage and inflammation markers

3.1 ANTIOXIDANT STATUS, OXIDATIVE STRESS AND DAMAGE IN ELITE TRAINED KAYAKERS AND CANOEISTS AND SEDENTARY CONTROLS

Teixeira VH[1], Valente H[1,2], Casal S[3], Marques F[4,5], Moreira P[1,2]

[1] Faculty of Nutrition and Food Sciences, University of Porto

[2] Research Centre in Physical Activity, Health and Leisure, University of Porto

[3] REQUIMTE, Bromatology Service, Faculty of Pharmacy, University of Porto

[4] Department of Clinical Analysis, Faculty of Pharmacy, University of Porto

[5] Institute for Molecular and Cell Biology – IBMC, University of Porto

SUBMITTED FOR PUBLICATION

ABSTRACT

Strenuous physical activity is known to generate reactive oxygen species to a point that can exceed the antioxidant defence system and lead to oxidative stress. Dietary intake of antioxidants, and plasma enzymatic (superoxide dismutase - SOD, glutathione reductase - Gr, and glutathione peroxidase - GPx) and non-enzymatic (total antioxidant status - TAS, uric acid, α-tocopherol, retinol, α-carotene, β-carotene, lycopene and lutein + zeaxanthin) antioxidants, and markers of lipid peroxidation (thiobarbituric acid reactive substances - TBARS) and muscle damage (creatine kinase - CK) were determined in 17 elite male kayakers/canoeists under resting conditions and in an equal number of age- and sex-matched sedentary individuals. Athletes showed increased plasma values of α-tocopherol (P = 0.037), α-carotene (P = 0.003), β-carotene (P = 0.007) and superoxide dismutase activity (P = 0.002), and a lower TAS level (P = 0.030). Antioxidant intake (α-tocopherol, vitamin C and β-carotene), and plasmatic GPx, Gr, lycopene, lutein + zeaxanthin, retinol and uric acid levels were similar in both groups. Nevertheless, TBARS (P < 0.001) and CK (P = 0.011) levels were found to be significantly higher in the kayakers/canoeists. Our work suggest that despite the enhanced levels of antioxidants, athletes undergoing regular strenuous exercise exhibited increased oxidative stress compared to sedentary controls.

INTRODUCTION

Exhaustive physical exercise is characterized by a dramatic increase in oxygen consumption by the whole body (10-15 fold) and particularly by the skeletal muscle (up to 100-fold), of which 2% to 5% is not completely reduced to water in the mitochondria, instead undergoing an univalent reduction to produce superoxide radicals, that could subsequently leak out of the electron transport chain [1]. Although oxidative stress has been linked to the pathophysiology of several diseases, such as atherosclerosis and cancer [2], there is, paradoxically, irrefutable evidence that regular physical activity is an important deterrent of such conditions [3]. Actually, chronic exercise (training) causes physiologic adaptations that enhance antioxidant defences and could minimize oxidative stress either in resting conditions and in response to acute exercise [4, 5]. It has been described that aerobic [4, 6, 7] and anaerobic [5, 6] training induces an enhancement in the activity of antioxidant enzymes, mainly of superoxide dismutase (SOD) and glutathione-peroxidase (GPx) [5, 6]. Thus, athletes generally present an up-regulated antioxidant enzymes activity in comparison with non-trained subjects [8, 9]. The published data on the impact of training in non-enzymatic antioxidants is not consensual, with the some studies [8, 10, 11] but not all [12] showing an increase in their plasmatic concentrations, that could result from its mobilization from tissue pools [10, 12]. Consequently, individuals who train regularly may exhibit an overall improved antioxidant status and protection against oxidative stress when compared with healthy sedentary subjects [4, 11].

However, those adaptations might not be sufficient to correspond in absolute to the increase in pro-oxidant events [13] and very intense physical activity may disrupt the fine physiological balance between oxidative reactions and antioxidant defences [4, 6]. If the generation of ROS overwhelms the antioxidant capacity of tissues to detoxify them, eliciting oxidative stress, these highly reactive molecules can attack proteins, DNA and, especially, lipids [1]. It has been reported that intense training produces a decrease in antioxidant status and an increase of lipoperoxidative damage [14]. The lipid peroxidation of polyunsaturated fatty acids in the sarcolemma can compromise its integrity resulting in the leakage of muscle-derived enzymes into the blood. In fact, lipid peroxidation appears to be an important mechanism underlying exercise-induced muscle damage and delayed-onset muscle soreness [9]. Oxidative stress has been shown to influence factors that adversely affect exercise performance, such as reduced immune function, and muscle damage and fatigue [15]. Moreover, athletes under a strenuous training may have difficulty in maintaining optimal plasmatic vitamins levels even

when consuming the dietary reference intakes [12]. The aim of the present study was to compare dietary intake of antioxidants, antioxidant status, lipid peroxidation and muscle damage of a group of elite kayakers with that of sex- and age-matched sedentary individuals.

MATERIAL AND METHODS

Subjects

Seventeen elite male athletes under regular training and 17 sedentary male controls were enrolled in the study. Athletes were kayakers (n=15) and canoeists (n=2) members of and candidates for the Portuguese Kayaking and Canoeing National Team. The study was performed in a week of June when the athletes were in the Team Campus engaged in a training period to the World Championship. The controlled physical training consisted of 12 sessions per week lasting approximately 1-1.5h each (~15h/week), comprising on-water (n=9), weight training (n=2), and aerobic running (n=1) sessions. All athletes had a short (50min) skills training session in the day before the experiment. The age-matched control subjects had a sedentary lifestyle, and did not practice formal physical exercise for more than 1h per week during the preceding 3 months. All participants had a body mass index (BMI) <30 kg/m^2 and were healthy (self-reported), non-smokers, not receiving non-steroidal anti-inflammatory medication or anabolic drugs, and had not used antioxidants supplements for at least 3 months, as recommended [16]. Informed consent was previously obtained from all the subjects (or their parents, if they were under 18 years old).

Dietary intake

In order to estimate average energy and nutritional intake, subjects recorded their dietary intake during four consecutive days, one being a weekend day. A trained nutritionist gave detailed verbal and written instructions about proper dietary recording. A full description of foods and fluids consumed was requested, including the commercial brand names of packaged food, cooking or processing methods, and food items and ingredients added in preparation. Subjects had to estimate the amount of food or fluids consumed, by referring to the weight or volume information provided on food package or by using standardized household measures. Participants were instructed to continue their habitual dietary intake during this period, and to avoid the omission and replacement of foods that are hard to record or that they feel that

shouldn't be eating [17], and to abstain from beverages with ethanol or caffeine for at least 24h prior to blood draws. Dietary records' information was converted to energy and nutrients using ESHA Food Processor 8.0 for Windows (Salem, OR, USA). This program was added with information for composite dishes, commercial foods, and sports foods, whenever reliable nutritional composition data could be obtained. Unfortunately, this software package does not provide estimates of α-carotene, lycopene, lutein and zeaxanthin intakes; another limitation is that vitamin A is reported as retinol equivalents instead of retinol activity equivalents, as recently recommended [18]. The estimated nutritional intake was assessed by reference to the Food and Nutrition Board's Dietary Reference Intakes [19]. For nutrients with established Estimated Average Requirement (EAR), the prevalence of inadequacy was calculated as the proportion of athletes whose intakes were below EAR; for those nutrients without EAR, we compared the mean intake of the group with Adequate Intake (AI) [19].

Physical activity and estimated energy expenditure

Subjects recorded all their physical activities in 15-min intervals in activity diaries over a four-day period. Athletes were also asked to detail the type, duration and intensity of their training session activities. The estimative of the energy expenditure for each recorded activity was calculated by multiplying the amount of time spent in that activity by the corresponding metabolic equivalents, according the intensity reported by subjects [20]. Total energy expenditure was the sum of energy expenditure calculated for each activity. Physical activity level (PAL) was determined by dividing total energy expenditure by the basal metabolic rate (BMR) calculated with the Cunningham equation, that has been shown to be the best prediction equation for BMR for athletes [21].

Body composition and anthropometry

Subjects' standing height and weight (lightly dressed and barefooted) were measured using a calibrated Seca stadiometer and electronic scale (model 701, UK), with a precision of 0.5cm and 100g respectively, according to the international recommended methodology [22]. Thereafter, body mass index (BMI) was calculated.

Skinfold thicknesses were measured at four sites (bicep, tricep, subscapula, iliac crest) using a Harpenden caliper (John Bull, UK), and body fat percentage was calculated using the formula suggested by Siri [23].

Blood collection and handling

Antecubital venous blood samples (10mL) were collected at rest between 08h00 and 09h00 after an overnight fast (8-12 hours) by a trained phlebotomist. In order to minimize potentially enhanced oxidative stress induced by ischemia-reperfusion mechanism, no tourniquet constriction was used. Blood samples were drawn into EDTA-treated vacutainer tubes and non-additive serum vacutainer tubes and immediately placed on ice in the dark until centrifugation. An aliquot of whole blood was separated to measure hematocrit and haemoglobin. Whole blood in serum tubes was allowed to clot for 30min at room temperature and then centrifuged at 2000 rpm for 10 minutes for serum separation. To obtain the plasma fraction, the remaining whole blood in EDTA-containing tubes was immediately centrifuged. Erythrocytes were washed and centrifuged three times with a 0.9% sodium chloride solution and lysed with ice-cold distilled deionized water. Serum, plasma and washed erythrocytes were separated into several aliquots and frozen at -80ºC for later biochemical analysis. Samples were analysed in duplicate, and the mean value was used for statistical analysis. A major limitation of present study is that, due to the technical problems, ascorbic acid was not measured.

Biochemical analysis

Haemoglobin and hematocrit were assessed from EDTA-treated blood using an automated analyzer (Horiba ABX Micros 60, ABX Diagnostic, Montpellier, France).

The concentrations of cholesterol and triglycerides in plasma and lipoprotein sub-fractions were determined by enzymatic colorimetric assays (kit 07 3680 5 for triglycerides, and 07 3664 3 for cholesterol; Hoffman-La Roche, Basel, Switzerland) in an autoanalyzer (Cobas Mira; Hoffmann-La Roche). LDL cholesterol (LDL-C) was calculated by means of Friedewald equation.

Uric acid was determined by an enzymatic method at 550 nm using a commercial kit (Horiba ABX A11A01670, ABX diagnostic, Montpellier, France), according to the manufacturer's specifications.

Serum total antioxidant status (TAS) was measured spectrophotometrically using a commercial kit (Randox NX2332, Randox, Crumlin, UK). In brief, the assay is based on the reduction of free radicals (2,2'-azino-di-(3-ethylbenzothiazoline-6-sulphonate – $ABTS^{\bullet+}$),

measuring the decreased of absorbance at 600nm. The radical cation $ABTS^{\bullet+}$ is formed by the interaction of ABTS with ferrylmyoglobin radical species generated by the activation of metmyoglobin with hydrogen peroxide (H_2O_2). The suppression of the absorbance of the radical cation $ABTS^{\bullet+}$ by plasma antioxidants was compared with that from Trolox. Samples were expressed in antioxidant capacity in millimoles per liter of Trolox equivalents.

Enzyme activities were analyzed according to the standard spectrophotometric–colorimetric procedures provided with the commercial kits in a Cobas Mira Plus analyzer (Roche Diagnostic Systems, Switzerland), at 37°C. Whole-blood GPX activity was determined spectrophotometrically using cumene hydroperoxide as the oxidant of glutathione (GSH) (Ransel RS 505, Randox, Crumlin, UK). The oxidised glutathione (GSSG) is immediately reduced to GSH by glutathione reductase, with a concomitant oxidation of NADPH to $NADP^+$. GPx activity was measure by the decrease in absorbance at 340 nm, and expressed in U/L. The plasmatic activity of Gr was measured by monitoring the oxidation of NADPH to $NADP^+$ during the reduction of GSSG (Ransel GR 2368; Randox, Crumlin, UK), and expressed in U/L. A value of 10 U/L was considered as the detection limit. Cu, Zn-SOD activity was measured in washed erythrocytes after lysis by using a commercial kit (Ransod SD 125, Randox, Crumlin, UK). For this purpose, xanthine and xanthine oxidase were used to generate superoxide anion which reacts with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye. SOD activity was calculated by assessing the extent of inhibition of the reaction, on the basis of the change in the absorbance over 3 min at 505 nm, and data from the standard curve generated from purified SOD obtained from the manufacturer. SOD activity was expressed in U/g Hb.

Plasma liposoluble vitamins were extracted with n-hexane, after protein precipitation with ethanol, using β-apo-carotenal and tocol as internal standards [(24)]. The chromatographic analyses were performed by HPLC (Jasco, Japan) on a Chromolith performance RP-18 column (100x3mm, Merck), using methanol and acetonitrile (50:50) as mobile phase. Lutein (co-eluting with zeaxanthin), α-carotene, β-carotene and the carotenoids internal standard (β-apo-8'-carotenal) were quantified at 450 nm, retinol at 325 nm and lycopene at 425 nm, using a diode-array detector. Alpha-tocopherol quantification was based on the fluorescence readings (ex: 290 nm; em: 330 nm), using tocol as internal standard.

Serum creatine kinase (CK) and uric acid were determined at 37ºC using commercially available methods (Roche Products, UK), and an automated system (COBAS Mira Plus, Roche Diagnostic Systems, Switzerland).

The determination of thiobarbituric acid reactive substances (TBARS) in serum was performed using a commercial kit (Oxi-tek TBARS assay kit, Zeptometrix Corporation, Buffalo, NY) according to the manufacturer's instructions. Briefly, serum (100 μL) was mixed with an equal volume of 8.1% sodium dodecyl sulfate (SDS) and 2.5 mL of 5% thiobarbituric acid/acetic acid reagent. Sample was incubated at 95ºC in capped tubes for 60 minutes and, thereafter, cooled to room temperature in an ice bath for 10 min before being centrifuged at 3000 rpm for 15 min. The supernatant was removed and its absorbance was read at 532 nm. The results are expressed as malondialdehyde (MDA) equivalents by interpolation from a MDA standard curve (0-100 nmol/mL).

Data and statistical analysis

All results were expressed as mean ± standard deviation. Data was statistically analysed using SPSS 13.0 (Chicago, IL, USA). All the data were tested for their normal distribution with the one-sample Kolmogorov-Smirnov test. Comparisons between the two groups were made using independent sample t-test, for parameters with normal distribution, and Mann–Whitney non-parametric test (U-test), when data did not follow the Gaussian distribution (namely for beta-carotene, energy-adjusted β-carotene and iron intakes). Correlations between variables were carried out by Pearson or Spearman tests depending on data distribution. Differences were considered significant at $P < 0.05$ in the bilateral situation.

RESULTS

Demographic, anthropometric and biochemical characteristics of study subjects are presented in Table 1. Kayakers and controls were of similar age and height. Athletes were heavier ($P = 0.012$), leaner ($P = 0.001$) and had a higher BMI ($P = 0.004$) than their sedentary counterparts. Muscularity, particularly of the arms and trunk, is a major contributor for successful kayaking performance at a high level [25]. The LDL-cholesterol level was significantly higher in the control subjects ($P = 0.049$), but no other statistically significant difference was observed in the other parameters between both groups.

The estimated energy expenditure, basal metabolic rate, physical activity level, and nutritional intakes of subjects are displayed in Table 2. Athletes had significantly higher energy requirements ($P < 0.001$), basal metabolic rates ($P < 0.001$) and PAL ($P < 0.001$) when

compared to controls. According, the estimated energy intakes were significantly higher in sportsmen ($P < 0.001$).

Table 1. Characteristics of the subjects

	Kayakers/canoeists (n = 17)	Controls (n = 17)	*P*
Age (years)	20.4 ± 3.9	22.1 ± 2.4	NS
Height (cm)	177.6 ± 5.5	176.8 ± 5.0	NS
Weight (kg)	77.3 ± 7.4	70.2 ± 8.2	0.012
BMI (kg/m^2)	24.48 ± 1.54	22.44 ± 2.21	0.004
Body fat (%)	13.4 ± 3.3	17.4 ± 3.2	0.001
Total cholesterol (mg/dL)	149 ± 46	172 ± 29	NS
LDL-cholesterol (mg/dL)	81.0 ± 37.6	103.0 ± 23.5	0.049
HDL-cholesterol (mg/dL)	48.8 ± 9.3	53.6 ± 6.3	NS
Triacylglycerols (mg/dL)	94 ± 34	79 ± 38	NS
Hemoglobin (g/dL)	15.4 ± 0.6	15.3 ± 0.8	NS
Hematocrit (L/L)	48.2 ± 1.8	47.3 ± 2.6	NS

NS: Non-significant

Nutritional analysis showed that macronutrient ($P < 0.001$ for protein, fat and carbohydrates intake) and cholesterol intakes ($P = 0.004$) were significantly greater in kayak paddlers in comparison with the control group. However, all of these differences lost their statistical significance when adjusted for energy intake, except for the percentage of energy ingested as fat ($P = 0.014$) which was lower. Regarding micronutrients, a significant higher intake of vitamin A, selenium, zinc, copper, iron, magnesium, and manganese ($P < 0.001$) was found in athletes. On the other hand, the two groups did not differ in vitamin antioxidant intakes (vitamins C, α-tocopherol and β-carotene). When micronutrient intake was adjusted for energy intake, only zinc ($P = 0.014$) and iron ($P = 0.026$) remained statistical different, while α-tocopherol difference reached statistical significance ($P = 0.045$). The estimated prevalence of inadequacy was 100% for α-tocopherol, and 0% for selenium, zinc, copper and iron, in both groups. For vitamins A and C, and for magnesium, the estimated prevalence of inadequacy was 0% and 18%, 41% and 76%, and 18% and 82%, respectively for kayakers and sedentary individuals. There is probably a low prevalence of inadequacy for manganese in athletes, but

we cannot evaluate controls' intake for this micronutrient because their mean intake was below the AI.

Table 2. Estimated daily energy expenditure and nutritional intake of subjects

	Kayakers/canoeists (n = 17)	Controls (n = 17)	*P*
Energy expenditure (kcal)	3645 ± 223	2401 ± 247	< 0.001
Basal metabolic rate (kcal)	1970 ± 122	1770 ± 123	< 0.001
Physical activity level	1.85 ± 0.06	1.36 ± 0.09	< 0.001
Energy intake (kcal)	3386 ± 557	2331 ± 283	< 0.001
Proteins (g)	156 ± 25	106 ± 13	< 0.001
Proteins (% of energy intake)	18.6 ± 2.9	18.2 ± 1.5	NS
Carbohydrates (g)	431 ± 106	275 ± 33	< 0.001
Carbohydrates (% of energy intake)	50.2 ± 6.3	47.3 ± 2.3	NS
Fibre (g)	24.3 ± 7.2	17.0 ± 3.9	0.001
Fat (g)	116.1 ± 19.7	89.5 ± 14.2	< 0.001
Fat (% of energy intake)	31.1 ± 4.6	34.5 ± 2.4	0.012
Saturated fat (g)	39.8 ± 8.3	33.7 ± 7.3	0.029
Monounsaturated fat (g)	45.3 ± 9.9	33.8 ± 5.5	< 0.001
Polyunsaturated fat (g)	17.7 ± 4.5	12.4 ± 3.4	0.001
Cholesterol (mg)	427 ± 98	332 ± 80	0.004
Vitamin C (mg)	86.1 ± 37.8	68.3 ± 27.6	NS
α-tocopherol (mg)	6.3 ± 2.0	5.6 ± 2.0	NS
Vitamin A (RE μg)	1289 ± 327	859 ± 309	< 0.001
β-carotene (μg)	867 ± 1092	793 ± 1379	NS
Selenium (μg)	128.6 ± 24.7	96.5 ± 28.7	0.001
Zinc (mg)	22.5 ± 4.5	13.4 ± 1.8	< 0.001
Copper (mg)	1.89 ± 0.45	1.32 ± 0.25	< 0.001
Iron (mg)	25.2 ± 8.7	14.0 ± 2.6	< 0.001
Magnesium (mg)	396 ± 72	289 ± 53	< 0.001
Manganese (mg)	3.44 ± 0.94	2.09 ± 0.32	< 0.001

NS: Non-significant

Table 3 presents antioxidant status, lipid peroxidation and muscle damage markers in this study. None of the subjects had plasma α-tocopherol concentrations below the cut-off point for deficiency (9.3 μmol/L). For β-carotene, 1 athlete and 9 sedentary subjects had plasma concentrations below 0.30 μmol/L [24]. Kayak paddlers were found to have significantly elevated erythrocyte superoxide dismutase activity, α-tocopherol, β-carotene and α-carotene plasmatic concentrations. Even when β-carotene, α- carotene and α-tocopherol levels were adjusted for lipoproteins [26], the differences remained statistically significant (P = 0.001, P < 0.001 and P = 0.002, respectively). Sedentary individuals revealed increased TAS values by 14% as compared to the athletes (P = 0.030). No statistically significant differences were observed between groups for whole blood glutathione peroxidise and plasmatic glutathione reductase activities, and plasmatic lycopene, and lutein + zeaxanthin levels. Sportsmen revealed statistically significant greater muscle damage and lipid peroxidation than their sedentary counterparts, as indicated by the respectively indices (CK and TBARS).

Table 3. Antioxidant status, lipid peroxidation and muscle damage markers of subjects

	Kayakers/canoeists (n = 17)	Controls (n = 17)	*P*
TAS (mmol/L)	1.03 ± 0.17	1.18 ± 0.20	0.030
Uric acid (mg/dL)	4.97 ± 1.37	5.44 ± 0.81	NS
Glutathione peroxidase (U/L)	6586.8 ± 2119.9	7096.1 ± 1660.1	NS
Superoxide dismutase (U/g Hg)	1234.4 ± 212.1	1040.8 ± 116.6	0.002
Glutathione reductase (U/L)	73.11 ± 14.53	75.41 ± 7.15	NS
α-tocopherol (μmol/L)	25.70 ± 3.87	22.95 ± 3.34	0.037
Retinol (μmol/L)	1.99 ± 0.36	1.87 ± 0.36	NS
α-carotene (μmol/L)	0.15 ± 0.10	0.06 ± 0.04	0.003
β-carotene (μmol/L)	0.65 ± 0.37	0.34 ± 0.22	0.007
Lycopene (μmol/L)	0.39 ± 0.10	0.40 ± 0.15	NS
Lutein + zeaxanthin (μmol/L)	0.33 ± 0.14	0.29 ± 0.08	NS
Creatine kinase (IU/L)	382.6 ± 249.2	103.5 ± 47.4	< 0.001
TBARS (μmol/L)	4.93 ± 1.32	3.75 ± 1.24	0.011

NS: Non-significant

DISCUSSION

The main aim of this work was to investigate the antioxidant status and lipoperoxidative and muscular damages in athletes under intense training and comparing them with sedentary individuals. It is well documented in the literature that training, either aerobic or anaerobic, if sufficiently long and intense, induces an increase in the antioxidant enzymes activities that reduces the basal state of oxidative stress and strengthens the organism's antioxidant capacity for the subsequent physical efforts [6, 27]. However, if athletes are subjected to a strenuous training and competitive schedule, the excessive production of ROS could surpass the antioxidant system efficiency and impose an oxidative stress [13], that may be involved in overtraining [27]. In the present study, sportsmen were in an intensive training program, encompassing endurance and resistance exercises, in order to prepare themselves for the forthcoming international championships as described earlier. In line with this, we found that kayak paddlers exhibited higher oxidative stress and muscle damage at basal state than untrained controls, despite showing increased antioxidant levels in plasma.

The levels of plasma antioxidants and the activities of antioxidant enzymes were within normal physiological ranges for general population [28] and those described in other studies in athletes [24], either professionals or amateurs [29]. In accordance with other studies, trained subjects presented augmented plasmatic levels of α-tocopherol [8, 10, 11, 13, 15, 30] and β-carotene [15, 30] than controls. Data on other carotenoids plasma concentrations in sportsmen is very scarce and did not consider the comparison with sedentary individuals [24, 29]. In relation to non-trained subjects, we found an increase in serum α-carotene in athletes, but similar concentrations of lycopene and lutein + zeaxanthin. Despite the discrepancies in plasmatic levels, kayakers' intakes of α-tocopherol and β-carotene were not significantly higher than that of the controls, in agreement with what has been previously described [11, 15, 30]. The increment of athletes' plasmatic concentrations of fat-soluble antioxidant vitamins in resting conditions could be explained by the cumulative result of the regularly repeated transient mobilization from tissue stores that occur during acute exercise [10-12, 24]. Actually, it is well recognized that athletes have greater abilities to mobilize and use lipid reserves as a fuel subtract for exercise [29]. We can also argue that plasma levels could not be truly reflective of tissue pools, or that the increased availability of other antioxidants, such as SOD activity in the present study, could spare antioxidant vitamins [15].

Exercise-induced changes in the redox status of tissues may initiate intracellular signal transduction processes that trigger antioxidant protein expression [11]. It has been reported that regular exercise training causes an up-regulation of the activity of antioxidant enzymes at rest and immediately after exercise [6]. While it is not perfectly established which and in what conditions enzymes can be activated [4], the response seems to depend on the duration and intensity of exercise [2, 8]. In the present study, no enhanced Gr or GPx activities were found at rest in the plasma of athletes. In contrast, our data evidenced that plasma SOD activity was significantly augmented in kayakers when compared with controls. This tendency has already been described by other authors for sportsmen [6, 8, 10, 12, 13, 31, 32], and could result from the training-induced post-transcriptional modulation of its activity, since enzyme protein and mRNA levels did not seem to be altered [33]. Although opposite effects of exercise training in GPx have been published, either toward higher [2, 6, 9, 32] or lower [12] activity levels, we found unaltered values on our sample in accordance with other studies [4, 5, 7, 30, 34]. It is noteworthy that kayak paddlers had significantly higher SOD activity and similar GPx activity that controls subjects, as Cazzola *et al*. [11] also found. Although Gr plays an important role in the antioxidant defence machinery there is a paucity of data in athletes. We did not found any difference in the activity of this enzyme in plasma of both groups, while Banfi and colleagues did [35]. The equivocal data reported in the literature on antioxidant enzymes activities could be linked to differences in the athletes' nutritional intakes and status, the type, intensity and duration of their training program, or the biological material analysed [15]. Actually, trained subjects presented higher resting activities of GPx, Gr and SOD in muscle but not in plasma, in comparison with non-trained individuals [5].

Although regular training may lead to a raised serum uric acid levels [13, 27], which could contribute to a higher antioxidant defence of plasma [10], our athletes presented a non-statistically significant tendency to have smaller concentrations than their non-trained counterparts, as described by others [9]. Three months of intense training demonstrated to cause a diminishment in uric acid levels that was inversely correlated with VO_{2max} [36].

The lower uric acid levels seen in sportsmen could, at least partially, explain their lower TAS value in comparison with sedentary individuals, resembling the results obtained by other researchers [14, 15]. Other possible explanations include minor plasmatic concentrations of other antioxidant molecules not measured in the present work, as ascorbic acid, glutathione and bilirubin, and/or an increased ROS generation in kayakers due to the intense physical training program. Actually, it has been recognized that overloaded training reduces TAS [7] and that ex-athletes exhibit higher serum TAS than athletes [37]. It has also been described that sportsmen

did not significantly differ from controls for antioxidant capacity [30, 38], even when taking vitamins C and E supplements [35]. Yet, we can find studies describing significantly higher TAS in athletes [8, 10].

As mentioned above, we found a strengthened antioxidant defense in kayakers compared with sedentary subjects, as has already been shown for runners and soccer, basketball and rugby players [8-11, 31, 39]. However, other studies have evidenced a low [12, 14] or unchanged antioxidant status in sportsmen [40]. The increased levels of plasma antioxidant vitamins (α-tocopherol, β-carotene and α-carotene) and SOD activity observed in the present study were insufficient to counterbalance the rise in lipid peroxidation in kayakers and protect their muscles from being oxidative damaged. Although the authors have not statistically compared both groups, it is noteworthy that a higher SOD activity and a lower GPx activity in erythrocytes has already been described in Polish Olympic kayakers, albeit plasma TBARS levels were quite similar to those of the untrained subjects [41]. A higher basal lipoperoxidation in athletes than in non-trained subjects was also previously found in other studies [6, 12-14, 32, 38]. Although this may suggest that the stress imposed by strenuous training exceeds the higher individual's capacity to detoxify ROS, several published data failed to find a difference in the peroxidation status between athletic and non-athletic groups [2, 7-9, 24, 30, 34, 35], or even described a lower oxidant damage in the former [2, 4, 11, 15, 31]. The inconsistent results among studies which compared resting lipid peroxidation levels between trained and untrained individuals might be attributed to differences in the sports examined, the time athletes spent training, their training and nutritional status, and the biomarkers used to detect oxidative stress [2, 15]. Chang *et al*. [34] found higher conjugated dienes levels in rugby players than in control subjects, but similar values of TBARS. Despite TBARS is one of the most studied procedures to evaluate plasmatic pro-oxidant status, it has been criticized for the lack of specificity and sensitivity [2, 12], and it would have been preferable to determine isoprostane levels [14]. Those differences could also be related with the season in which the studies were carried out. Actually, diverse circannual rhythms of this lipid peroxidation marker for untrained and trained subjects were described [42]. Ice hockey players presented significantly lower TBARS in autumn compared to the sedentary controls, while in spring (the season the present study was done) the effect was the opposite [42]. The season with the highest TBARS concentrations was summer for athletes and sedentary men, which could explain the relative high concentration of TBARS that we observed in both groups. Furthermore, as the current study was performed near the end of the competitive period, the TBARS level could reflect an increase in lipid peroxidation through the season even though antioxidant status remain unchanged, as has already been observed in

American football players [43]. It was shown that the oxidant insult (superoxide anion release) only returned to pre-season levels 3 weeks after the end of competition in professional basketball players [44]. Thus, the lower lipoperoxides levels exhibited by soccer players in the work of Cazzola *et al*. [11] could be related to their competitive moment (pre-season). This is consistent with the data that demonstrate that intense periods of training induce a higher oxidative stress in rugby players [45] and triathletes [7]. Chronic high level exercise also evidenced to increase lipid peroxidation in cyclists [32], while 4 weeks of overloaded training in triathletes did not caused an increased TBARS levels in rest conditions, but lead to increased exercise-induce lipid peroxidation [7]. In the present work, athletes' TBARS levels were significantly elevated in comparison with controls subjects under resting conditions, indicating a potential basal oxidative stress caused by daily training sessions. The permanent oxidative stress during the competition period, although not clinically overt, could translate into harmful effects to sportsmen, such as muscle damage and soreness, since a ROS mechanism may be involved [9]. Actually, although the adaptation to training involves, to some extension, a degree of strengthening of antioxidant defense (as the increase of SOD activity in this study), an intensive training could cause muscle cells to lose membrane integrity, with loss of intramuscular enzymes, even in highly-trained athletes [7]. As it was expected from other studies' data [8, 9], resting plasma CK activity was significantly increased in sportsmen in comparison with controls.

CONCLUSIONS

In conclusion, we report that elite kayak paddlers exhibited augmented lipid peroxidation and muscle damage despite having higher plasma antioxidant capacity at rest (SOD, α-tocopherol, β-carotene and α-carotene) when compared to sedentary individuals. No difference in the dietary intake of those antioxidants was observed between groups.

ACKOWLEDEGMENTS

The authors would like to thank all the athletes and sedentary individuals for their voluntary participation in the study.

REFERENCES

1. Banerjee AK, Mandal A, Chanda D, Chakraborti S. Oxidant, antioxidant and physical exercise. Mol Cell Biochem. 2003; 253(1-2):307-12

2. Knez WL, Jenkins DG, Coombes JS. Oxidative stress in half and full Ironman triathletes. Med Sci Sports Exerc. 2007; 39(2):283-88

3. Warburton DER, Nicol CW, Bredin SSD. Health benefits of physical activity: The evidence. CMAJ. 2006; 174(6):801-09

4. Selamoglu S, Turgay F, Kayatekin BM, Gönenc S, Yslegen C. Aerobic and anaerobic training effects on the antioxidant enzymes of the blood. Acta Physiol Hung. 2000; 87(3):267-73

5. Ørtenblad N, Madsen K, Djurhuus MS. Antioxidant status and lipid peroxidation after short-term maximal exercise in trained and untrained humans. Am J Physiol. 1997; 272(4 Pt 2):R1258-63

6. Marzatico F, Pansarasa O, Bertorelli L, Somenzini L, Della Valle G. 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. 1997; 37(4):235-9

7. Palazzetti S, Richard MJ, Favier A, Margaritis I. Overloaded training increases exercise-induced oxidative stress and damage. Can J Appl Physiol. 2003; 28(4):588-604

8. Evelson P, Gambino G, Travacio M, Jaita G, Verona J, Maroncelli C, et al. Higher antioxidant defences in plasma and low density lipoproteins from rugby players. Eur J Clin Invest. 2002; 32(11):818-25

9. Robertson JD, Maughan RJ, Duthie GG, Morrice PC. Increased blood antioxidant systems of runners in response to training load. Clin Sci. 1991; 80(6):611-18

10. Brites FD, Evelson PA, Christiansen MG, Nicol MF, Basilico MJ, Wikinski RW, et al. Soccer players under regular training show oxidative stress but an improved plasma antioxidant status. Clin Sci (Lond). 1999; 96(4):381-5

11. Cazzola R, Russo-Volpe S, Cervato G, Cestaro B. Biochemical assessments of oxidative stress, erythrocyte membrane fluidity and antioxidant status in professional soccer players and sedentary controls. Eur J Clin Invest. 2003; 33(10):924-30

12. Balakrishnan SD, Anuradha CV. Exercise, depletion of antioxidants and antioxidant manipulation. Cell Biochem Funct. 1998; 16(4):269-75

13. Lekhi C, Gupta PH, Singh B. Influence of exercise on oxidant stress products in elite Indian cyclists. Br J Sports Med. 2007; 41(10):691-93

14. Gougoura S, Nikolaidis MG, Kostaropoulos IA, Jamurtas AZ, Koukoulis G, Kouretas D. Increased oxidative stress indices in the blood of child swimmers. Eur J Appl Physiol. 2007; 100(2):235-39

15. Watson TA, MacDonald-Wicks LK, Garg ML. Oxidative stress and antioxidants in athletes undertaking regular exercise training. Int J Sport Nutr Exerc Metab. 2005; 15(2):131-46

16. 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. 2004; 36(10):1329-41

17. Ballew C, Killingsworth R. Estimation of food and nutrient intakes of Athletes. In: Wolinsky I, Driskell J, editores. Nutritional Assessment of Athletes. London: CRC Press; 2002. p. 17-42.

18. Food and Nutrition Board, Institute of Medicine. Dietary reference intakes for vitamin C, vitamin E, selenium, and carotenoids. Washington, DC: National Academy Press; 2000.

19. Food and Nutrition Board, Institute of Medicine. Dietary Reference Intakes: Applications in Dietary Assessment. Washington, D.C.: National Academic Press; 2000.

20. Ainsworth BE, Haskell WL, Whitt MC, Irwin ML, Swartz AM, Strath SJ, et al. Compendium of physical activities: an update of activity codes and MET intensities. Med Sci Sports Exerc. 2000; 32(9 Suppl):S498-504

21. Thompson J, Manore M. Predicted and Measured Resting Metabolic Rate of Male and Female Endurance Athletes. J Am Diet Assoc. 1996; 96(1):30-34

22. Frisancho R. Anthropometric standards for the assessment of growth and nutritional status. Michigan: University of Michigan Press; 1990.

23. Siri W. Body composition from fluid spaces and density: analysis of methods. In: Brozek J, Henschel A, editores. Techniques for Measuring Body Composition. Washington, D.C: National Academy of Sciences-National Research Council; 1961. p. 223-44.

24. Rousseau AS, Hininger I, Palazzetti S, Faure H, Roussel AM, Margaritis I. Antioxidant vitamin status in high exposure to oxidative stress in competitive athletes. Br J Nutr. 2004; 92(3):461-8

25. Humphries B, Abt GA, Stanton R, Sly N. Kinanthropometric and physiological characteristics of outrigger canoe paddlers. J Sports Sci. 2000; 18(6):395-99

26. Traber MG, Jialal I. Measurement of lipid-soluble vitamins - Further adjustment needed? Lancet. 2000; 355(9220):2013-14

27. Finaud J, Lac G, Filaire E. Oxidative stress: Relationship with exercise and training. Sports Med. 2006; 36(4):327-58

28. Winklhofer-Roob BM, Van'T Hof MA, Shmerling DH. Reference values for plasma concentrations of vitamin E and A and carotenoids in a Swiss population from infancy to adulthood, adjusted for seasonal influences. Clin Chem. 1997; 43(1):146-53

29. Aguiló A, Tauler P, Guix MP, Villa G, Córdova A, Tur JA, et al. Effect of exercise intensity and training on antioxidants and cholesterol profile in cyclists. J Nutr Biochem. 2003; 14(6):319-25

30. Watson TA, MacDonald-Wicks LK, Garg ML. Regular exercise training does not elevate oxidative stress or deplete antioxidant defences. Asia Pacific J Clin Nutr. 2002; 11(SUPPL.)

31. Metin G, Atukeren P, Alturfan AA, Gulyasar T, Kaya M, Gumustas MK. Lipid peroxidation, erythrocyte superoxide-dismutase activity and trace metals in young male footballers. Yonsei Med J. 2003; 44(6):979-86

32. Mena P, Maynar M, Gutierrez JM, Maynar J, Timon J, Campillo JE. Erythrocyte free radical scavenger enzymes in bicycle professional racers. Adaptation to training. Int J Sports Med. 1991; 12(6):563-66

33. Oh-ishi S, Kizaki T, Nagasawa J, Izawa T, Komabayashi T, Nagata N, et al. Effects of endurance training on superoxide dismutase activity, content and mRNA expression in rat muscle. Clin Exp Pharmacol Physiol. 1997; 24(5):326-32

34. Chang CK, Tseng HF, Hsuuw YD, Chan WH, Shieh LC. Higher LDL oxidation at rest and after a rugby game in weekend warriors. Ann Nutr Metab. 2002; 46(3-4):103-07

35. Banfi G, Malavazos A, Iorio E, Dolci A, Doneda L, Verna R, et al. Plasma oxidative stress biomarkers, nitric oxide and heat shock protein 70 in trained elite soccer players. Eur J Appl Physiol. 2006; 96(5):483-86

36. Bergholm R, Mäkimattila S, Valkonen M, Liu ML, Lahdenperä S, Taskinen MR, et al. Intense physical training decreases circulating antioxidants and endothelium-dependent vasodilatation in vivo. Atherosclerosis. 1999; 145(2):341-9

37. García-Unzueta MT, Gutiérrez-Sánchez JB, de Mier I, Amado JA, Berrazueta JR. Autoantibodies against oxidized LDL and serum total antioxidant status in active cyclists and ex-cyclists. Horm Metab Res. 2003; 35(9):541-5

38. Santos-Silva A, Rebelo MI, Castro EMB, Belo L, Guerra A, Rego C, et al. Leukocyte activation, erythrocyte damage, lipid profile and oxidative stress imposed by high competition physical exercise in adolescents. Clin Chim Acta. 2001; 306(1-2):119-26

39. Pincemail J, Lecomte J, Castiau JP, Collard E, Vasankari T, Cheramy-Bien JP, et al. Evaluation of autoantibodies against oxidized LDL and antioxidant status in top soccer and basketball players after 4 months of competition. Free Radic Biol Med. 2000; 28(4):559-65

40. Akova B, Sürmen-Gür E, Gür H, Dirican M, Sarandöl E, Küçükoglu S. Exercise-induced oxidative stress and muscle performance in healthy women: role of vitamin E supplementation and endogenous oestradiol. Eur J Appl Physiol. 2001; 84(1-2):141-7

41. Wozniak A, Wozniak B, Drewa G, Mila-Kierzenkowska C. The effect of whole-body cryostimulation on the prooxidant - Antioxidant balance in blood of elite kayakers after training. Eur J Appl Physiol. 2007; 101(5):533-37

42. Balog T, Sobocanec S, Sverko V, Krolo I, Rocic B, Marotti M, et al. The influence of season on oxidant-antioxidant status in trained and sedentary subjects. Life Sci. 2006; 78(13):1441-7

43. Schippinger G, Wonisch W, Abuja PM, Fankhauser F, Winklhofer-Roob BM, Halwachs G. Lipid peroxidation and antioxidant status in professional American football players during competition. Eur J Clin Invest. 2002; 32(9):686-92

44. Benoni G, Bellavite P, Adami A, Chirumbolo S, Lippi G, Brocco G, et al. Changes in several neutrophil functions in basketball players before, during and after the sports season. Int J Sports Med. 1995; 16(1):34-37

45. Finaud J, Scislowski V, Lac G, Durand D, Vidalin H, Robert A, et al. Antioxidant status and oxidative stress in professional rugby players: evolution throughout a season. Int J Sports Med. 2006; 27(2):87-93

3.2 ANTIOXIDANT STATUS, OXIDATIVE STRESS AND DAMAGE AFTER A 1000M KAYAK SPRINT IN ELITE KAYAKERS

Teixeira VH[1], Valente H[1,2], Casal S[3], Marques F[4,5], Moreira P[1,2]

[1] Faculty of Nutrition and Food Sciences, University of Porto

[2] Research Centre in Physical Activity, Health and Leisure, University of Porto

[3] REQUIMTE, Bromatology Service, Faculty of Pharmacy, University of Porto

[4] Department of Clinical Analysis, Faculty of Pharmacy, University of Porto

[5] Institute for Molecular and Cell Biology – IBMC, University of Porto

SUBMITTED FOR PUBLICATION

ABSTRACT

Kayaking is a physically demanding exercise involving both anaerobic and aerobic metabolism which potentially could induce oxidative stress. The aim of this study was to determine the plasmatic response of antioxidants and biomarkers of lipid peroxidation, muscle damage and inflammation to a 1000m kayak trial in well-trained kayakers. Enzymatic (superoxide dismutase (SOD), glutathione reductase (Gr) and glutathione peroxidase (GPx) activities) and non-enzymatic (total antioxidant status (TAS), uric acid, α-tocopherol, α-carotene, β-carotene, lycopene and lutein + zeaxanthin) antioxidants, thiobarbituric acid reactive substances (TBARS), creatine kinase (CK), interleukin-6 (IL-6) and cortisol were determined in 21 elite kayakers (15 men) before and 15min after a simulated 1000m kayak race. Both enzymatic and non-enzymatic antioxidants were unaffected by exercise, with the exception of α-carotene (P = 0.013), in men, and lycopene (P = 0.003), in women. There was a tendency to decrease activities of GPx (P = 0.084), in women, and Gr (P = 0.069), in men, in post-exercise samples. Uric acid levels were incremented following exercise in all kayakers (P = 0.016, in men, and P = 0.034, in women). The acute exercise resulted in a decrease in TAS and in an augment in CK and TBARS, although only statistically significant in male athletes (P = 0.001, P = 0.023 and P < 0.001, respectively). After the bout, IL-6 rose significantly in men (P = 0.028), and cortisol tended to (P = 0.076) in women. Our study suggests that a 1000m kayak race induces oxidative stress and damage, eventually more markedly in male athletes.

INTRODUCTION

Davies *et al.* [1] were the first to report that skeletal muscle contractile activity produces reactive oxygen species (ROS). Since that pioneer work, the scientific evidence that associates exhaustive exercise with ROS generation, and subsequent macromolecule damage, has become increasingly consistent [2]. Because of their molecular instability, ROS are highly reactive and can oxidatively damage biomolecules, such as proteins, nucleic acids and, specially, lipids. Under normal physiologic conditions, ROS are rendered inactive by a complex antioxidant defence system, that includes antioxidant enzymes, such as catalase, SOD, GR and GPx, and non-enzymatic compounds, such as ascorbic acid, α-tocopherol, carotenoids, uric acid, thiols, coenzyme Q10, heat shock proteins, ferritin, albumin, caeruloplasmin, bilirubin and flavonoids [2]. However, exhaustive exercise can generate ROS to a level that overwhelms antioxidant defence system's capacity and imposes an acute cellular oxidative stress [3]. An increase in oxidative stress and damage has been demonstrated following both aerobic and/or anaerobic exercises of sufficient intensity [2]. The main ROS source in skeletal muscles during prolonged submaximal aerobic exercise appears to be the leakage from the mitochondrial electron transport chain [4, 5]. Exercise can generate ROS in anaerobic conditions by additional pathways, including catecholamine autoxidation, ischemic reperfusion conditions, phagocytic respiratory burst activity, prostanoid release, disturbance of intracellular calcium homeostasis, disruption of iron-containing proteins, xanthine and NADPH oxidase production, and lactate production [6-8].

Therefore, we have hypothesized that a 1000m kayak trial, in which both aerobic and anaerobic metabolism are relevant, may induce oxidative stress even in highly trained kayakers. The estimated relative average aerobic contribution for a simulated 1000m race effort was found to be 82.0 $\pm$ 5.0% (76.0-90.0%) in a work with male kayak paddlers of the USA National Team [9]. Glycolytic processes also play an important role in the energy supply, as evidenced by the elevated blood lactate concentrations at the end of trial (10.4 mmol/L) we have found in preliminary tests (data not published). To the best of our knowledge, this is the first study that has analysed the impact of acute kayak exercise on plasma oxidative and damage markers, since only the effect of chronic exercise (training) has been studied [10].

Although valuable, the majority of investigations on exercise-induced oxidative stress are performed under laboratory settings in untrained or recreationally active individuals, often involving exercise protocols unrealistic in sport. As this approach do not truly recreate the

conditions encountered by elite sportsmen during competition or training, the applicability of the findings to athletic populations is, somewhat, limited. Moreover, there is a paucity of information concerning oxidative stress in high-level athletes, who regularly perform strenuous exercise in their daily specific training sessions. Regular training induces a strengthening of enzymatic antioxidants defences and/or a diminished ROS production that, consequently, might attenuate the oxidative response to an acute exercise bout [2]. Actually, trained and non-trained individuals respond differently to the same exercise protocol [11].

The purpose of this investigation was to determine the response of enzymatic and non-enzymatic antioxidants, and markers of lipid peroxidation, muscle damage, inflammation and physiological stress to a 1000m kayak race in elite athletes.

MATERIAL AND METHODS

Subjects

Fifteen male and six female elite kayakers of the Portuguese National Senior and Junior Teams volunteered to participate in this study. Sportsmen were concentrated in the National Team Campus in order to be selected for the European Championship. The training regime was similar to all the participants and included resistance, endurance and kayaking skills training sessions. Athletes were asked to avoid intense physical activity during the day before testing to avoid acute effects of exercise. The last training was a short (50min) skills session 24 h prior the experiment. All sportsmen were healthy, non-smokers, and none was taking non-steroidal anti-inflammatory medication or antioxidants supplements for at least 3 months [12]. Subjects, or their parents if they were under 18 years old, signed an informed consent to participate in the study after the aims and procedures had been explained to them.

Exercise

The experience was carried out in the morning (between 08h30 and 09h00), with the participants in an overnight-fasted state, following a standardised low-intensity warm-up comprehending stretching (5 min) and gentle kayaking (10 min). The trial consisted of a 1000m flatwater kayaking race, where sportsmen were asked to cover the distance in the least time possible. On the day of the experiment, temperature was of 20 °C.

Dietary intake

Athletes completed food records intake for the four days prior to the exercise session. Subjects were asked to fully describe the foods and fluids consumed and estimate the amount ingested, by referring to the weight or volume information provided on food package or by using standardized household measures. Detailed verbal and written instructions regarding portion sizes and recording had been previously given by a trained nutritionist in food consumption methodology. Kayak paddlers were instructed not to do appreciable deviations from their habitual dietary intake, and to abstain from beverages with ethanol or caffeine for at least 24h prior to blood draws. Dietary records' information was converted to energy and nutrients using ESHA Food Processor 8.0 for Windows (Salem, OR, USA). Unfortunately, this package does not quantify α-carotene, lycopene, lutein and zeaxanthin intakes. The adequacy of nutritional intake was assessed by reference to the Food and Nutrition Board's Dietary Reference Intakes [13].

Body composition and anthropometry

Athletes' height and weight (lightly dressed and barefooted) were measured using a Seca stadiometer and electronic scale (model 701, UK), with a precision of 0.5cm and 100g respectively, by standard methods [14]. Body fat percentage was predicted from 4 skinfold thicknesses (bicipital, tricipital, subscapular, suprailiac) using a Harpenden caliper (John Bull, UK) [15].

Blood collection and handling

Blood samples (10mL) were collected by venous puncture from the antecubital vein with the subject seated, after an overnight fast (8-12h), both at rest and 15 min after finishing the kayak trial. No tourniquet constriction was used, to minimize potentially enhanced oxidative stress induced by an ischemia-reperfusion manoeuvre. Blood samples were drawn into EDTA-treated vacutainer tubes and non-additive serum vacutainer tubes and immediately placed on ice in the dark until centrifugation. An aliquot of whole blood was separated to measure hematocrit and haemoglobin. Whole blood in serum tubes was allowed to clot for 30min at room temperature and then centrifuged at 2000 rpm for 10 minutes for serum separation. To obtain the plasma fraction, the remaining whole blood in EDTA-containing tubes was immediately centrifuged. Erythrocytes were washed and centrifuged three times with a 0.9% sodium chloride solution and lysed with ice-cold distilled deionized water. Serum, plasma and

washed erythrocytes were separated into several aliquots and frozen at -80ºC for later biochemical analysis.

Even brief and intense exercises can induce hemoconcentration due to the shift of water to the active muscles [7, 16]. Therefore, all post-exercise variables were corrected for changes in plasma volume following the procedures of Dill and Costill [17]. Moreover, as eventual changes in erythrocyte number with exercise may also occur, SOD and GPx activities were expressed as U/g Hg. The pre-exercise analytical values were expressed without a correction for plasma volume changes. All assay procedures were performed in duplicate. Due to technical problems, ascorbic acid was not determined in the present work.

Biochemical analysis

Haemoglobin and hematocrit were assessed from EDTA-treated blood using an automated analyzer (Horiba ABX Micros 60, ABX Diagnostic, Montpellier, France).

The concentrations of cholesterol and triglycerides in plasma and lipoprotein subfractions were determined by enzymatic colorimetric assays (kit 07 3680 5 for triglycerides, and 07 3664 3 for cholesterol; Hoffman-La Roche, Basel, Switzerland) in an autoanalyzer (Cobas Mira; Hoffmann-La Roche). LDL cholesterol (LDL-C) was calculated by means of Friedewald equation.

Uric acid was determined by an enzymatic method at 550 nm using a commercial kit (Horiba ABX A11A01670, ABX diagnostic, Montpellier, France), according the manufacturer's specifications.

Serum total antioxidant status (TAS) was measured spectrophotometrically using a commercial kit (Randox NX2332, Randox, Crumlin, UK). In brief, the assay is based on the reduction of free radicals (2,2'-azino-di-(3-ethylbenzothiazoline-6-sulphonate – $ABTS^{\bullet+}$), measuring the decreased of absorbance at 600nm. The radical cation $ABTS^{\bullet+}$ is formed by the interaction of ABTS with ferrylmyoglobin radical species generated by the activation of metmyoglobin with hydrogen peroxide (H_2O_2). The suppression of the absorbance of the radical cation $ABTS^{\bullet+}$ by plasma antioxidants was compared with that from Trolox. Samples were expressed in antioxidant capacity in millimoles per liter of Trolox equivalents.

Enzyme activities were analyzed according to the standard spectrophotometric–colorimetric procedures provided with the commercial kits in a Cobas Mira Plus analyzer (Roche Diagnostic Systems, Switzerland), at 37°C. Whole-blood GPX activity was determined spectrophotometrically using cumene hydroperoxide as the oxidant of glutathione (GSH) (Ransel RS 505, Randox, Crumlin, UK). The oxidised glutathione (GSSG) is immediately reduced

to GSH by glutathione reductase, with a concomitant oxidation of NADPH to $NADP^+$. GPx activity was measure by the decrease in absorbance at 340 nm, and expressed in U/L. The plasmatic activity of Gr was measured by monitoring the oxidation of NADPH to $NADP^+$ during the reduction of GSSG (Ransel GR 2368; Randox, Crumlin, UK), and expressed in U/L. A value of 10 U/L was considered as the detection limit. Cu, Zn-SOD activity was measured in washed erythrocytes after lysis by using a commercial kit (Ransod SD 125, Randox, Crumlin, UK). For this purpose, xanthine and xanthine oxidase were used to generate superoxide anion which reacts with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye. SOD activity was calculated by assessing the extent of inhibition of the reaction, on the basis of the change in the absorbance over 3 min at 505 nm, and data from the standard curve generated from purified SOD obtained from the manufacturer. SOD activity was expressed in U/g Hb.

Plasma liposoluble vitamins were extracted with n-hexane, after protein precipitation with ethanol, using β-apo-carotenal and tocol as internal standards [(18)]. The chromatographic analyses were performed by HPLC (Jasco, Japan) on a Chromolith performance RP-18 column (100x3mm, Merck), using methanol and acetonitrile (50:50) as mobile phase. Lutein (co-eluting with zeaxanthin), α-carotene, β-carotene and the carotenoids internal standard (β-apo-8'-carotenal) were quantified at 450 nm, and lycopene at 425 nm, using a diode-array detector. Alpha-tocopherol quantification was based on the fluorescence readings (ex: 290 nm; em: 330 nm), using tocol as internal standard.

Serum creatine kinase (CK) and uric acid were determined at 37ºC using commercially available methods (Roche Products, UK), and an automated system (COBAS Mira Plus, Roche Diagnostic Systems, Switzerland).

The determination of thiobarbituric acid reactive substances (TBARS) in serum was performed using a commercial Kit (Oxi-tek TBARS assay kit, Zeptometrix Corporation, Buffalo, NY) according to the manufacturer's instructions. Briefly, serum (100 µL) was mixed with an equal volume of 8.1% sodium dodecyl sulfate (SDS) and 2.5 mL of 5% thiobarbituric acid/acetic acid reagent. Sample was incubated at 95ºC in capped tubes for 60 minutes and, thereafter, cooled to room temperature in an ice bath for 10 min before being centrifuged at 3000 rpm for 15 min. The supernatant was removed and its absorbance was read at 532 nm. The results are expressed as malondialdehyde (MDA) equivalents by interpolation from a MDA standard curve (0-100 nmol/mL).

Plasma IL-6 was measured with a commercially available solid-phase high-sensitivity ELISA Kit (Human Quantikine IL-6 Immunoassay D6050, R & D Systems, UK). The limit of detection of IL-6 was < 0.70 pg/mL.

Serum cortisol was measured with an ELFA (enzyme linked fluorescent assay - Vidas) technique using a commercial kit (cat. Nº30417, Biomerieux).

Data and statistical analysis

The values are presented as mean ± standard deviation. All the data were tested for their normal distribution with the one-sample Kolmogorov-Smirnov test. Significant differences between both pre- and post-exercise and female and male values were analyzed using t-tests. Pearson's correlation coefficients were determined to study the relationship between two variables. The SPSS 13.0 (Chicago, IL, USA) was used for all statistical analysis. Significance was set at $P < 0.05$ in the bilateral situation.

RESULTS

Kayakers' anthropometric characteristics and race times are presented in Table 1. Male athletes were heavier ($P < 0.001$), higher ($P < 0.001$), leaner ($P < 0.001$) and performed the 1000m kayaking trial faster (3:54.02 vs. 4:29.22) than their female counterparts. All kayak paddlers were of national to international level, with most of them being regularly selected to represent the National Senior and Junior Teams in Championships. All the participants had an average of 8.9 years of experience and have been regularly training and competing in regional and national kayaking events since then.

Table 1 Characteristics of the kayakers and race time

	Male kayakers (n=15)	Female kayakers (n=6)
Age (years)	20.3 ± 3.8	19.8 ± 3.9
Height (cm)	179.7 ± 5.3	165.4 ± 4.8
Weight (kg)	77.5 ± 6.6	64.2 ± 4.2
BMI (kg/m^2)	23.9 ± 1.4	23.2 ± 2.0
Body fat (%)	12.4 ± 3.1	24.9 ± 3.8
Race time	3:54.02 ± 0:05.77	4:29.22 ± 0:07.06

The estimated energy expenditure and energy and nutrient intakes of athletes during the 4 days before the exercise session are presented in Table 2. The estimated prevalences of inadequacy were 100% in both sexes, for α-tocopherol, 27% in men and 0% in women, for vitamin C, and 0% in men and 50% in women, for magnesium. The inadequacy prevalence was 0% for vitamin A, selenium, zinc, copper and iron, either in male and female subjects. There is probably a low prevalence of inadequacy for manganese in both genders, because their mean intakes were above the Adequate Intake reference.

Table 2. Estimated daily energy expenditure, and energy and nutrient intake of kayakers

	Male kayakers (n=15)	Female kayakers (n=6)
Energy expenditure (kcal)	3618 ± 281	2821 ± 182
Basal metabolism (kcal)	1990 ± 96	1558 ± 43
Physical Activity Level	1.82 ± 0.11	1.81 ± 0.16
Energy intake (kcal)	3455 ± 451	2321 ± 294
Proteins (g)	155 ± 18	109 ± 8
Proteins (% of energy intake)	18.0 ± 2.2	18.8 ± 1.8
Carbohydrates (g)	432 ± 84	311 ± 49
Carbohydrates (% of energy intake)	49.5 ± 3.8	53.1 ± 3.5
Fibre (g)	24.1 ± 4.8	20.1 ± 4.0
Fat (g)	124 ± 15	73 ± 13
Fat (% of energy intake)	32.4 ± 3.1	28.1 ± 2.6
Saturated fat (g)	42.3 ± 6.5	23.0 ± 3.5
Monounsaturated fat (g)	51.6 ± 6.2	30.1 ± 6.9
Polyunsaturated fat (g)	17.9 ± 3.8	11.6 ± 3.5
Cholesterol (mg)	436 ± 55	313 ± 30
Vitamin C (mg)	93.1 ± 33.3	84.3 ± 21.5
α-tocopherol (mg)	6.58 ± 1.78	6.50 ± 1.45
Vitamin A (RE μg)	1427 ± 529	1093 ± 229
β-carotene (μg)	1555 ± 917	1240 ± 689
Selenium (μg)	143 ± 18	117 ± 33
Zinc (mg)	22.5 ± 4.5	15.7 ± 1.5
Copper (mg)	1.77 ± 0.24	1.28 ± 0.19
Iron (mg)	23.2 ± 4.6	16.0 ± 3.2
Magnesium (mg)	402 ± 62	295 ± 61
Manganese (mg)	3.68 ± 1.16	3.01 ± 1.88

The exercise-induced alterations in haemoglobin, hematocrit, lipids and cholesterol profile are shown in Table 3. Despite plasma volume only decreased slightly after exercise (-1.3%), all plasma measurements following exercise were corrected for the effects of hemoconcentration, which has attenuated the changes. Plasma triglycerides, total cholesterol,

VLDL-cholesterol, HDL-cholesterol and LDL-cholesterol did not change with 1000m kayaking in both sexes.

Table 3. Effect of 1000m kayaking on lipoproteins and hematologic parameters of kayakers

	Male kayakers (n=15)			Female kayakers (n=6)		
	Before exercise	After exercise	*P*	Before exercise	After exercise	*P*
Total cholesterol (mg/dL)	152.6 ± 40.3	147.5 ± 28.1	NS	166.5 ± 44.0	156.1 ± 33.0	NS
VLDL-cholesterol (mg/dL)	8.5 ± 2.6	9.6 ± 3.4	NS	8.2 ± 4.3	8.4 ± 4.4	NS
LDL-cholesterol (mg/dL)	92.0 ± 32.0	86.0 ± 21.5	NS	103.0 ± 37.8	93.9 ± 29.1	NS
HDL-cholesterol (mg/dL)	52.0 ± 9.9	51.9 ± 9.4	NS	56.6 ± 7.8	54.1 ± 9.1	NS
Triglycerides (mg/dl)	42.7 ± 12.9	47.8 ± 17.2	NS	34.8 ± 22.2	40.9 ± 21.3	NS
Hemoglobin (g/dL)	15.4 ± 0.7	15.4 ± 0.6	NS	13.5 ± 0.8	13.7 ± 0.6	NS
Hematocrit (L/L)	48.2 ± 2.4	48.9 ± 2.5	NS	42.0 ± 2.4	43.0 ± 2.3	NS

NS: Non-significant

Enzymatic and non-enzymatic antioxidant capacity and markers of lipid peroxidation, muscle damage, inflammation and physiological stress at rest and after the exercise are expressed in Table 4. There was a reduction of TAS with exercise, but only with statistically significance in men (P = 0.001, for men, vs. P = 0.107, for women). The kayak trial caused an increase in uric acid concentrations in both sexes (P = 0.016, for men, and P = 0.034, for women). In comparison with men, female athletes exhibited a lower TAS value before exercise (P = 0.005), that could partially be explained by their diminished uric acid content (P = 0.010). There is no evidence of statistically significant differences between pre- and post-exercise conditions regarding antioxidant enzyme activities, in both groups. However, it was found a tendency to an exercise-induced reduction in the activity of GPx in women (P = 0.084) and Gr in men (P = 0.069). Interestingly, compared to male kayakers, sportswomen presented a higher GPx activity at rest (P = 0.025) and a lower GPX activity after the exercise (P = 0.006). There were no significant changes in plasmatic α-tocopherol (P = 0.243, for men, and P = 0.482, for women), β-carotene (P = 0.8296, for men, and P = 0.315, for women) and lutein + zeaxanthin (P = 0.957, for men, and P = 0.211, for women) concentrations after the kayak race. While α-carotene concentration did not significantly (P = 0.658) change in female kayakers, it decreased significantly (P = 0.013) in males by 19.2%. On the contrary, lycopene concentrations rose significantly (P = 0.003) in women with the exercise bout, although they diminished non-significantly in men (P = 0.176). Increases in CK (19%, P = 0.023) and TBARS (66%, P < 0.001) contents were observed 15min after the cessation of the kayak trial for male athletes, while for women the augmentations were not only smaller (10% for CK and 32% for

TBARS) but also non-significant (P = 0.082 for CK and P = 0.085 for TBARS). However, an erroneous and significant increment in CK with the 1000m trial (P = 0.021) was noted without the adjustment for hemoconcentration in women. IL-6 rose (34%, P = 0.028) in males after the experiment, but remained unchanged in female kayakers (P = 0.859). Cortisol concentrations did not exhibit any difference due to exercise either in male (P = 0.824) and female athletes (P = 0.076). We can speculate that a lack of statistical power due to the small size of the women group was responsible for the non-significant changes seen for cortisol (P = 0.076), GPx (P = 0.084), CK (P = 0.082) and TBARS (P = 0.085).

Table 4. Effect of 1000m kayaking on antioxidant status, lipid peroxidation, muscle damage, inflammation and physiological stress markers of kayakers

	Male kayakers (n=15)			Female kayakers (n=6)		
	Before exercise	After exercise	*P*	Before exercise	After exercise	*P*
TAS (mmol/L)	1.46 ± 0.16	1.13 ± 0.30	0.001	1.23 ± 0.12*****	1.03 ± 0.14	NS
Uric acid (mg/dL)	5.04 ± 0.89	5.42 ± 0.99	0.016	3.92 ± 0.52*****	4.29 ± 0.53*****	0.034
GPx (U/L)	34.9 ± 9.7	32.7 ± 3.7	NS	55.9 ± 30.8*****	25.8 ± 6.6*****	NS
SOD (U/gHg)	1246 ± 230	1244 ± 143	NS	1277 ± 105	1305 ± 85	NS
Gr (U/L)	73.5 ± 10.9	67.8 ± 15.4	NS	66.5 ± 8.1	64.0 ± 19.2	NS
α-tocopherol (mmol/L)	21.0 ± 4.1	22.9 ± 4.0	NS	23.1 ± 5.4	21.0 ± 2.2	NS
α-carotene (mmol/L)	0.22 ± 0.12	0.18 ± 0.10	0.013	0.22 ± 0.13	0.23 ± 0.12	NS
β-carotene (mmol/L)	0.44 ± 0.18	0.46 ± 0.26	NS	0.58 ± 0.27	0.43 ± 0.11	NS
Lycopene (mmol/L)	0.54 ± 0.27	0.46 ± 0.15	NS	0.55 ± 0.25	0.66 ± 0.30	0.003
Lutein + zeaxanthin (mmol/L)	0.39 ± 0.14	0.39 ± 0.15	NS	0.45 ± 0.17	0.44 ± 0.18	NS
Creatine kinase (IU/L)	256 ± 107	304 ± 85	0.023	156 ± 58*****	171 ± 69*****	NS
TBARS (mmol/L)	5.40 ± 1.43	9.00 ± 2.36	<0.001	5.95 ± 0.75	7.86 ± 2.03	NS
IL-6 (pg/mL)	1.80 ± 0.59	2.41 ± 0.69	0.028	1.60 ± 0.82	1.53 ± 0.46	NS
Cortisol (nmol/L)	188 ± 35	193 ± 73	NS	199 ± 88	255 ± 127	NS

***** Significantly different (P < 0.05) from males' values

DISCUSSION

To our knowledge, the effects of acute exercise on the redox status of kayakers have not been studied yet. Our data showed for the first time that a single kayak bout results in an impaired antioxidant capacity and an increased expression of lipid peroxidation, muscle damage and inflammation markers in plasma, more pronounced and only statistically significant in male athletes.

Due to the synergistic action of antioxidants it is useful to quantify the total ROS quenching capacity of plasma. The decrease in the plasma antioxidant status that we have found, despite being only significant in men, suggests that non-enzymatic antioxidants were strongly consumed during exercise. This lowering in antioxidant protection is consistent with the observed increased in oxidative damage in male athletes. Likewise, Watson *et al*. [19] have described a decline in antioxidant capacity after an incremental running test to exhaustion. Considering that TAS assay is an indicator of aqueous antioxidant defenses, which relies mostly on protein (10–28%), uric acid (7–58%) and ascorbic acid (3–27%) [19-21], the observed decrement immediately after kayak trial may reflect a reduction in ascorbic acid (unfortunately not measured herein), previously observed following acute short-term maximal exercises [20, 22, 23], since there was a significant increase in uric acid with exercise. The discrepancies between the published data regarding TAS can be explained by differences in data analysis. Some authors have described a rise in antioxidant capacity after anaerobic [24] or aerobic [25] protocols, but those differences lost their statistical significance after having been corrected for exercise-induced plasma volume shifts. Moreover, the type of exercise studied can impact differentially the usage or mobilization of the antioxidants pool. Actually, it were described significantly augmentations in TAS after exercise protocols involving ischemia-reperfusion conditions, either of isometric [26] or resistance nature [27], while in contrast aerobic bouts did not significantly change this parameter [26, 27]. The blood sampling time also seems relevant, since, for example, Steinberg and colleagues [20] found a significant decline in TAS 5min after the end of a cycling exercise until exhaustion that gave rise to an augment at the 20th min of the recovery period.

The increased post-exercise uric acid concentrations, which is found after aerobic [3] and anaerobic [16] exercises, provided they are sufficiently intense [2, 28], most likely resulted from an enhancement of energy-rich purine phosphates catabolism within skeletal muscle [16, 29]. The rise in uric acid may also result from the lactate-induced inhibition of its renal clearance [21, 26]. Xanthine oxidase, that catalyzes the conversion of hypoxanthine to uric acid, uses molecular oxygen as the electron acceptor, generating the superoxide anion as a by-product [3, 26]. Thus, the activation of xanthine oxidase might have a role in the oxidative damage found. On the other hand, uric acid is a potent antioxidant, acting by forming stable complexes with iron and copper ions and by directly scavenging ROS (hydroxyl radical, superoxide anion and singlet oxygen) [16]. Uric acid metabolism seems to be a double-edge sword. Baseline and post-exercise levels of uric acid were lower in women, which may have contributed to their lower

plasma antioxidant capacity compared to men, resembling the data of Kaikkonen and colleagues [30].

Decreases in α-tocopherol levels with exercise have been described [16, 31], due to its consumption or faster lipoprotein turnover, but also increases [3], explained by its mobilization from tissue storages, via *de novo* synthesis of VLDL, and release from adipose tissue concomitantly with triglycerides [3]. The stability of α-tocopherol concentrations after the kayaking trial in both sexes we observed is in accordance with other published data [8, 11], and consistent with the maintenance of triglycerides levels, since the exercise duration was not enough to significantly mobilize peripheral fat reserves as fuel as observed in longer exercises [3]. Moreover, the decrease in α-tocopherol induced by a sprint exercise [16] was restored to resting values within 20min of recovery (approximately the sampling time in our study). Plasmatic α-tocopherol, the most important fat-soluble chain-breaking antioxidant, appears to be well-regulated, namely by the liver, resulting in small [32]. Few works [3, 8, 31] investigated the effects of exercise on carotenoids other than β-carotene and none studied short aerobic exercise. The lack of change in carotenoids levels after the kayak trial we found is coincident with the maintenance of LDL, their main carrier, and is in agreement with other authors' findings [3]. Interestingly, the only published study about the effect of acute exercise on α-carotene [31] reported a drop just like we have observed in male athletes. The significant rise in lycopene in women was also noted in the work of Ramel *et al.* [8], but only before the correction for plasma volume changes.

Antioxidant enzyme activities were unchanged after the kayak race test in both genders. The data on enzymes' response to acute exercise is equivocal, with increases [33], decreases [34] and no changes [25] being published. The disagreements may result from the type of exercise, fitness levels of participants and timing of blood samples [34-36]. Elosua *et al*. [35] have shown diverse time course responses in GPx, Gr and SOD, which were further modified by a 16-week training period. The activity of GPx in female (P = 0.084) and Gr in male athletes (P = 0.069) revealed a tendency to decrease after exercise. The loss of enzyme activities was also found by other researchers [3, 34, 37], and may be attributable to ROS-stimulated proteolysis or oxidative damage of the enzymes' catalytic centre [3, 37]. The higher GPx in women at rest was already noticed [38], and could be related to its positive correlation with estrogen levels which, consequently, induces fluctuations on their activity across the menstrual cycle [39].

The augmented post-exercise TBARS we observed may reflect the efflux of peroxides from tissues, especially active muscle, into plasma, subsequent to ROS attack of polyunsaturated fatty acid in cell membranes. It has been consistently demonstrated that single bouts of

various types of exercise induce lipid peroxidation, including those mainly anaerobic (either isometric sessions [20, 23, 26] or running [4] and cycling sprints [40, 41]), aerobic (either of short- [20, 25, 41, 42] or long-duration [4, 5, 34, 43]) and mixed aerobic-anaerobic exercises [36, 41, 44]. Some data [4, 41] suggests that anaerobic or mixed exercises increase lipid peroxidation to a greater extent compared with aerobic exercises. Even though there were no significant gender based differences in lipid peroxidation at baseline, in agreement with other studies [12, 23, 35, 42], the TBARS' exercise-induced changes were greater (67% vs. 32%) and only significant ($P < 0.001$ vs. $P = 0.085$) in male athletes. In agreement, Cavas [33] only found post-exercise significantly increased lipid peroxidation levels in male players, while that of sportswomen remained stable. Higher TBARS values in men compared with women were described immediately after anaerobic and aerobic exercises [41]. Moreover, women's F_2-isoprostanes recovered to rest levels more rapidly after an ultramarathon than those of men [12]. Although we cannot exclude that the lack of statistical significance could result from the small number of women engaged in our study, it can also be alleged that women are more resistant to oxidative stress. One explanation for the observed gender difference in susceptibility to ROS lipid damage is that men have, habitually, more lean body tissue which leads to a higher metabolic rate and, consequently, to an increased oxygen mitochondrial flux and production of ROS [12]. Another possible explanation relies on the higher oestrogen levels female have in circulation [33], which are known to exhibit remarkable antioxidant properties, functioning as chain-breaking agents due to the presence of the phenolic group in the steroid structure [33, 45]. In light of this, it would have been interesting if we had measured sex hormones' levels, particularly because female athletes were, likely, evaluated at various stages of the menstrual cycle. Due to training program constraints, we could not schedule women's tests to a low oestrogen phase of their menstrual cycle. Interestingly, we found an inverse correlation between post-exercise TBARS and TAS levels in men ($r = -0.569$, $P = 0.027$).

Lipid peroxidation elicits an increase in the permeability of membranes resulting in the efflux of miocellular enzymes, such as CK, into plasma. However, no correlation between lipid peroxidation and muscle damage indices was detected in this study, in accordance with some [5, 40] but not all studies [43]. This scenario may indicate that mechanical factors, instead of oxidative damage, are the main contributors to muscle damage. We have found a slight increase in CK activity after kayaking, despite being only significant in men. Exercise-induced muscle injuries have been repeatedly described after short-term anaerobic exercises [6, 40, 46] and more prolonged protocols [5, 44]. The concentric nature of kayaking may help to explain the modest rise in CK we found, since it is established that eccentric contractions induce greater muscle injury than concentric ones [46]. Also, we were probably not able to detect the peak

activity of CK, that may continuously increase in the first hours after exercise [44], since it takes time to reach the circulation through the lymphatic system. Moreover, we studied well-trained athletes that were familiarized with the exercise, a condition which recognizably attenuates the muscle damage in subsequent bouts of the same exercise stimuli [11].

Exercise-induced muscle injury elicits an acute-phase local inflammatory response, modulated through the action of cytokines. IL-6 release, stimulated by the pro-inflammatory cytokines tumor necrosis factor α (TNF-α) and IL-1β, triggers the synthesis of anti-inflammatory cytokines (IL-1ra and IL-10) and acute-phase proteins [23]. This immune response plays a role in the repair and regeneration of damaged muscular tissue [46]. We found a slight rise (34%) in plasma IL-6 after exercise in men, while women's levels remained unchanged. The magnitude of the augment was, however, considerably lower than those described previously for anaerobic [46, 47] and short [23] and prolonged [48] aerobic exercise protocols. The discrepancies can rely on the duration and intensity of the exercise, the muscle mass recruited, the subjects' training status and the blood sampling time [23, 48]. Also, IL-6 increments are expected to be greater after eccentric than concentric exercises, as a result of a superior muscle injury [46]. Edwards *et al.* [48] were the first to report different IL-6 responses to exercise between sexes, with women exhibiting an augmented production. Estrogens might not explain the divergence, since similar IL-6 levels in both genders and throughout women's menstrual cycle have been demonstrated [49]. Our results were very close to support the hypothesis that subjects exhibiting greater cortisol responses to exercise, as female kayakers tended to (P = 0.072), will have blunted IL-6 responses [48], as they did (P = 0.859). This glucocorticoid might attenuate the inflammatory response in order to prevent damage to tissues [48]. In agreement with Willoughby *et al*. [46], the increase in cortisol was not related to differences in IL-6 levels, as had been suggested [50]. Since exercise activates the hypothalamic-pituitary-adrenal axis in an intensity dependent manner [51, 52], higher cortisol levels were seen in response to maximal than to submaximal exercises [48]. Augmented serum cortisol was described either after short duration anaerobic exercises [47] and prolonged triathlon races [53]. As cortisol is co-released, from the adrenal glands, along with ascorbic acid [5, 16, 54], we can speculate that this phenomena could have contributed, at least in part, to the women's minor TAS decline with the kayak trial.

CONCLUSIONS

In conclusion, our data indicate that a 1000m kayak trial induces oxidative stress and damage, as evidence by a decrease in TAS and increases in CK, TBARS and IL-6, of a superior degree in male athletes. Neither enzymatic nor non-enzymatic antioxidants were significantly changed with exercise.

ACKOWLEDEGMENTS

The authors would like to thank all the athletes for their voluntary participation in the study.

REFERENCES

1. Davies KJ, Quintanilha AT, Brooks GA. Free radicals and tissue damage produced by exercise. Biochem Biophys Res Commun. 1982; 107(4):1198-205
2. Finaud J, Lac G, Filaire E. Oxidative stress: Relationship with exercise and training. Sports Med. 2006; 36(4):327-58
3. Aguiló A, Tauler P, Fuentespina E, Tur JA, Córdova A, Pons A. Antioxidant response to oxidative stress induced by exhaustive exercise. Physiol Behav. 2005; 84(1):1-7
4. Marzatico F, Pansarasa O, Bertorelli L, Somenzini L, Della Valle G. 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. 1997; 37(4):235-9
5. Child RB, Wilkinson DM, Fallowfield JL, Donnelly AE. Elevated serum antioxidant capacity and plasma malondialdehyde concentration in response to a simulated half-marathon run. Med Sci Sports Exerc. 1998; 30(11):1603-7
6. Bloomer RJ, Falvo MJ, Fry AC, Schilling BK, Smith WA, Moore CA. Oxidative stress response in trained men following repeated squats or sprints. Med Sci Sports Exerc. 2006; 38(8):1436-42

7. Groussard C, Rannou-Bekono F, Machefer G, Chevanne M, Vincent S, Sergent O, et al. Changes in blood lipid peroxidation markers and antioxidants after a single sprint anaerobic exercise. Eur J Appl Physiol. 2003; 89(1):14-20

8. Ramel A, Wagner KH, Elmadfa I. Correlations between plasma noradrenaline concentrations, antioxidants, and neutrophil counts after submaximal resistance exercise in men. Br J Sports Med. 2004; 38(5):E22

9. Byrnes WC, Kearney JT. Aerobic and anaerobic contributions during simulated canoe/kayak sprint events 1256. Med Sci Sports Exerc. 1997; 29(5):220

10. Wozniak A, Drewa G, Chesy G, Rakowski A, Rozwodowska M, Olszewska D. Effect of altitude training on the peroxidation and antioxidant enzymes in sportsmen. Med Sci Sports Exerc. 2001; 33(7):1109-13

11. Ørtenblad N, Madsen K, Djurhuus MS. Antioxidant status and lipid peroxidation after short-term maximal exercise in trained and untrained humans. Am J Physiol. 1997; 272(4 Pt 2):R1258-63

12. 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. 2004; 36(10):1329-41

13. Food and Nutrition Board, Institute of Medicine. Dietary Reference Intakes: Applications in Dietary Assessment. Washington, D.C.: National Academic Press; 2000.

14. Frisancho R. Anthropometric standards for the assessment of growth and nutritional status. Michigan: University of Michigan Press; 1990.

15. Siri W. Body composition from fluid spaces and density: analysis of methods. In: Brozek J, Henschel A, editores. Techniques for Measuring Body Composition. Washington, D.C: National Academy of Sciences-National Research Council; 1961. p. 223-44.

16. Groussard C, Machefer G, Rannou F, Faure H, Zouhal H, Sergent O, et al. Physical fitness and plasma non-enzymatic antioxidant status at rest and after a wingate test. Can J Appl Physiol. 2003; 28(1):79-92

17. Dill DB, Costill DL. Calculation of percentage changes in volumes of blood, plasma, and red cells in dehydration. J Appl Physiol. 1974; 37(2):247-8

18. Rousseau AS, Hininger I, Palazzetti S, Faure H, Roussel AM, Margaritis I. Antioxidant vitamin status in high exposure to oxidative stress in competitive athletes. Br J Nutr. 2004; 92(3):461-8

19. Watson TA, Callister R, Taylor RD, Sibbritt DW, MacDonald-Wicks LK, Garg ML. Antioxidant restriction and oxidative stress in short-duration exhaustive exercise. Med Sci Sports Exerc. 2005; 37(1):63-71

20. Steinberg JG, Delliaux S, Jammes Y. Reliability of different blood indices to explore the oxidative stress in response to maximal cycling and static exercises. Clin Physiol Funct Imaging. 2006; 26(2):106-12

21. Ascensão A, Ferreira R, Marques F, Oliveira E, Azevedo V, Soares J, et al. Effect of off-road competitive motocross race on plasma oxidative stress and damage markers. Br J Sports Med. 2007; 41(2):101-05

22. Quindry JC, Stone WL, King J, Broeder CE. The effects of acute exercise on neutrophils and plasma oxidative stress. Med Sci Sports Exerc. 2003; 35(7):1139-45

23. Steinberg JG, Ba A, Brégeon F, Delliaux S, Jammes Y. Cytokine and oxidative responses to maximal cycling exercise in sedentary subjects. Med Sci Sports Exerc. 2007; 39(6):964-68

24. Ihara H, Shino Y, Morita Y, Kawaguchi E, Hashizume N, Yoshida M. Is skeletal muscle damaged by the oxidative stress following anaerobic exercise? J Clin Lab Anal. 2001; 15(5):239-43

25. Vider J, Lehtmaa J, Kullisaar T, Vihalemm T, Zilmer K, Kairane C, et al. Acute immune response in respect to exercise-induced oxidative stress. Pathophysiology. 2001; 7(4):263-70

26. Magalhães J, Ferreira R, Marques F, Olivera E, Soares J, Ascensão A. Indoor climbing elicits plasma oxidative stress. Med Sci Sports Exerc. 2007; 39(6):955-63

27. Vincent HK, Morgan JW, Vincent KR. Obesity Exacerbates Oxidative Stress Levels after Acute Exercise. Med Sci Sports Exerc. 2004; 36(5):772-79

28. Green HJ, Fraser IG. Differential effects of exercise intensity on serum uric acid concentration. Med Sci Sports Exerc. 1988; 20(1):55-59

29. Westing Y, Ekblom B, Sjödin B. The metabolic relation between hypoxanthine and uric acid in man following maximal short-distance running. Acta Physiol Scand. 1989; 137(3):341-45

30. Kaikkonen J, Porkkala-Sarataho E, Tuomainen TP, Nyyssönen K, Kosonen L, Ristonmaa U, et al. Exhaustive exercise increases plasma/serum total oxidation resistance in moderately trained men and women, whereas their VLDL + LDL lipoprotein fraction is more susceptible to oxidation. Scand J Clin Lab Invest. 2002; 62(8):599-608

31. Oostenbrug GS, Mensink RP, Hardeman MR, De Vries T, Brouns F, Hornstra G. Exercise performance, red blood cell deformability, and lipid peroxidation: effects of fish oil and vitamin Eur J Appl Physiol. 1997; 83(3):746-52

32. Hütler M, Woweries S, Leithäuser R, Böning D, Beneke R. Exercise-induced changes in blood levels of α-tocopherol. Eur J Appl Physiol. 2001; 85(1-2):151-56

33. Cavas L. Does underwater rugby stimulate the over-production of reactive oxygen species? Cell Biochem Funct. 2005; 23(1):59-63

34. Knez WL, Jenkins DG, Coombes JS. Oxidative stress in half and full Ironman triathletes. Med Sci Sports Exerc. 2007; 39(2):283-88

35. Elosua R, Molina L, Fito M, Arquer A, Sanchez-Quesada JL, Covas MI, et al. Response of oxidative stress biomarkers to a 16-week aerobic physical activity program, and to acute physical activity, in healthy young men and women. Atherosclerosis. 2003; 167(2):327-34

36. Chang CK, Tseng HF, Hsuuw YD, Chan WH, Shieh LC. Higher LDL oxidation at rest and after a rugby game in weekend warriors. Ann Nutr Metab. 2002; 46(3-4):103-07

37. Hessel E, Haberland A, Müller M, Lerche D, Schimke I. Oxygen radical generation of neutrophils: A reason for oxidative stress during marathon running? Clin Chim Acta. 2000; 298(1-2):145-56

38. Rush JWE, Sandiford SD. Plasma glutathione peroxidase in healthy young adults: Influence of gender and physical activity. Clin Biochem. 2003; 36(5):345-51

39. Massafra C, Gioia D, De Felice C, Picciolini E, De Leo V, Bonifazi M, et al. Effects of estrogens and androgens on erythrocyte antioxidant superoxide dismutase, catalase and glutathione peroxidase activities during the menstrual cycle. J Endocrinol. 2000 167(3):447-52

40. Baker JS, Bailey DM, Hullin D, Young I, Davies B. Metabolic implications of resistive force selection for oxidative stress and markers of muscle damage during 30 s of high-intensity exercise. Eur J Appl Physiol. 2004; 92(3):321-7

41. Ilhan N, Kamanli A, Ozmerdivenli R, Ilhan N. Variable effects of exercise intensity on reduced glutathione, thiobarbituric acid reactive substance levels, and glucose concentration. Arch Med Res. 2004; 35(4):294-300

42. Jammes Y, Steinberg JG, Bregéon F, Delliaux S. The oxidative stress in response to routine incremental cycling exercise in healthy sedentary subjects. Respir Physiol Neurobiol. 2004; 144(1):81-90

43. Kanter MM, Lesmes GR, Kaminsky LA, La Ham-Saeger J, Nequin ND. Serum creatine kinase and lactate dehydrogenase changes following an eighty kilometer race. Relationship to lipid peroxidation. Eur J Appl Physiol. 1988; 57(1):60-63

44. Thompson D, Williams C, Kingsley M, Nicholas CW, Lakomy HK, McArdle F, et al. Muscle soreness and damage parameters after prolonged intermittent shuttle-running following acute vitamin C supplementation. Int J Sports Med. 2001; 22(1):68-75

45. Akova B, Sürmen-Gür E, Gür H, Dirican M, Sarandöl E, Küçükoglu S. Exercise-induced oxidative stress and muscle performance in healthy women: role of vitamin E supplementation and endogenous oestradiol. Eur J Appl Physiol. 2001; 84(1-2):141-7

46. Willoughby DS, Vanenk C, Taylor L. Effects of concentric and eccentric contractions on exercise-induced muscle injury, inflammation, and serum IL-6. J Exerc Physiol Online. 2003; 6(4):8-15

47. Meyer T, Gabriel HH, Rätz M, Müller HJ, Kindermann W. Anaerobic exercise induces moderate acute phase response. Med Sci Sports Exerc. 2001; 33(4):549-55

48. Edwards KM, Burns VE, Ring C, Carroll D. Individual differences in the interleukin-6 response to maximal and submaximal exercise tasks. J Sports Sci. 2006; 24(8):855-62

49. O'Brien SM, Fitzgerald P, Scully P, Landers AMT, Scott LV, Dinan TG. Impact of gender and menstrual cycle phase on plasma cytokine concentrations. NeuroImmunoModulation. 2007; 14(2):84-90

50. Steensberg A, Fischer CP, Keller C, Møller K, Pedersen BK. IL-6 enhances plasma IL-1ra, IL-10, and cortisol in humans. Am J Physiol Endocrinol Metab. 2003; 285(2 48-2):E433-E37

51. Singh A, Papanicolaou DA, Lawrence LL, Howell EA, Chrousos GP, Deuster PA. Neuroendocrine responses to running in women after zinc and vitamin E supplementation. Med Sci Sports Exerc. 1999; 31(4):536-42

52. Schröder H, Navarro E, Mora J, Galiano D, Tramullas A. Effects of alpha-tocopherol, beta-carotene and ascorbic acid on oxidative, hormonal and enzymatic exercise stress markers in habitual training activity of professional basketball players. Eur J Nutr. 2001; 40(4):178-84

53. McAnulty SR, McAnulty LS, Nieman DC, Morrow JD, Shooter LA, Holmes S, et al. Effect of alpha-tocopherol supplementation on plasma homocysteine and oxidative stress in highly trained athletes before and after exhaustive exercise. J Nutr Biochem. 2005; 16(9):530-7

54. Davison G, Gleeseon M. Influence of acute vitamin C and/or carbohydrate ingestion on hormonal, cytokine, and immune responses to prolonged exercise. Int J Sport Nutr Exerc Metab. 2005; 15(5):465-79

3.3 ANTIOXIDANT STATUS, OXIDATIVE STRESS AND DAMAGE IN ELITE KAYAKERS AFTER 1 YEAR OF TRAINING AND COMPETITION IN TWO SEASONS

Teixeira VH[1], Valente H[1,2], Pereira L[3], Casal S[3], Marques F[4,5], Moreira P[1,2]

[1] Faculty of Nutrition and Food Sciences, University of Porto

[2] Research Centre in Physical Activity, Health and Leisure, University of Porto

[3] REQUIMTE, Bromatology Service, Faculty of Pharmacy, University of Porto

[4] Department of Clinical Analysis, Faculty of Pharmacy, University of Porto

[5] Institute for Molecular and Cell Biology – IBMC, University of Porto

SUBMITTED FOR PUBLICATION

ABSTRACT

Strenuous exercise can generate reactive oxygen species (ROS) to a level that surpasses the strengthened antioxidant defences of athletes. Due to the large volume of training performed regularly by elite sportsmen throughout the season, these acute effects can translate into a chronic oxidative insult. Therefore, we determined markers of oxidative stress, muscle damage and inflammation in well-trained kayakers in two different periods of the same season and in two different seasons, in order to study the effects that chronically high training loads have on athletes' redox status. Superoxide dismutase (SOD), glutathione reductase (Gr), glutathione peroxidase (GPx) and creatine kinase (CK) activities, and total antioxidant status (TAS), uric acid, retinol, α-tocopherol, α-carotene, β-carotene, lycopene, lutein + zeaxanthin, vitamin C, thiobarbituric acid reactive substances (TBARS), interleukin-6 (IL-6) and cortisol levels were determined in 9 kayakers (6 men) in a competitive period of the first season (June, T1), and in a pre-competitive (March, T2) and a competitive period (June, T3) of the following season. There were no significant differences between male and female kayakers concerning the above-mentioned parameters, except for α-tocopherol (P = 0.041), CK (P = 0.044) and uric acid (P = 0.049) in T1, TAS (P = 0.007) and uric acid (P = 0.015) in T2, and CK (P = 0.041), α-carotene (P = 0.020) and uric acid (P = 0.003) in T3. Taking the entire sample in consideration, TAS diminished from the first (T1) to the second season (T1 vs. T2, $P < 0.001$; T1 vs. T3, $P < 0.001$). Alpha-tocopherol augmented (P = 0.001) and TBARS (P = 0.024) declined from T1 to T2. GPx (P = 0.003) increased and SOD ($P < 0.001$) and uric acid (P = 0.032) decreased from T2 to

T3. Cortisol levels declined significantly throughout the study (T1 vs. T2, *P* = 0.042; T2 vs. T3, *P* = 0.018; T1 vs. T3, *P* = 0.002). No significant differences were observed for all the other parameters studied during the course of this work. Antioxidant status changed more within the same season than from one season to another. Redox markers should be monitored throughout the season to detect athletes at an increased oxidative risk.

INTRODUCTION

Acute exercise is known to increase the generation of reactive oxygen species (ROS) and inflammation which are involved in the aetiology of several chronic diseases [(1)]. However, there is convincing evidence that regular physical activity reduces the incidence of some oxidative stress-associated diseases, like cardiovascular disease, rheumatic arthritis, neurodegenerative diseases and certain cancers [(2)]. It was suggested that the health benefits of exercise may be associated with reduced levels of inflammation and oxidative stress [(3)]. Regular training has been claimed to reinforce the antioxidant potential, as it causes an enhancement in the activity of antioxidant enzymes [(4, 5)], and, consequently, augment the resistance to oxidative stress [(2)]. The permanent production of low doses of ROS induced by regular moderate exercise appears to play a role in the training-induced beneficial adaptations. Actually, hindering their formation could hamper the adaptations to exercise [(1)]. However, an increment in ROS that surpasses the antioxidant defence capacity can cause oxidative stress and damage. If moderate exercise can be regarded as an antioxidant, the beneficial effects seem to be lost when it is exhaustive [(1)], resembling the hormesis theory [(2)].

Enrolment in aerobic physical activity training appears to be beneficial for previously sedentary individuals, as it increases antioxidant potential and decreases oxidative stress [(4)]. In return, intense physical training seems to result in a decline in circulating antioxidants in moderate- and well-trained athletes [(6, 7)]. Curiously, Groussard and colleagues [(8)] found that athletes who exhibited a higher performance were those with the lowest antioxidant status. In accordance, high level sportsmen following a regular training programme presented diminished antioxidant capacities in comparison with matched sedentary controls [(9)]. Thus, it is likely that the large volume of training performed by elite sportsmen could lead to a chronic oxidative stress state, particularly if is not accompanied by an increase in antioxidant dietary intake, which is sometimes inadequate within the athletic population [(10-14)]. Physical exercise can be considered a double-edged sword, *i.e.*, if practiced in moderation it can induce a

strengthening in antioxidant capacity, but if strenuous enough it can cause oxidative damage to cellular molecules perturbing their homeostasis [1].

In order to have a performance at a high standard, elite paddlers should have high aerobic and anaerobic capabilities, good technical skills and great upper body strength [15]. To achieve such physical demands, athletes must undertake heavy training loads, involving on-water, resistance and endurance training sessions. Thus, training for kayaking can lead to increased ROS generation through diverse pathways, such as leakage from mitochondrial electron transport chain, transient hypoxia and reoxygenation, raised intracellular calcium concentrations, activated invading leukocytes or elevation in the activities of cyclooxygenase, nitric oxide synthase and/or xanthine oxidase [7].

Therefore, our aim was to investigate longitudinally the effects of intense exercise training on antioxidant status and lipoperoxidative and muscle damage in elite sportsmen. In order to study how long-term exercise affects athletes' redox status, we evaluated them in two different periods (pre-competitive, in March, and competitive, in June) of the same season and in two competitive periods (June) of two different seasons.

MATERIAL AND METHODS

Subjects

Data were collected on 9 members (6 men and 3 women) of the Portuguese Kayaking Team regularly attending training in National Campus. All athletes were healthy, non-smokers and reported no use of non-steroidal anti-inflammatory drugs and antioxidants supplements for at least the 3 months prior to the blood collections. The experimental procedures were approved by the Scientific Committee of the Faculty of Nutrition and Food Sciences at the University of Porto. Subjects, or their parents, were informed about the purpose and procedures of the experiment and provided written consent prior to enrolment in the study.

Study design

The experience was performed in June, within the period of competition, in two consecutive seasons (T1 and T3, respectively), and in March, a pre-competitive training period (T2). This specific preparatory phase precede the competitive period of the second season,

from May until the end of August, when the volume and intensity of training differed. At those three moments, kayak paddlers were concentrated in the National Team Campus and followed the training regime set by the National Coach.

The present research was planned to not interfere with the training programmed. However, blood collections were scheduled for the day after (~24h) a standardized short-duration (50min) technical session, in each one of the 3 moments studied (T1 to T3), after which athletes were asked to refrain from intense physical activity until blood sampling.

In the T1 and T2 moments, athletes were anthropometrically evaluated and were asked to record their dietary intake and physical activities in diaries over the four days prior to each measurement. An experienced nutritionist gave detailed verbal explanations and written instructions concerning recording tasks. After recording, diet and activity dairies were reviewed individually to ensure completeness and resolve ambiguities.

Dietary intake

Subjects were asked to fully describe the foods and fluids consumed and record their commercial name whenever possible. They were persuaded to be as accurate as possible in estimating the amount of food consumed, by recording the information on quantity displayed on package or by using standardized household measures. Sportsmen were instructed to maintain their habitual dietary intake and to abstain from alcoholic or caffeinated beverages for at least 24h prior to blood draws. Dietary records' information was converted to nutrients using ESHA Food Processor 8.0 for Windows (Salem, OR, USA), which does not allow the quantification of α-carotene, lycopene, lutein and zeaxanthin intakes. The Food and Nutrition Board's Dietary Reference Intakes were used to evaluate the adequacy of athletes' nutritional intake [(16)]. These recommendations do not take into account that micronutrients requirements might be higher for sportsmen than for sedentary individuals [(17)].

Physical activity and estimated energy expenditure

Subjects recorded all their physical activities in 15-min intervals throughout the day with the exception of the training sessions, whose information about the duration and intensity was given by their coach and enabled us to estimate the training volume (hours in 4 days). The daily energy expenditure was calculated as the sum of energy expenditure for each activity,

obtained by multiplying the amount of time spent in that activity by the corresponding metabolic equivalents [18]. The ratio of total energy expenditure energy expenditure on basal metabolic rate, calculated with the Cunningham equation [19], corresponds to the Physical Activity Level.

Body composition and anthropometry

Kayakers' height and weight (lightly dressed and barefooted) were measured using a calibrated Seca stadiometer and electronic scale (model 701, UK), with a precision of 0.5cm and 100g respectively, by standard methods [20]. Thereafter, body mass index (BMI) was calculated. Siri's formula [21] was used to estimate the body fat percentage, after measuring the skinfold thicknesses at four sites (bicep, tricep, subscapula and iliac crest) using a Harpenden caliper (John Bull, UK).

Blood collection and handling

Venous blood samples were drawn from the antecubital vein by a trained phlebotomist in the morning, between 08h30 and 09h00, after an overnight fast (8-12 hours), with athletes in resting conditions. In order to minimize potentially enhanced oxidative stress induced by ischemia-reperfusion mechanism, no tourniquet constriction was used. Blood samples were drawn into using EDTA-treated vacutainer tubes and non-additive serum vacutainer tubes and immediately placed on ice in the dark until centrifugation. An aliquot of whole blood was separated to measure hematocrit and haemoglobin. Whole blood in serum tubes was allowed to clot for 30min at room temperature and then centrifuged at 2000 rpm for 10 minutes for serum separation. To obtain the plasma fraction, the remaining whole blood in EDTA-containing tubes was immediately centrifuged. Erythrocytes were washed and centrifuged three times with a 0.9% sodium chloride solution and lysed with ice-cold distilled deionized water. Serum, plasma and washed erythrocytes were separated into several aliquots and frozen at -80ºC for later biochemical analysis. All assay procedures were performed in duplicate. Due to the technical problems, vitamin C was not measured in T2 and T3 moments.

Biochemical analysis

Haemoglobin and hematocrit were assessed from EDTA-treated blood using an automated analyzer (Horiba ABX Micros 60, ABX Diagnostic, Montpellier, France).

Uric acid was determined by an enzymatic method at 550 nm using a commercial kit (Horiba ABX A11A01670, ABX diagnostic, Montpellier, France), according to the manufacturer's specifications.

Serum total antioxidant status (TAS) was measured spectrophotometrically using a commercial kit (Randox NX2332, Randox, Crumlin, UK). In brief, the assay is based on the reduction of free radicals (2,2'-azino-di-(3-ethylbenzothiazoline-6-sulphonate – $ABTS^{\bullet+}$), measuring the decrease of absorbance at 600nm. The radical cation $ABTS^{\bullet+}$ is formed by the interaction of ABTS with ferrylmyoglobin radical species generated by the activation of metmyoglobin with hydrogen peroxide (H_2O_2). The suppression of the absorbance of the radical cation $ABTS^{\bullet+}$ by plasma antioxidants was compared with that from Trolox. Samples were expressed in antioxidant capacity in millimoles per liter of Trolox equivalents.

Enzyme activities were analyzed according to the standard spectrophotometric–colorimetric procedures provided with the commercial kits in a Cobas Mira Plus analyzer (Roche Diagnostic Systems, Switzerland), at 37°C. Whole-blood GPX activity was determined spectrophotometrically using cumene hydroperoxide as the oxidant of glutathione (GSH) (Ransel RS 505, Randox, Crumlin, UK). The oxidised glutathione (GSSG) is immediately reduced to GSH by glutathione reductase, with a concomitant oxidation of NADPH to $NADP^+$. GPx activity was measured by the decrease in absorbance at 340 nm, and expressed in U/L. The plasmatic activity of Gr was measured by monitoring the oxidation of NADPH to $NADP^+$ during the reduction of GSSG (Ransel GR 2368; Randox, Crumlin, UK), and expressed in U/L. A value of 10 U/L was considered as the detection limit. Cu, Zn-SOD activity was measured in washed erythrocytes after lysis using a commercial kit (Ransod SD 125, Randox, Crumlin, UK). For this purpose, xanthine and xanthine oxidase were used to generate superoxide anion which reacts with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye. SOD activity was calculated by assessing the extent of inhibition of the reaction, on the basis of the change in the absorbance over 3 min at 505 nm, and data from the standard curve generated from purified SOD obtained from the manufacturer. SOD activity was expressed in U/g Hb.

Plasma liposoluble vitamins were extracted with n-hexane, after protein precipitation with ethanol, using β-apo-carotenal and tocol as internal standards [(22)]. The chromatographic analyses were performed by HPLC (Jasco, Japan) on a Chromolith performance RP-18 column (100x3mm, Merck), using methanol and acetonitrile (50:50) as mobile phase. Lutein (co-eluting with zeaxanthin), α-carotene, β-carotene and the carotenoids internal standard (β-apo-8'-

carotenal) were quantified at 450 nm, retinol at 325 nm and lycopene at 425 nm, using a diode-array detector. Alpha-tocopherol quantification was based on the fluorescence readings (ex: 290 nm; em: 330 nm), using tocol as internal standard.

After deproteinization with metaphosphoric acid (0.75 M), serum vitamin C was determined by atomic absorption spectrometry (AAS), at 520nm using a Perkin Elmer Lambda 2 UV-Visible Spectrophotometer.

Serum creatine kinase (CK) and uric acid were determined at 37ºC using commercially available methods (Roche Products, UK), and an automated system (COBAS Mira Plus, Roche Diagnostic Systems, Switzerland).

The determination of thiobarbituric acid reactive substances (TBARS) in serum was performed using a commercial Kit (Oxi-tek TBARS assay kit, Zeptometrix Corporation, Buffalo, NY) according to the manufacturer's instructions. Briefly, serum (100 µL) was mixed with an equal volume of 8.1% sodium dodecyl sulfate (SDS) and 2.5 mL of 5% thiobarbituric acid/acetic acid reagent. Sample was incubated at 95ºC in capped tubes for 60 minutes and, thereafter, cooled to room temperature in an ice bath for 10 min before being centrifuged at 3000 rpm for 15 min. The supernatant was removed and its absorbance was read at 532 nm. The results are expressed as malondialdehyde (MDA) equivalents by interpolation from a MDA standard curve (0-100 nmol/mL).

Plasma IL-6 was measured with a commercially available solid-phase high-sensitivity ELISA Kit (Human Quantikine IL-6 Immunoassay D6050, R & D Systems, UK). The limit of detection of IL-6 was < 0.70 pg/mL.

Serum cortisol was measured with an ELFA (enzyme linked fluorescent assay - Vidas) technique using a commercial kit (cat. Nº30417, Biomerieux).

Data and statistical analysis

Values are expressed as mean ± standard deviations. All results were tested for their normal distribution with the one-sample Kolmogorov-Smirnov test. Significant differences in anthropometric and nutritional parameters between the competitive periods were analyzed using t-tests. Pearson's correlation coefficients were determined to study the relationship between two variables. Biochemical data were analysed using General Linear Model with Repeated Measures. Where significant main effects were observed, Bonferroni multiple

comparisons procedures were used to determine differences between the time-points. All statistical analysis was performed using SPSS 13.0 (Chicago, IL, USA). The level of significance was set at $P < 0.05$, in the bilateral situation.

RESULTS

The training program over the 4 days of the first competitive period studied (T1) consisted of 9 sessions, lasting 450min, comprising on-water (n=5, 52km, 285min), weight training (n=2, 115min) and running (n=2, 10km, 50min) sessions. Physical training amounted to 500min during the 4 days of the pre-competitive phase investigated (T2), and encompassed 2 resistance (140min), 1 running (8km, 30min) and 5 kayak training sessions (64km, 330min). In the 4 days of the subsequent competitive period (T3), athletes trained 3 times (180min), comprising 2 kayaking sessions (20km, 115min) and 1 muscular strength sessions (65min), and competed twice (in 2 consecutive days).

Anthropometric characteristics of subjects are summarized in Table 1. Although we have not laboratoriallly demonstrated their high fitness level (e.g. VO_{2max}), all kayakers were Portuguese international representatives, regularly training and competing since their official affiliation on the Portuguese National Federation (on average, 10.3 years). From T1 to T2, athletes' weight ($P < 0.001$), and consequently BMI ($P = 0.001$), increased at the expense of both fat mass ($P = 0.052$) and fat-free mass ($P = 0.084$).

Table 1 Characteristics of the kayakers

	T1	T2
Age (years)	21.9 ± 3.3	22.7 ± 3.1*
Height (cm)	172.9 ± 7.9	173.0 ± 7.3
Weight (kg)	70.0 ± 7.0	73.1 ± 7.4*
BMI (kg/m^2)	23.1 ± 1.3	24.4 ± 1.2*
Body fat (%)	16.2 ± 6.6	17.6 ± 6.7

*Significantly different from T1 ($P < 0.05$)

The athletes' estimated energy expenditure and nutritional intake are displayed in Table 2. The only statistical significant nutritional difference between T1 and T2 was the monounsaturated fatty acid intake ($P = 0.019$). The mean intake of all micronutrients were noted to exceed the RDA standards, with the exception of females' iron intake in T1, vitamin C intake of men in T2, and α-tocopherol intake of both sexes in the two time-points. The estimated prevalence of inadequacy was 100% for α-tocopherol, in both sexes and moments,

0% for vitamin A, selenium, zinc, copper and iron, in both sexes and moments, 33% for magnesium in T1 and T2, in women, and 16.7% in T1 and 33% in T2 for vitamin C, in men.

Energy intake (P = 0.015 in T1; P = 0.017 in T2) and expenditure (P = 0.006 in T1; P < 0.001 in T2), and protein (P = 0.005 in T1; P = 0.006 in T2), fat (P = 0.005 in T1; P = 0.015 in T2), cholesterol (P = 0.010 in T1), saturated (P = 0.009 in T1) and monounsaturated fat absolute intakes (P = 0.004 in T1; P = 0.010 in T2) were significantly greater in male than in female athletes. Concerning micronutrients, only copper (P = 0.038 in T1; P = 0.020 in T2), and zinc (P = 0.022 in T2) were ingested in greater amounts by sportsmen in comparison with sportswomen.

Table 2. Estimated daily energy expenditure and energy and nutrient intakes of kayakers

	T1	T2
Energy expenditure (kcal)	3248 ± 357	3319 ± 441
Basal metabolism (kcal)	1795 ± 197	1835 ± 209
Physical Activity Level	1.81 ± 0.14	1.81 ± 0.07
Energy intake (kcal)	3135 ± 782	3180 ± 747
Proteins (g)	140 ± 30	146 ± 35
Proteins (% of energy intake)	18.1 ± 2.8	18.5 ± 2.8
Carbohydrates (g)	408 ± 113	431 ± 125
Carbohydrates (% of energy intake)	51.8 ± 5.1	53.6 ± 5.1
Fibre (g)	23.9± 5.8	26.2± 6.7
Fat (g)	106 ± 32	98 ± 22
Fat (% of energy intake)	30.1 ± 4.0	27.9 ± 3.2
Saturated fat (g)	36.0 ± 12.3	31.7 ± 10.4
Monounsaturated fat (g)	44.8 ± 14.0	36.9 ± 7.6*
Polyunsaturated fat (g)	15.4 ± 4.8	17.2 ± 5.0
Cholesterol (mg)	391 ± 91	382 ± 93
Vitamin C (mg)	90.4 ± 31.2	82.3 ± 29.5
α-tocopherol (mg)	6.61 ± 2.18	6.78 ± 1.87
Vitamin A (RE μg)	1303 ± 463	1155 ± 383
β-carotene (μg)	1110 ± 994	1112 ± 1285
Selenium (μg)	137 ± 24	133 ± 30
Zinc (mg)	20.2 ± 5.0	21.5 ± 6.0
Copper (mg)	1.68 ± 0.34	1.93 ± 0.55
Iron (mg)	21.0 ± 5.9	23.3 ± 4.1
Magnesium (mg)	389 ± 85	400 ± 82
Manganese (mg)	3.87 ± 1.87	3.93 ± 1.57

*Significantly different from T1 (P < 0.05)

Table 3 presents data on antioxidant status and oxidative stress and damage parameters. Concerning the antioxidant enzymes activities, GPx (P = 0.003) raised and SOD (P < 0.001)

diminished from the pre-competitive (T2) to the competitive period (T3). TAS was greatest in the first season (T1) in comparison with both time-points of the second season (T1 vs. T2, $P < 0.001$; T1 vs. T3, $P < 0.001$). Alpha-tocopherol concentrations increased significantly from T1 to T2 ($P = 0.001$), while TBARS ($P = 0.024$) followed in the opposite direction. A higher uric acid value was evidenced in T2 compared to that measured at the last sampling time (T3, $P = 0.032$). Although the muscle damage tended to be elevated during the pre-competition period (T2), as indicated by the plasma activity of CK, the differences were not statistically significant ($P = 0.184$). Cortisol levels declined significantly throughout the study (time main effect, $P < 0.001$; T1 vs. T2, $P = 0.042$; T2 vs. T3, $P = 0.018$; T1 vs. T3, $P = 0.002$). No significant differences were observed for all the other parameters studied during the course of this research.

Table 3. Antioxidant status, lipid peroxidation, muscle damage, inflammation and physiological stress markers of kayakers throughout the study

	T1	T2	T3
TAS (mmol/L)	1.43 ± 0.21	0.99 ± 0.07*	0.95 ± 0.19*
Uric acid (mg/dL)	4.61 ± 1.25	4.96 ± 1.25	4.01 ± 1.12#
GPx (U/L)	6224 ± 2420	5235 ± 1142	7602 ± 1704#
SOD (U/g Hg)	1347 ± 159	1396 ± 133	1013 ± 77*#
Gr (U/L)	68.8 ± 9.5	67.1 ± 6.7	68.3 ± 12.1
α-tocopherol (μmol/L)	22.1 ± 5.5	29.3 ± 3.6*	25.5 ± 6.0
Retinol (μmol/L)	2.02 ± 0.50	2.29 ± 0.64	2.02 ± 0.31
α-carotene (μmol/L)	0.19 ± 0.07	0.15 ± 0.07	0.18 ± 0.07
β-carotene (μmol/L)	0.49 ± 0.21	0.57 ± 0.28	0.74 ± 0.43
Lycopene (μmol/L)	0.62 ± 0.20	0.46 ± 0.17	0.48 ± 0.15
Lutein + zeaxanthin (μmol/L)	0.41 ± 0.12	0.41 ± 0.21	0.31 ± 0.16
Vitamin C (μmol/L)	63.0 ± 24.2	ND	ND
Creatine kinase (IU/L)	172 ± 69	317 ± 221	181 ± 90
TBARS (μmol/L)	6.00 ± 1.21	4.64 ± 1.68*	5.28 ± 0.66
IL-6 (pg/mL)	1.47 ± 0.71	1.97 ± 0.30	2.15 ± 1.44
Cortisol (nmol/L)	223 ± 55	188 ± 33*	165 ± 35*#

* Significantly different from T1 ($P < 0.05$)
# Significantly different from T2 ($P < 0.05$)
ND Not determined

Female kayakers displayed no significant differences compared with male kayakers with regard to the majority of the above-mentioned parameters, except for α-tocopherol (P = 0.041), CK (P = 0.044) and uric acid (P = 0.049) in T1; TAS (P = 0.007) and uric acid (P = 0.015) in T2; and CK (P = 0.041), α-carotene (P = 0.020) and uric acid (P = 0.003) in T3. Therefore, and because the main objective of our study was to monitor those markers longitudinally, data of both sexes were pooled to increase the power of analysis.

DISCUSSION

Our aim was to longitudinally monitor the evolution of antioxidant status and oxidative stress and damage markers in well-trained athletes during routine training. In fact, it was suggested that periods of high intensity training and/competition could decrease the efficiency of antioxidant system [6, 23] and induce an increase in oxidative stress both at rest [24, 25] an in response to exercise [26].

Our nutritional data agree well with the insufficient intakes of antioxidant vitamins that have been reported in the literature in moderate to well-trained athletes, particularly for vitamin E [10-14]. Vitamin C intake of our kayakers was in the lower limit of the values described, being similar to that of professional rugby players [25].

Despite the inadequate intake of antioxidant vitamins, the correspondent plasmatic concentrations were not deficient, in agreement with what was observed by others [11]. None of the athletes presented plasma α-tocopherol and vitamin C concentrations below the cut-off point for deficiency (9.3 and 20 μmol/L respectively), while 2 kayakers, in T2, and 1 kayaker, in T3, had β-carotene plasma levels below 0.30 μmol/L [22]. Overall, concentration of antioxidant vitamins were all within reference values whatever the moment analysed (T1-T3) [27-29]. Serum antioxidant vitamin levels were quite similar to those previously published in athletes [8, 11, 13, 14, 22, 24, 30, 31] and were not associated with dietary intake, in accordance with previous works [11, 32]. Actually, the plasmatic concentrations reflect the influence not only from intake, but also from transport, storage and use [11]. Athletes can, inclusively, have higher plasmatic antioxidant levels than those of non-athletes even when their intake is similar [9].

To the best of our knowledge, only one work [25] has determined antioxidant status and oxidative stress in 2 different seasons as we did. The authors [25] did not find any significant difference in muscle damage, oxidative stress and damage, and antioxidant status parameters between similar training and competing periods in two seasons. However, they observed

fluctuations during the course of one season, namely a decreased oxidative stress and an elevated α-tocopherol concentration in the non-competitive period in comparison with the phases of increased volume of training and competition [25]. Other authors also described a diminished α-tocopherol status during the periods of more intense training, probably as a result of its consumption, even when dietary intakes exceeded the recommendations standards [7]. Schippinger *et al*. [24] found that peroxide levels were significantly higher after 4 months of competition than in pre-season, but only in half of the sportsmen studied [24]. Interestingly, also only half the soccer and basketball players studied by Pincemail *et al*. [33] and the majority of soccer players investigated by Klapcinska *et al*. [31] presented high levels of autoantibodies against oxidized LDL during regular training and competition. This high between-athletes variability in susceptibility to oxidative stress was not determined by baseline antioxidant vitamin concentrations, antioxidant enzyme activities or oxidative stress markers, which limits the predictability of detecting those at greater risk [24, 31, 33].

We have observed higher levels of α-tocopherol and lower values of TBARS in pre-competition (T2), although it was the phase with the greatest training volume. The transient increment of serum α-tocopherol caused by acute exercise [34], as a result of its mobilization from adipose tissue along with triglycerides [22], could have cumulative effects and result in chronically augmented resting levels in those exercising regularly. A non-significant increase in α-tocopherol concentrations from pre-season to competitive season has been described in American football players [24]. Moreover, athletes under regular training have improved α-tocopherol status in comparison with sedentary individuals [9, 35-37]. Our data support the notion that the increase in α-tocopherol levels could primarily be the result of its mobilization from tissue storage to circulation, as triglycerides levels were coincidently increased in T2 in comparison with T1 (51.7 vs. 106.9 mg/dL, respectively; $P < 0.001$), and excludes diet as a likely explanation, since α-tocopherol intake was not different between those moments.

This increase in plasma α-tocopherol levels could have attenuated exercise-induced lipid peroxidation by scavenging ROS. However, augmented uric acid levels in T2 could have also played a role in the decrease in TBARS levels, as previously stated by other authors [38]. Serum uric acid levels have been found to rise after intense acute aerobic [34] and anaerobic [8] exercises and with chronic exercise (training) [7, 25], as a result of heightened purine metabolism or decreased renal clearance [7, 8]. The production of uric acid from hypoxanthine is dependent on both duration and intensity of exercise [24]. A lowered uric acid synthesis, consecutive to a lesser activation of xanthine oxidase, could be responsible for a decrease in resting plasma TAS [13]. In accordance, we observed diminished uric acid levels in the period of minor training

volume in the second season (T3), which may have contributed to the lowest value of TAS at this time-point. These parameters were highly correlated at this moment (r = 0.75, *P* = 0.020), but not in any other. Actually, despite uric acid concentrations were greater in T2 than in T3, TAS values were not different. We can theorize that the augmented training load in T2 could potentially have resulted into an increased oxidant insult which might have compromised the status of other aqueous antioxidants, such as vitamin C that, unfortunately, was not measured in T2 and T3. Schröder *et al*. [23] observed a decline in vitamin C status in basketball players during regular training and competing. In fact, the lower value of cortisol in this period, which is known to be co-released with vitamin C from adrenal glands with exercise [8] and training [6], reinforces this theory. Cortisol levels in T2 and T3 are very similar to those reported in kayakers during habitual training [39]. The augmented levels in T1 suggest a higher physical stress caused by exercise, since cortisol increase appears to reflect the intensity of exercise [40]. Greater cortisol increases with exercise may blunt IL-6 responses [40]. Although these parameters were not correlated in the present study, mean values of cortisol significantly diminished across the study (*P* < 0.001), while that of IL-6 showed a non-significant increase (*P* = 0.193). Elevated plasmatic levels of IL-6 after exercise have been found consistently [41], and the response seems to be influenced by the duration and intensity of exercise and the muscle mass involved [40]. However, we found longitudinally stable levels of this acute-phase mediator.

Kayakers' retinol levels and their stability across the different training periods resemble the findings of other authors [31]. β-carotene levels were unchanged throughout the study in accordance with other investigations, either in athletic [24] and non-athletic populations [28]. Although we observed a significant main effect of time for plasma lycopene (*P* = 0.023), pairwise post hoc comparisons of means at different moments were only almost significant between T1 and T2 (*P* = 0.050). In other studies, lycopene tended to decrease after a 7-day extreme running competition [42] and significantly decreased with a time trial of 1 h on a cycle ergometer [43].

Concerning antioxidant enzymes, we observed that the activity of GPx was elevated in T3 compared to T2, while SOD activity was reduced in T3 in comparison with T1 and T2. Twelve weeks of high-intensity endurance training significantly increased SOD activity in a sample of previously sedentary subjects [5]. In this context, athletes presented higher levels of this enzyme than their sedentary counterparts during habitual training [35-37]. The decline in SOD activity in the period of minor training load (T3) we observed may reflect its training responsive nature. Actually, the time spent training was positively related to resting SOD activity in triathletes [44]. A decrease in physical activity in a tapering training period induced a lowering in SOD activity at rest, which was attributed to the minor formation of superoxide [13].

SOD activity seems to require a higher training load to be up-regulated [5]. On the other hand, increased activity of GPx at T3 suggests an augmented hydrogen peroxide generation, which, in high concentrations, inhibits SOD [45].

Some data supports the hypothesis that exercise-induced oxidative stress is involved in the peroxidative damage of miocellular membranes, augmenting its permeability, which causes the leakage of intracellular proteins, such as CK [31]. Despite this, no correlation between lipid peroxidation and muscle damage indices was detected in this study (data not shown), in accordance with the findings of other investigators [46].

The activity of CK in our sample was in agreement with that reported in kayakers of the Polish Olympic Team [39]. Our data support the findings of other investigators [25] that found a greater muscle damage in the more intense training periods. CK activity was significantly correlated with the estimated energy expenditure in T1 (r =0.71, P = 0.033) and almost in T2 (r = 0.66, P = 0.054). The tendency to higher levels of CK in T2 could be related to the greater volume of training, namely resistance training, and the minor time between the last weight session and blood sampling. This seems to be relevant because eccentric contractions induce greater muscle injury than concentric ones [47]. On the other time-points studied, kayaking sessions predominated, which are not eccentric-based activities. The increase of CK activity in T2 was not accompanied by an elevation of TBARS levels which indicates that muscle damage is not due to an increased peroxidation of muscle cells membranes [39]. As expected [48], CK values were lower in female than in male kayakers, but there were no gender-associated differences in the pattern of response to chronic training. Correcting CK values for lean body mass (kg) eliminated gender differences, but not changes over time [49]. Assuming they trained at the same relative intensity as men, perhaps this is explained by the stabilizing effect estrogens have on cell membranes [48].

The decrease in TAS from the first to the second season we observed can indicate that the sustained high volume of training over the season may have cumulatively stressed the athletes' antioxidant capacity. Increases in oxidative stress markers during habitual training of sportsmen have been reported, in spite of normal antioxidant levels [24, 31, 33]. On the other hand, antioxidant status could be compromised during an intense training period without any measurable elevation of oxidative stress and damage [7]. Regular intensive training appears to be a latent and permanent stressor to the antioxidant system. In this context, oxidative stress only returned to resting levels 8 days after a marathon [50] and 3 weeks after the end of a basketball season [51]. Actually, some studies revealed diminished antioxidant capacities in athletes compared to sedentary individuals [9], which can be attenuated with antioxidant vitamins supplements [52]. Antioxidant supplementation contributed to the maintenance of

antioxidant status during an overloaded training period, especially in those athletes with lower antioxidant intakes [14]. It is, therefore, repeatedly stated that sportsmen would benefit from antioxidant supplementation, in order to assure an adequate supply and to lower oxidative damage, which is supported by some research data [23].

CONCLUSIONS

Overall, it was not evidenced neither a degradation of antioxidant status nor chronically elevated oxidative damage in kayakers with training. On the other hand, a strengthening of antioxidant defences was also not observed. This study demonstrated that antioxidant capacity and oxidative stress changed more within the same season than from one season to another. Redox markers should be monitored throughout the season to timely detect those athletes at increased risk and to individually fine-tune their antioxidant intake, by diet or supplements, in order to avoid consequences to athletes' health and sports performance. Therefore, oxidative stress and antioxidant parameters should be considered in the biological follow-up of all athletes.

ACKOWLEDEGMENTS

The authors would like to thank all the athletes for their voluntary participation in the study.

REFERENCES

1. Gomez-Cabrera M, Domenech E, Viña J. Moderate exercise is an antioxidant: Upregulation of antioxidant genes by training. Free Radic Biol Med. 2008; 44(2):126-31

2. Radak Z, Chung HY, Goto S. Systemic adaptation to oxidative challenge induced by regular exercise. Free Radic Biol Med. 2008; 44(2):153-59

3. Peake JM, Suzuki K, Coombes JS. The influence of antioxidant supplementation on markers of inflammation and the relationship to oxidative stress after exercise. J Nutr Biochem. 2007; 18(6):357-71

4. Elosua R, Molina L, Fito M, Arquer A, Sanchez-Quesada JL, Covas MI, et al. Response of oxidative stress biomarkers to a 16-week aerobic physical activity program, and to acute physical activity, in healthy young men and women. Atherosclerosis. 2003; 167(2):327-34

5. Miyazaki H, Oh-ishi S, Ookawara T, Kizaki T, Toshinai K, Ha S, et al. Strenuous endurance training in humans reduces oxidative stress following exhausting exercise. Eur J Appl Physiol. 2001; 84(1-2):1-6

6. Bergholm R, Mäkimattila S, Valkonen M, Liu ML, Lahdenperä S, Taskinen MR, et al. Intense physical training decreases circulating antioxidants and endothelium-dependent vasodilatation in vivo. Atherosclerosis. 1999; 145(2):341-9

7. Subudhi AW, Davis SL, Kipp RW, Askew EW. Antioxidant status and oxidative stress in elite alpine ski racers. Int J Sport Nutr Exerc Metab. 2001; 11(1):32-41

8. Groussard C, Machefer G, Rannou F, Faure H, Zouhal H, Sergent O, et al. Physical fitness and plasma non-enzymatic antioxidant status at rest and after a wingate test. Can J Appl Physiol. 2003; 28(1):79-92

9. Watson TA, MacDonald-Wicks LK, Garg ML. Oxidative stress and antioxidants in athletes undertaking regular exercise training. Int J Sport Nutr Exerc Metab. 2005; 15(2):131-46

10. Groussard C, MacHefer G, Rannou F, Gratas-Delamarche A. Estimation of the dietary intake in antioxidant vitamins of sportsmen. Sci Sports. 2004; 19(4):193-95

11. Machefer G, Groussard C, Zouhal H, Vincent S, Youssef H, Faure H, et al. Nutritional and plasmatic antioxidant vitamins status of ultra endurance athletes. J Am Coll Nutr. 2007; 26(4):311-16

12. Machefer G, Malarde L, Groussard C, Gratas-Delamarche A. Antioxidant vitamin intakes of endurance athletes. Sci Sports. 2006; 21(2):107-09

13. Margaritis I, Palazzetti S, Rousseau AS, Richard MJ, Favier A. Antioxidant supplementation and tapering exercise improve exercise-induced antioxidant response. J Am Coll Nutr. 2003; 22(2):147-56

14. 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. 2004; 91(1):91-100

15. Humphries B, Abt GA, Stanton R, Sly N. Kinanthropometric and physiological characteristics of outrigger canoe paddlers. J Sports Sci. 2000; 18(6):395-99

16. Food and Nutrition Board, Institute of Medicine. Dietary Reference Intakes: Applications in Dietary Assessment. Washington, D.C.: National Academic Press; 2000.

17. Martin A. Apports nutritionnels conseillés pour la population française. 3ème ed. Paris: Ed Tec & Doc; 2001.

18. Ainsworth BE, Haskell WL, Whitt MC, Irwin ML, Swartz AM, Strath SJ, et al. Compendium of physical activities: an update of activity codes and MET intensities. Med Sci Sports Exerc. 2000; 32(9 Suppl):S498-504

19. Thompson J, Manore M. Predicted and Measured Resting Metabolic Rate of Male and Female Endurance Athletes. J Am Diet Assoc. 1996; 96(1):30-34

20. Frisancho R. Anthropometric standards for the assessment of growth and nutritional status. Michigan: University of Michigan Press; 1990.

21. Siri W. Body composition from fluid spaces and density: analysis of methods. In: Brozek J, Henschel A, editores. Techniques for Measuring Body Composition. Washington, D.C: National Academy of Sciences-National Research Council; 1961. p. 223-44.

22. Rousseau AS, Hininger I, Palazzetti S, Faure H, Roussel AM, Margaritis I. Antioxidant vitamin status in high exposure to oxidative stress in competitive athletes. Br J Nutr. 2004; 92(3):461-8

23. 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. 2000; 21(2):146-50

24. Schippinger G, Wonisch W, Abuja PM, Fankhauser F, Winklhofer-Roob BM, Halwachs G. Lipid peroxidation and antioxidant status in professional American football players during competition. Eur J Clin Invest. 2002; 32(9):686-92

25. Finaud J, Scislowski V, Lac G, Durand D, Vidalin H, Robert A, et al. Antioxidant status and oxidative stress in professional rugby players: evolution throughout a season. Int J Sports Med. 2006; 27(2):87-93

26. Palazzetti S, Richard MJ, Favier A, Margaritis I. Overloaded training increases exercise-induced oxidative stress and damage. Can J Appl Physiol. 2003; 28(4):588-604

27. Food and Nutrition Board, Institute of Medicine. Dietary reference intakes for vitamin C, vitamin E, selenium, and carotenoids. Washington, DC: National Academy Press; 2000.

28. Winklhofer-Roob BM, Van'T Hof MA, Shmerling DH. Reference values for plasma concentrations of vitamin E and A and carotenoids in a Swiss population from infancy to adulthood, adjusted for seasonal influences. Clin Chem. 1997; 43(1):146-53

29. Food and Nutrition Board, Institute of Medicine. Dietary reference intakes for vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium and zinc. Washington, DC.: National Academy Press; 2001.

30. Schröder H, Navarro E, Mora J, Galiano D, Tramullas A. Effects of alpha-tocopherol, beta-carotene and ascorbic acid on oxidative, hormonal and enzymatic exercise stress markers in habitual training activity of professional basketball players. Eur J Nutr. 2001; 40(4):178-84

31. Klapcinska B, Kempa K, Sobczak A, Sadowska-Krepa E, Jagsz S, Szoltysek I. Evaluation of autoantibodies against oxidized LDL (oLAB) and blood antioxidant status in professional soccer players. Int J Sports Med. 2005; 26(1):71-8

32. Kardinaal AFM, Van 't Veer P, Brants HAM, Van den H, Jan van Schoonhoven B, Hermus RJJ. Relations between antioxidant vitamins in adipose tissue, plasma, and diet. Am J Epidemiol. 1995; 141(5):440-50

33. Pincemail J, Lecomte J, Castiau JP, Collard E, Vasankari T, Cheramy-Bien JP, et al. Evaluation of autoantibodies against oxidized LDL and antioxidant status in top soccer and basketball players after 4 months of competition. Free Radic Biol Med. 2000; 28(4):559-65

34. Aguiló A, Tauler P, Fuentespina E, Tur JA, Córdova A, Pons A. Antioxidant response to oxidative stress induced by exhaustive exercise. Physiol Behav. 2005; 84(1):1-7

35. Brites FD, Evelson PA, Christiansen MG, Nicol MF, Basilico MJ, Wikinski RW, et al. Soccer players under regular training show oxidative stress but an improved plasma antioxidant status. Clin Sci (Lond). 1999; 96(4):381-5

36. Evelson P, Gambino G, Travacio M, Jaita G, Verona J, Maroncelli C, et al. Higher antioxidant defences in plasma and low density lipoproteins from rugby players. Eur J Clin Invest. 2002; 32(11):818-25

37. Cazzola R, Russo-Volpe S, Cervato G, Cestaro B. Biochemical assessments of oxidative stress, erythrocyte membrane fluidity and antioxidant status in professional soccer players and sedentary controls. Eur J Clin Invest. 2003; 33(10):924-30

38. Mikami T, Yoshino Y, Ito A. Does a relationship exist between the urate pool in the body and lipid peroxidation during exercise? Free Radic Res. 2000; 32(1):31-9

39. Wozniak A, Drewa G, Wozniak B, Drewa T, Mila-Kierzenkowska C, Czajkowski R, et al. Effect of cryogenic temperatures and exercise on lipid peroxidation in kayakers. Biol Sport. 2005; 22(3):247-60

40. Edwards KM, Burns VE, Ring C, Carroll D. Individual differences in the interleukin-6 response to maximal and submaximal exercise tasks. J Sports Sci. 2006; 24(8):855-62

41. Sacheck JM, Blumberg JB. Role of vitamin E and oxidative stress in exercise. Nutrition. 2001; 17(10):809-14

42. Machefer G, Groussard C, Rannou-Bekono F, Zouhal H, Faure H, Vincent S, et al. Extreme running competition decreases blood antioxidant defense capacity. J Am Coll Nutr. 2004; 23(4):358-64

43. Oostenbrug GS, Mensink RP, Hardeman MR, De Vries T, Brouns F, Hornstra G. Exercise performance, red blood cell deformability, and lipid peroxidation: effects of fish oil and vitamin E. J Appl Physiol. 1997; 83(3):746-52

44. Knez WL, Jenkins DG, Coombes JS. Oxidative stress in half and full Ironman triathletes. Med Sci Sports Exerc. 2007; 39(2):283-88

45. Wozniak A, Drewa G, Wozniak B, Drewa T, Olszewska D, Mila-Kierzenkowska C, et al. Effect of cryogenic temperatures and exercise on antioxidant enzymes activity in erythrocytes of kayakers. Biol Sport. 2002; 19(1):63-72

46. Baker JS, Bailey DM, Hullin D, Young I, Davies B. Metabolic implications of resistive force selection for oxidative stress and markers of muscle damage during 30 s of high-intensity exercise. Eur J Appl Physiol. 2004; 92(3):321-7

47. Willoughby DS, Vanenk C, Taylor L. Effects of concentric and eccentric contractions on exercise-induced muscle injury, inflammation, and serum IL-6. J Exerc Physiol Online. 2003; 6(4):8-15

48. Dernbach AR, Sherman WM, Simonsen JC, Flowers KM, Lamb DR. No evidence of oxidant stress during high-intensity rowing training. J Appl Physiol. 1993; 74(5):2140-5

49. Mastaloudis A, Traber MG, Carstensen K, Widrick J. Antioxidants did not prevent muscle damage in response to an ultramarathon run. Med Sci Sports Exerc. 2006; 38(1):72-80

50. Hessel E, Haberland A, Müller M, Lerche D, Schimke I. Oxygen radical generation of neutrophils: A reason for oxidative stress during marathon running? Clin Chim Acta. 2000; 298(1-2):145-56

51. Benoni G, Bellavite P, Adami A, Chirumbolo S, Lippi G, Brocco G, et al. Changes in several neutrophil functions in basketball players before, during and after the sports season. Int J Sports Med. 1995; 16(1):34-37

52. Banfi G, Malavazos A, Iorio E, Dolci A, Doneda L, Verna R, et al. Plasma oxidative stress biomarkers, nitric oxide and heat shock protein 70 in trained elite soccer players. Eur J Appl Physiol. 2006; 96(5):483-86

3.4 EFFECTS OF COMBINED ANTIOXIDANT SUPPLEMENTATION ON EXERCISE-INDUCED LIPID PEROXIDATION, MUSCLE DAMAGE AND INFLAMMATION BIOMARKERS IN ELITE KAYAKERS

Teixeira VH[1], Valente H[1,2], Casal S[3], Marques F[4,5], Moreira P[1,2]

[1] Faculty of Nutrition and Food Sciences, University of Porto

[2] Research Centre in Physical Activity, Health and Leisure, University of Porto

[3] REQUIMTE, Bromatology Service, Faculty of Pharmacy, University of Porto

[4] Department of Clinical Analysis, Faculty of Pharmacy, University of Porto

[5] Institute for Molecular and Cell Biology – IBMC, University of Porto

SUBMITTED FOR PUBLICATION

ABSTRACT

Exhaustive exercise has the potential to induce oxidative stress and damage even in highly trained athletes. The aim of the study was to determine the effects of 4 weeks of antioxidant supplementation on exercise-induced lipid peroxidation, muscle damage and inflammation in 20 elite kayakers. Subjects were randomly assigned to receive a placebo (PLA) or an antioxidant capsule (AOX; 272 mg α-tocopherol, 400 mg vitamin C, 30 mg β-carotene, 2 mg lutein, 400 μg selenium, 30 mg zinc and 600 mg magnesium). Blood samples were collected at rest and 15min after a 1000m kayak race, both before and after the supplementation period, for analysis of α-tocopherol, α-carotene, β-carotene, lycopene, lutein plus zeaxanthin, vitamin C, uric acid, total antioxidant status (TAS), thiobarbituric reactive acid substances (TBARS) and interleukin-6 (IL-6) levels and creatine kinase (CK), superoxide dismutase (SOD), glutathione reductase (Gr) and glutathione peroxidase (GPx) activities. The post-exercise values were adjusted for changes in plasma volume. With supplementation, plasma α-tocopherol (P = 0.003) and β-carotene (P = 0.007) augmented significantly in the AOX group. IL-6 (Exercise, P = 0.039), TBARS (Exercise, $P < 0.001$) and uric acid (Exercise, P = 0.032) increased significantly in response to the exercise regardless of treatment group. Cortisol level raised more from pre- to post-supplementation period in the PLA group (Time $\times$ Supplementation, P = 0.002). While TAS declined after exercise before intervention, it increased above pre-exercise values after the 4-week period in the AOX group (Supplementation $\times$ Time $\times$ Exercise, P = 0.034). CK increased

after exercise in both groups (Exercise effect, $P < 0.001$), and decreased from week 0 to week 4 more markedly in the PLA group (Supplementation × Time, $P = 0.049$). Our data indicate that antioxidant supplementation do not offer protection against exercise-induced lipid peroxidation and inflammation and may hinder the recovery of muscle damage.

INTRODUCTION

Physical exercise of sufficient intensity or duration enhances, through several pathways, the generation of reactive oxygen species (ROS) which can oxidative damage proteins, nucleic acids and lipids, leading to impaired cellular function [1-4]. ROS-mediated sarcolemmal phospholipids peroxidation may play a role in the aetiology of exercise-induced muscle damage [5, 6]. A correlation was found between miocellular CK efflux, an indicator of muscle damage, and TBARS, a biomarker of lipid peroxidation [7].

Muscle damage and/or ROS increase after exercise promote an acute-phase local inflammatory response [8] characterized by the release of inflammatory cytokines (like IL-6) [9] from various cell types [10], with the aim of stimulating the recruitment of neutrophils and monocytes to inflammation areas in order to repair damaged tissue [3, 8]. The mobilization and activation of immune cells during exercise seems to be mediated by stress hormones, such as cortisol [11]. Infiltrated phagocytes produce additional superoxide ($O_2^{\bullet-}$) that may amplify muscle injury [12], which may be the cause of the delayed-onset muscle soreness [13]. There is also evidence that ROS produced during exhaustive exercise are responsible for protein oxidation, contributing to the development of muscle fatigue [14]. This ROS-mediated disturbance in cellular homeostasis might result in muscular injury, soreness and fatigue, and consequently decrements in physical performance [15].

In order to minimize exercise-related oxidative harmful effects, the body contains an intracellular and extracellular defence equipment composed by both enzymatic and non-enzymatic antioxidants [16-18]. Trained individuals appear to have an enhanced endogenous antioxidant system due to regular training [2, 4, 16, 19, 20]. However, intensive and sustained exercise can increase ROS to a level that outpaces tissues' antioxidant capacity [2, 17, 18]. Therefore, the high training load of elite athletes could impose a cellular oxidative stress even in highly adapted muscles [2].

As physiologic adaptations induced by training may not completely counter-balance the increased oxidant insult associated with exhaustive exercise, athletes' dietary antioxidant requirements may be increased [21], particularly during periods of intensive training and competition [4]. Strenuous physical training can diminish plasma antioxidants vitamins to a sub-optimal level even if dietary intakes respect the recommendations [22]. Athletes may have a greater likelihood of antioxidant vitamin deficiency [21]. Moreover, sportsmen's antioxidant dietary intake is frequently inadequate [4, 21]. Thus, a normal diet may not be always sufficient for athletes and supplementing them with antioxidants would probably have beneficial effects against exercise-induced oxidative stress [4, 22].

The use of antioxidant supplements in an attempt to minimize the extent of muscle injury or oxidative stress in response to exercise has attracted the interest of researchers and is a common practice among the athletic community [3]. However, the results of works that have evaluated its effectiveness are not unequivocal and conclusive [2, 3, 19]. Furthermore, the majority of these studies focused on exercise-unaccustomed subjects and/or used exercise protocols unrealistic for athletes [19]. The extrapolation of the findings of laboratory-based intervention studies in untrained individuals to the athletic population is questionable [23].

The proper functioning of the antioxidant system depends on the concerted action of antioxidants, as each one of them play a specific role and functionally complement the others. It has been suggested that the combination of several antioxidants may be more effective than a single-compound supplementation [4]. The association of antioxidants may maximize the biological antioxidant effect by allowing the scavenging of ROS generated in different environments, either lipid (such as in muscle cell lipid membranes and in plasma lipoproteins) or aqueous (such as in cytosol and in extracellular fluid) [2, 4, 24, 25].

The objective of this work was to evaluate the effects of combined antioxidant supplementation in attenuating the oxidative stress, lipid peroxidation, muscle damage and inflammation induced by a well-know exercise protocol for elite kayakers in a field situation, while following their habitual dietary pattern and training and competition programme.

MATERIAL AND METHODS

Subjects

Twenty volunteer athletes (14 men and 6 women) recruited from the Portuguese National Kayaking and Canoeing Senior and Junior Teams participated in this 4-week double-blind study. During most of the time frame (3 weeks), these elite athletes were in the National Team Sport Centre engaged in a controlled competitive period of training to the European Championship, consisting of resistance, endurance and kayaking skills training sessions. All kayakers were training and competing regularly since their official affiliation on the Portuguese National Federation (antioxidant group: 8.5 $\pm$ 4.4 years; placebo group: 8.0 $\pm$ 4.6 years; P = 0.807). Athletes, or their parents, gave written informed consent after having been explained verbally and in writing the purpose, demands and possible risks associated with the study. The protocol for this study was approved by the Scientific Committee of the Faculty of Nutrition and Food Sciences at the University of Porto. All sportsmen were healthy, non-smokers, reported no use of non-steroidal anti-inflammatory drugs or oral contraceptives, and did not take antioxidants supplements for at least 3 months [(3)]. Subjects were instructed to refrain from making any drastic changes in the diet, to abstain from anti-inflammatory or analgesic drugs throughout the study, and to not consume caffeine or alcoholic beverages for 24h prior to blood draws.

Study design

Supplementation

Subjects were randomly assigned in a double-blind fashion to two groups taking either an antioxidant complex supplement (AOX, n=10) or a placebo (PLAC, n=10) during the 4 weeks. Each antioxidant capsule contained 136mg of α-tocopherol, 200mg of vitamin C, 15mg of β-carotene, 1mg of lutein, of 200μg selenium, 15mg of zinc and 300mg of magnesium. Antioxidant and placebo (lactose) capsules were generously donated by a pharmaceutical company. Kayakers were told before the beginning of the study to comply carefully with the treatment and to take two capsules daily, one before lunch and the other before dinner. Supplementation was started after the completion of the first exercise bout, continued for 28 days and ended in the night before the second kayak trial. Capsules were counted upon return of the capsule bottles to assess compliance with the treatment. The average compliance was 90.1% with no differences between treatments groups (P = 0.873).

Exercise

Subjects were asked to complete in the least time possible a 1000m maximal flat-water kayaking trial on two occasions at an interval of 4 weeks. The exercises were carried out under the same equipment conditions, and in the same place, time of the day (between 08h30 and 09h00), day of the week and month to avoid circadian variations, and menstrual-cycle variations in women [(26)]. Before each test, all canoeists did a standardized warming up for 15 min by jogging, stretching and kayaking slowly. The training program in the day before both exercise trials was a short low intensity skills session (50min). Athletes were asked to avoid additional intense physical activity afterwards. Each subject served as self-control to eliminate any biological variability in the response to antioxidant supplementation. This design was chosen instead of a randomized crossover design due to storage kinetics of liposoluble antioxidants vitamins [(10)].

Dietary intake

Since 2 days before the first trial, athletes ate identical meals prepared and provided in the same local in almost all days throughout the study. In order to estimate average energy and nutritional intake, athletes recorded their dietary intake during seven consecutive days, starting 2 days before the first 1000m bout. A trained nutritionist gave detailed oral and written instructions about proper nutritional recording, including estimating portion sizes. Athletes were asked to describe the foods and fluids consumed and estimate the amount ingested by using standardized household measures or record the weight/volume and the commercial name of packaged food. Dietary records' information was transformed into energy and nutrients using ESHA Food Processor 8.0 for Windows (Salem, OR, USA), that does not allow the quantification of α-carotene, lycopene, lutein and zeaxanthin intakes. The adequacy of nutritional intake was assessed by reference to the Food and Nutrition Board Dietary Reference Intakes [(27)].

Physical activity and estimated energy expenditure

In order to estimate energy expenditure, athletes recorded their daily activities in physical activity records at 15-min intervals over a 7-d period, comprising the same days of their similar dietary counterpart (2 days before and 5 days after the first 1000m trial). The technical staff provided the detailed information about the training program over the 7 days, that consisted

of 11 sessions (~560min), comprising on-water (n=7, 74km, 395min), weight training (n=2, 115min) and running (n=2, 10km, 50min) sessions. The estimative of the mean energy expenditure for each recorded activity was calculated according its duration and correspondent physical activity level [28], that enables assessment of kayakers daily energy expenditure. The basal metabolic rate was calculated using the Cunningham equation [29].

Body composition and anthropometry

Weight (lightly dressed and barefooted) and height were measured with a stadiometer and electronic scale (SECA 701, UK), with a precision of 0.5cm and 100g respectively, according to the international recommended methodology [30]. Skinfold thicknesses were measured at four sites (bicep, tricep, subscapula, iliac crest) using a Harpenden caliper (John Bull, UK) [31], and body fat percentage was calculated using the formula of Siri [32].

Blood collection and handling

Antecubital venous blood samples were collected at the onset of the study (baseline) and after 4 weeks of supplementation (compliance), in pre-exercise and post-exercise conditions. The time of day for basal blood test was standardized to within 10 min for each subject, and all samples were taken between 08h00 and 09h30 after an overnight fast (>12 hours). The pre-exercise samples were drawn after subjects had been seated at rest for at least 15min and immediately before warming up. The post-exercise blood samples were obtained 15 min after the 1000m canoeing trial has ended, which allows time for subjects to get from the finish line to our research site. Although more blood collections in the post-exercise period would have been useful to provide additional information about the oxidative status response, the peak changes in oxidative stress biomarkers after relatively short-duration aerobic exercise seem to occur at this time point [2]. Blood samples were drawn into EDTA-treated vacutainer tubes and non-additive serum vacutainer tubes and immediately placed on ice in the dark until centrifugation. An aliquot of whole blood was separated to measure hematocrit and haemoglobin. Whole blood in serum tubes was allowed to clot for 30min at room temperature and then centrifuged at 2000 rpm for 10 minutes for serum separation. To obtain the plasma fraction, the remaining whole blood in EDTA-containing tubes was immediately centrifuged. Erythrocytes were washed and centrifuged three times with a 0.9% sodium chloride solution and lysed with ice-cold distilled deionized water. Serum, plasma and washed erythrocytes were separated into several aliquots and frozen at -80ºC for later biochemical analysis. In

evaluating the results, all plasma or serum post-exercise values were adjusted by the equation suggested by Dill and Costill [33], in order to correct for plasma volume shift, with the exception of SOD and GPx activities that were expressed relative to haemoglobin concentration. The pre-exercise analytical values were presented without a correction for plasma volume changes. All assay procedures were performed in duplicate.

Biochemical analysis

Haemoglobin and hematocrit were assessed from EDTA-treated blood using an automated analyzer (Horiba ABX Micros 60, ABX Diagnostic, Montpellier, France).

The concentrations of cholesterol and triglycerides in plasma and lipoprotein subfractions were determined by enzymatic colorimetric assays (kit 07 3680 5 for triglycerides, and 07 3664 3 for cholesterol; Hoffman-La Roche, Basel, Switzerland) in an autoanalyzer (Cobas Mira; Hoffmann-La Roche). LDL cholesterol (LDL-C) was calculated by means of Friedewald equation.

Uric acid was determined by an enzymatic method at 550 nm using a commercial kit (Horiba ABX A11A01670, ABX diagnostic, Montpellier, France), according the manufacturer's specifications.

Serum total antioxidant status (TAS) was measured spectrophotometrically using a commercial kit (Randox NX2332, Randox, Crumlin, UK). In brief, the assay is based on the reduction of free radicals (2,2'-azino-di-(3-ethylbenzothiazoline-6-sulphonate – $ABTS^{\bullet+}$), measuring the decreased of absorbance at 600nm. The radical cation $ABTS^{\bullet+}$ is formed by the interaction of ABTS with ferrylmyoglobin radical species generated by the activation of metmyoglobin with hydrogen peroxide (H_2O_2). The suppression of the absorbance of the radical cation $ABTS^{\bullet+}$ by plasma antioxidants was compared with that from Trolox. Samples were expressed in antioxidant capacity in millimoles per liter of Trolox equivalents.

Enzyme activities were analyzed according to the standard spectrophotometric–colorimetric procedures provided with the commercial kits in a Cobas Mira Plus analyzer (Roche Diagnostic Systems, Switzerland), at 37°C. Whole-blood GPX activity was determined spectrophotometrically using cumene hydroperoxide as the oxidant of glutathione (GSH) (Ransel RS 505, Randox, Crumlin, UK). The oxidised glutathione (GSSG) is immediately reduced to GSH by glutathione reductase, with a concomitant oxidation of NADPH to $NADP^+$. GPx activity was measured by the decrease in absorbance at 340 nm, and expressed in U/L. The

plasmatic activity of Gr was measured by monitoring the oxidation of NADPH to $NADP^+$ during the reduction of GSSG (Ransel GR 2368; Randox, Crumlin, UK), and expressed in U/L. A value of 10 U/L was considered as the detection limit. Cu, Zn-SOD activity was measured in washed erythrocytes after lysis by using a commercial kit (Ransod SD 125, Randox, Crumlin, UK). For this purpose, xanthine and xanthine oxidase were used to generate superoxide anion which reacts with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye. SOD activity was calculated by assessing the extent of inhibition of the reaction, on the basis of the change in the absorbance over 3 min at 505 nm, and data from the standard curve generated from purified SOD obtained from the manufacturer. SOD activity was expressed in U/g Hb.

Plasma liposoluble vitamins were extracted with n-hexane, after protein precipitation with ethanol, using β-apo-carotenal and tocol as internal standards [(21)]. The chromatographic analyses were performed by HPLC (Jasco, Japan) on a Chromolith performance RP-18 column (100x3mm, Merck), using methanol and acetonitrile (50:50) as mobile phase. Lutein (co-eluting with zeaxanthin), α-carotene, β-carotene and the carotenoids internal standard (β-apo-8'-carotenal) were quantified at 450 nm, retinol at 325 nm and lycopene at 425 nm, using a diode-array detector. Alpha-tocopherol quantification was based on the fluorescence readings (ex: 290 nm; em: 330 nm), using tocol as internal standard.

After deproteinization with metaphosphoric acid (0.75 M), serum vitamin C was determined by atomic absorption spectrometry (AAS), at 520nm using a Perkin Elmer Lambda 2 UV-Visible Spectrophotometer.

Serum creatine kinase (CK) and uric acid were determined at 37ºC using commercially available methods (Roche Products, UK), and an automated system (COBAS Mira Plus, Roche Diagnostic Systems, Switzerland).

The determination of thiobarbituric acid reactive substances (TBARS) in serum was performed using a commercial Kit (Oxi-tek TBARS assay kit, Zeptometrix Corporation, Buffalo, NY) according to the manufacturer's instructions. Briefly, serum (100 µL) was mixed with an equal volume of 8.1% sodium dodecyl sulfate (SDS) and 2.5 mL of 5% thiobarbituric acid/acetic acid reagent. Sample was incubated at 95ºC in capped tubes for 60 minutes and, thereafter, cooled to room temperature in an ice bath for 10 min before being centrifuged at 3000 rpm for 15 min. The supernatant was removed and its absorbance was read at 532 nm. The results are expressed as malondialdehyde (MDA) equivalents by interpolation from a MDA standard curve (0-100 nmol/mL).

Plasma IL-6 was measured with a commercially available solid-phase high-sensitivity ELISA Kit (Human Quantikine IL-6 Immunoassay D6050, R & D Systems, UK). The limit of detection of IL-6 was < 0.70 pg/mL.

Serum cortisol was measured with an ELFA (enzyme linked fluorescent assay - Vidas) technique using a commercial kit (cat. Nº30417, Biomerieux).

Data and statistical analysis

Statistical analyses were performed with the software SPSS 13.0 (Chicago, IL, USA). Group data were expressed as mean ± standard deviation. By convention, the a priori level of significance was set at $\alpha < 0.05$. All data were assessed for normality (one-sample Kolmogorov-Smirnov test). Baseline subjects' characteristics, nutritional parameters and plasma antioxidant levels between treatment groups were compared using independent sample t tests. Significant changes in pre-exercise plasma antioxidant levels before and after supplementation were analyzed using paired sample t tests. The other biochemical data were analyzed using mixed-model repeated-measures General Linear Model, with treatment (antioxidants vs. placebo) as the between-subject factor, and time (pre-supplementation vs. post-supplementation) and exercise (pre-exercise vs. post-exercise) as the 2 within-subject factors. Effect of each factor and combined factors (interaction) on each parameter were analyzed.

RESULTS

Subject's descriptive characteristics are presented in Table 1 for the antioxidant (n = 10, 7 males and 3 females) and placebo (n = 10, 7 males and 3 females) groups. There were no significant differences between treatment groups, also within each gender, with respect to these characteristics at baseline.

The dietary data is listed in Table 2. The supplemented and placebo groups did not differ in the estimated average energy expenditure and energetic and nutritional intakes ($P > 0.05$).

Table 1. General characteristics, lipoproteins and hematologic parameters of kayakers

	Placebo (n=10)	Supplement (n=10)
Age (years)	20.3 ± 3.3	19.1 ± 3.8
Height (cm)	176.1 ± 7.2	174.2 ± 9.7
Weight (kg)	72.7 ± 8.3	71.4 ± 9.1
BMI (kg/m^2)	23.2 ± 1.6	23.3 ± 1.9
Body fat (%)	16.1 ± 6.4	14.8 ± 8.2
Total cholesterol (mg/dL)	150.6 ± 45.9	154.3 ± 35.3
Triglycerides (mg/dL)	41.6 ± 17.4	38.3 ± 15.3
LDL-cholesterol (mg/dL)	89.5 ± 38.2	92.3 ± 27.2
HDL-cholesterol (mg/dL)	52.8 ± 9.9	54.3 ± 9.9
Haemoglobin (g/dL)	14.8 ± 1.0	14.9 ± 1.3
Hematocrit (L/L)	46.1 ± 3.3	46.8 ± 4.4

Table 2. Estimated daily energy expenditure, and energy and nutrient intake of kayakers

	Placebo (n=10)	Supplement (n=10)
Energy expenditure (kcal)	3351 ± 401	3417 ± 453
Energy intake (kcal)	3176 ± 583	3054 ± 678
Proteins (g)	146 ± 26	138 ± 31
Proteins (% of energy intake)	18.4 ± 2.0	18.1 ± 1.2
Carbohydrates (g)	400 ± 68	381 ± 76
Carbohydrates (% of energy intake)	50.4 ± 3.7	50.3 ± 4.5
Fibre (g)	24.9 ± 4.4	21.1 ± 4.0
Fat (g)	112 ± 29	109 ± 33
Fat (% of energy intake)	31.2 ± 3.4	31.6 ± 4.5
Saturated fat (g)	37.5 ± 11.1	36.5 ± 10.8
Monounsaturated fat (g)	44.5 ± 12.4	44.6 ± 14.9
Polyunsaturated fat (g)	15.8 ± 3.8	17.1 ± 6.1
Cholesterol (mg)	412 ± 75	383 ± 95
Vitamin C (mg)	101 ± 31	78 ± 35
α-tocopherol (mg)	7.3 ± 1.7	6.4 ± 1.3
Vitamin A (RE μg)	1285 ± 330	1147 ± 507
β-carotene (μg)	1701 ± 1135	1308 ± 803
Selenium (μg)	133 ± 33	130 ± 31
Zinc (mg)	19.8 ± 3.9	18.8 ± 4.7
Copper (mg)	1.64 ± 0.25	1.56 ± 0.34
Iron (mg)	20.2 ± 3.4	20.0 ± 5.2
Magnesium (mg)	367 ± 70	348 ± 77
Manganese (mg)	3.28 ± 1.11	3.17 ± 0.99

Prior supplementation, plasma antioxidant levels were similar in both groups (Table 3). In response to 4 weeks of supplementation, plasma α-tocopherol and β-carotene augmented in the antioxidant supplement group (P = 0.003 and P = 0.007, respectively) becoming higher than in the placebo group (P = 0.041 and P = 0.007, respectively), while the levels in the placebo group were unchanged. The levels of the other vitamins and the activities of antioxidant enzymes were similar in both groups, either in pre- and post-supplementation conditions, and were not modified significantly over the time course of the study and/or by supplementation. We observed a non-significant decrease in GPx after supplementation in the placebo group. There were no statistically significant differences in race performance between groups, either before or after intervention.

Table 3. Antioxidant vitamin concentrations, enzymatic activities and race time of kayakers before and after supplementation

	Placebo (n=10)			Supplement (n=10)		
	Before supplementation	After supplementation	*P*	Before supplementation	After supplementation	*P*
Vitamin C (μmol/L)	58.5 ± 20.9	57.3 ± 19.6	NS	53.9 ± 26.7	62.5 ± 22.2	NS
α-tocopherol (μmol/L)	20.5 ± 4.0	25.1 ± 8.5	NS	23.4 ± 5.1	33.1 ± 7.6*	0.003
Retinol (μmol/L)	2.08 ± 0.48	2.15 ± 0.54	NS	1.95 ± 0.19	2.20 ± 0.35	NS
β-carotene (μmol/L)	0.48 ± 0.13	0.60 ± 0.33	NS	0.57 ± 0.29	1.25 ± 0.59*	0.007
Lycopene (μmol/L)	0.61 ± 0.27	0.69 ± 0.24	NS	0.48 ± 0.24	0.59 ± 0.314	NS
Lutein + zeaxanthin (μmol/L)	0.42 ± 0.14	0.41 ± 0.17	NS	0.41 ± 0.17	0.34 ± 0.12	NS
GPx (U/L)	43.2 ± 25.9	30.9 ± 10.8	NS	43.4 ± 13.2	39.3 ± 10.0	NS
SOD (U/g Hg)	1330 ± 100	1231 ± 406	NS	1201 ± 149	1222 ± 318	NS
Gr (U/L)	72.0 ± 9.4	71.7 ± 12.7	NS	70.6 ± 9.8	67.8 ± 13.2	NS
Race time (s)	245.8 ± 19.7	246.9 ± 21.4	NS	248.9 ± 17.0	249.0 ± 17.3	NS

NS : non-significant; * significantly different from Placebo ($P < 0.05$); s: seconds

A significant interaction effect among Supplementation, Time and Exercise on plasma TAS was found (P = 0.034), with elevated values above pre-exercise after the kayak race in antioxidant supplemented athletes after the 4-week period, whereas a decrease in response to exercise was observed in all the other conditions. Uric acid and IL-6 increased significantly 15-minutes post-exercise (P = 0.032 and P = 0.039, respectively) and did not appear to be affected by time or treatment, as no statistical significant treatment and time main or interaction effects were noted ($P > 0.05$).

Table 4. Levels of markers of antioxidant status, muscle damage, lipid peroxidation and inflammation, and hormones at rest and after the exercise, before and after supplementation

	Before supplementation				After supplementation				
	Placebo (n=10)		Supplement (n=10)		Placebo (n=10)		Supplement (n=10)		
	Before exercise	After exercise	Before exercise	After exercise	Before exercise	After exercise	Before exercise	After exercise	*ANOVA*
TAS (mmol/L)	1.35 ± 0.16	1.23± 0.19	1.40 ± 0.18	1.07 ± 0.28	1.22 ± 0.09	1.19 ± 0.13	1.19 ± 0.12	1.23 ± 0.26	T × E × S (0.034)
Uric acid (mg/dL)	4.85 ± 1.03	5.03± 0.74	4.87 ± 1.03	5.51 ± 1.21	5.20 ± 1.07	5.31 ± 0.86	4.75 ± 1.43	5.13 ± 1.72	E (0.032)
Creatine kinase (IU/L)	269 ± 109	340 ± 135	221 ± 100	258 ± 110	182 ± 86	228 ± 103	195 ± 110	225 ± 119	E (<0.001) T × S (0.049)
TBARS (µmol/L)	5.8 ± 1.6	7.7 ± 1.9	5.5 ± 1.0	10.3 ± 2.6	6.1 ± 1.2	9.8 ± 2.8	7.3 ± 4.2	9.5 ± 1.9	E (<0.001)
IL-6 (pg/mL)	1.77 ± 0.75	2.20 ± 0.58	1.62 ± 0.72	1.83 ± 0.58	1.41 ± 0.45	1.83 ± 1.08	1.48 ± 1.32	1.93 ± 0.98	E (0.039)
Cortisol (nmol/L)	167 ± 40	188 ± 86	213 ± 60	240 ± 95	273 ± 78	284 ± 67	244 ± 68	247 ± 91	T × S (0.002)

E: exercise main effect; T: Time main effect; S: supplementation main effect; T × E: time and exercise interaction effect; T × S: time and supplementation interaction effect; T × E × S: time, exercise and supplementation interaction effect

Muscle protein leakage enhanced in response to the kayak trial (Exercise main effect, $P < 0.001$) and decreased from week 0 to week 4 (Time main effect, $P = 0.001$) more markedly in the antioxidant group (Supplementation × Time interaction effect, $P = 0.049$). Serum concentrations of TBARS rose significantly following the kayak bout (Exercise main effect, $P < 0.001$) and showed a tendency to augment over the study (Time main effect, $P = 0.069$) in all participants, although the difference was not statistical significant. Analysis of variance revealed an almost significant Supplementation × Time × Exercise interaction effect ($P = 0.085$), with a lower TBARS' increase in response to exercise in the antioxidant supplemented athletes after intervention. With regard to cortisol, participants responded differently to the treatment over the time course of the study (Treatment × Time interaction effect, $P = 0.002$), with the placebo group experiencing a greater increase from pre- to post-supplementation.

DISCUSSION

As expected from other works' findings [14], we observed a significant elevation of α-tocopherol and β-carotene concentrations after the administration of the antioxidant supplement, whereas the levels of those receiving the placebo remained unchanged. As previously found [22], retinol levels did not augment with carotenoids supplementation. In accordance to our data, lutein (1 mg) administration for 24 days was also unable to induce an increase in its plasma levels. The non-significant trend ($P = 0.149$) we found for decreased lutein concentrations in the supplemented group after supplementation might be explained by its competition for intestinal absorption with β-carotene. Actually, β-carotene supplementation demonstrated to significantly lower plasma lutein status [34, 35]. The supplementation led to a small increase in serum vitamin C concentrations according with other study using the same amount (400 mg) [5]. However, we did not observe a statistically significant increase as they did, in accordance with other authors findings' [22, 36], which may reflect the hydrosoluble nature of this vitamin and the existence of homeostatic control of its plasma levels [37]. Schröder and colleagues supplemented professional basketball players with 1000mg of vitamin C for 32 days [22] and 35 days [36] and their plasma levels also did not increment, though have avoided the drop seen in the placebo group [22].

We observed an increase in TAS with exercise in the post-supplementation period in the athletes that have received antioxidants. Despite antioxidant supplements did not have

ameliorated plasmatic antioxidant capacity at rest, in agreement with other works [18, 22], they seemed to help to counterbalance the exercise oxidative insult. In line with this, a supplementation with selenium, retinol, vitamin C and α-tocopherol has been shown to significantly augment the exercise-induced TAS increase, whereas a non-significantly reduction in TAS in resting conditions was observed [4]. The TAS response to kayak trial may have been influenced, at least partially, by the significant augmented uric acid synthesis (Exercise main effect, $P = 0.032$) consecutive to an enhanced activation of xanthine oxidase. Consistently with others studies' data [3, 5, 13], the post-kayaking increase above pre-exercise levels of this aqueous antioxidant did not differ significantly between treatment groups.

The higher TBARS levels at baseline we observed reflect the high physical stress sportsmen are exposed to during training [22] and the tendency to increase from pre- to post-supplementation period (Time main effect, $P = 0.069$) may be due to the cumulative augment through the season [38]. Although several studies indicate that antioxidant supplementation attenuate oxidative damage to lipids caused by exercise [3, 9, 24, 39-41], there are, likewise, published literature that suggests their ineffectiveness [5, 13, 15, 25, 42] or that even report a pro-oxidative effect [12, 43, 44]. The discrepancy in the research outcomes do not appear to be significantly influenced by the type, form (natural vs. synthetic) and dose of the antioxidant, the exercise protocol and the training level of subjects [45]. We noticed a trend of supplementation to lower the increase in TBARS after kayaking (Supplementation $\times$ Time $\times$ Exercise interaction effect, $P = 0.085$). A limitation of this study is that we only have a single blood sampling time (15min after exercise) and, since TBARS may follow specific time courses, it is unknown whether differences may have occurred latter [19]. Zembron-Lacny *et al*. [41] found lowered erythrocytes TBARS levels in the supplemented group only 30min after the exercise ended, that were not statistically evident immediately after. Despite TBARS is the most widely used biomarker of lipid peroxidation for it is inexpensive and easy to assay, there is some concern about its specificity and sensitivity. It has been demonstrated that antioxidant supplements significantly influenced lipid hydroperoxides and F_2-isoprostanes levels in response to exercise, while they did not alter TBARS [12].

Oxidative stress and inflammation have been proposed to be involved in muscle soreness and impaired recovery following damaging exercise [5, 11]. Antioxidant supplements can potentially attenuate inflammatory responses to exercise by neutralizing ROS, which are able to activate redox-sensitive signal transduction pathways that control cytokine production [9, 11]. Nieman *et al*. [43] found that the triathletes with the highest plasma F_2-isoprostanes levels also presented the highest plasma concentrations of IL-6. However, very few studies have studied

the effects of antioxidants on both exercise-induced oxidative stress and inflammation. In our work, antioxidants were ineffective in blunting TBARS increase with exercise and, consequently, did not influence IL-6 levels, in accordance with previous results [42]. By contrast, vitamin C and E supplementation demonstrated to be efficient in repressing exercise-induced lipid peroxidation as well as in attenuating cytokine (IL-6) translocation from contracting skeletal muscle into the circulation [9]. However, antioxidant supplementation did not down-regulate the cytokine response to an ultramarathon, despite having blunted the exercise-induced rise in F_2-isoprostanes levels seen in the placebo group [3]. Actually, the findings of studies involving antioxidant supplementation on alterations in markers of inflammation in response to exercise are equivocal. It has been suggested [11, 39] that differences in the subjects' training status, the type, dose an timing of supplementation, and/or the mode of exercise may explain the discrepant responsiveness to antioxidant supplementation. Nevertheless, conflicting data have been described either after supplementation with a combination of [9, 39] or single [5, 43] antioxidants, in athletic [3, 43] on non-athletic populations [5, 9, 12], and in response to concentric [9, 39], high-intensity intermittent running [5, 13] or ultraendurance [3, 43] exercise protocols. Our findings do not support that antioxidant supplements are useful in limiting the increase in cytokines levels in response to exercise in highly-trained athletes. The exercise studied was that kayakers' actually do in competition, and the inflammation it elicited was relatively small in comparison with earlier investigation. The response pattern of IL-6 to exercise is influenced by the type, duration and intensity of the exercise, the muscle mass recruited and damaged, the subjects' training status and the blood sampling time [8, 46, 47]. It is possible that the exercise used in the present investigation was not strenuous enough to induce significant inflammation, potentially disallowing for any effect to be noted. However, the magnitude of increment of cytokines did not appear to be crucial for an effect of antioxidant supplements [11]. Carbohydrate intake may modulate the cytokine release with exercise [3], but is not expected to have influenced our findings since their intakes were not different between treatment groups.

Resting cortisol concentrations were similar to those of the Polish Olympic kayaking team during training [48], and did not significantly increase after the kayak trial. Plasma cortisol responds to exercise in an intensity and duration dependent manner [36]. Interestingly, augmented cortisol levels were described after repeated short anaerobic cycling bouts but not after a single 1-min all-out test on a cycling ergometer [49]. The lack of effect might also be related to the very modest increase observed in IL-6 in response to exercise, since this cytokine is, recognizably, a stimulus for cortisol secretion [50]. In the present study a significant

Supplementation × Time interaction (P = 0.002) was observed, with the placebo group experiencing an augment in cortisol levels from the pre- to the post-supplementation period. The effects of antioxidant supplements on cortisol responses to exercise are variable, with some [9, 39] but not all [5, 13, 36, 43, 44] studies demonstrating an attenuation. The mechanism could involve a direct action on the hypothalamic–pituitary–adrenal axis [20, 39] or indirectly by modulating IL-6 [9]. However, even when antioxidant supplements significantly blunt cortisol, it was not always evidenced the desirable effect on the modulation of post-exercise neutrophilia and neutrophil function [39].

An extensive lipid peroxidation of membrane unsaturated fatty acids could lead to augmented permeability eliciting a CK efflux from the muscle cells. The similar increases in serum activity of CK with exercise in both groups indicate that antioxidants had no effect on sarcolemmal integrity, which is supported by the similar TBARS response to exercise with or without supplementation. The exercise employed in the present investigation produced similar muscle damage (CK: AOX ↑ 16%, PLA ↑ 25%) and lipid peroxidation (TBARS: AOX ↑ 30%, PLA ↑ 59%) to that rowers exhibited after performing a laboratory 2000m rowing trial which lasted ~ 407 seconds (CK: AOX ↑ 19%, PLA ↑ 32%; TBARS: AOX ↑ 70%, PLA ↑ 71%). Our observation that supplementation had no effect on exercise-related increases in CK is in agreement with the literature [5, 6, 13, 40, 41], and this outcome do not seem to depend of the amount, duration and type of antioxidant supplemented, as well as the subjects' fitness status and the mode of exercise investigated. This calls into question the role of ROS in the etiology of muscle damage, that may instead be a by-product of muscle damage [11]. ROS play a dual mission regarding to the regulation of muscle contraction. While the low ROS levels continuously generated by skeletal muscle under basal conditions are involved in force production, higher concentrations, as those produced during strenuous exercise, can contribute to the development of muscle fatigue [51], by inhibiting calcium sensitivity [52]. Thus, antioxidant interventions have the potential to delay muscle fatigue, as was demonstrated by the prolonged time to fatigue by 26% during intense cycling in trained individuals after the intravenous infusion of N-acetylcysteine [53]. However, research generally does not support that nutritional antioxidants supplementation have ergogenic effects, with very few exceptions [14]. Antioxidant supplementation does not appear to enhance exercise performance in non-deficient athletes [16]. However, Aguiló *et al*. [14] showed that administration of vitamin E and β-carotene, for 3 months, and vitamin C, for 15 days, induces a lower maximal blood lactate concentration after a maximal exercise and might improve the efficiency of aerobic metabolism. We must considerer that ROS-mediated decline in contractile function can be a protective mechanism in

order to limit further muscle injury, and the use of antioxidant supplements can override that protection, causing greater exercise-induced damage and increasing recovery times [52]. We theorize that the minor decrease in the resting CK levels post-supplementation we observed in the antioxidant supplemented athletes (Supplementation × Time interaction effect, $P = 0.041$) can be illustrative of this phenomenon. Interestingly, significantly [15] and non-significantly [12, 19, 54] elevated plasma CK activities in the supplement vs. placebo groups have already been encountered in the days (1 to 10) following exercise, even when had been initially minor [54]. Our data biochemically support the previously reported observations of a delayed recovery in muscle function [24] and an attenuated muscle torque at specific velocities following vitamin C supplementation [5]. ROS may have an important physiological role in muscle regeneration and attempts to prevent their post-exercise production through antioxidant intervention can be detrimental to the recovery process, by hampering the phagocytes ROS-mediated removal of degraded tissue proteins [24] and posterior muscle fiber regeneration [54]. An antioxidant supplementation after muscle injury caused by eccentric exercise has been shown to increase further tissue damage in the following days [12]. Furthermore, ROS are recognized modulators of cell signaling and gene expression and might, this way, be involved in the adaptive responses to exercise [1]. Actually, there is evidence that decreasing the exercise-induced ROS formation suppresses activation of important cellular signals implicated in cellular adaptations to training [55, 56]. Furthermore, vitamin C administration mitigated, albeit non-significantly, the training-induced increases in VO_{2max} [57]. Moreover, the International Olympic Committee warned that as many as one in four supplements may result in a positive test [58]. Not to mention that a recent paper associates β-carotene, vitamin A, and vitamin E supplementation with increased mortality [59]. Thus, the recommendation that athletes should take antioxidant supplements needs to be seriously reconsidered, either for health- and performance-related issues. Actually, exercise itself may be already an antioxidant [1].

CONCLUSIONS

The results of the present research indicate that the supplementation with a combination of antioxidants does not afford protection against lipid peroxidation, muscle damage and inflammation elicited by a 1000m kayaking bout in highly trained athletes. Our findings do not recommend the use of the antioxidant supplements for purposes of attenuating exercise-induced oxidative stress and muscle injury in already well-trained individuals. Further

experiments are needed to explore the hypothesis that acute antioxidant administration before damaging exercise can alleviate lipid peroxidation and muscle injury, whereas chronic supplementation may delay muscle recovery.

ACKOWLEDEGMENTS

We gratefully acknowledge to the Portuguese Canoeing Federation, and respective kayakers, coaches and physician who participated in this study.

REFERENCES

1. Gomez-Cabrera M, Domenech E, Viña J. Moderate exercise is an antioxidant: Upregulation of antioxidant genes by training. Free Radic Biol Med. 2008; 44(2):126-31
2. Bloomer RJ, Goldfarb AH, McKenzie MJ. Oxidative stress response to aerobic exercise: Comparison of antioxidant supplements. Med Sci Sports Exerc. 2006; 38(6):1098-105
3. 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. 2004; 36(10):1329-41
4. Margaritis I, Palazzetti S, Rousseau AS, Richard MJ, Favier A. Antioxidant supplementation and tapering exercise improve exercise-induced antioxidant response. J Am Coll Nutr. 2003; 22(2):147-56
5. Thompson D, Williams C, McGregor SJ, Nicholas CW, McArdle F, Jackson MJ, et al. Prolonged vitamin C supplementation and recovery from demanding exercise. Int J Sport Nutr Exerc Metab. 2001; 11(4):466-81
6. Mastaloudis A, Traber MG, Carstensen K, Widrick J. Antioxidants did not prevent muscle damage in response to an ultramarathon run. Med Sci Sports Exerc. 2006; 38(1):72-80
7. Maughan RJ, Donnelly AE, Gleeson M, Whiting PH, Walker KA, Clough PJ. Delayed-onset muscle damage and lipid peroxidation in man after a downhill run. Muscle Nerve. 1989; 12(4):332-6

8. Willoughby DS, Vanenk C, Taylor L. Effects of concentric and eccentric contractions on exercise-induced muscle injury, inflammation, and serum IL-6. J Exerc Physiol Online. 2003; 6(4):8-15

9. Fischer CP, Hiscock NJ, Penkowa M, Basu S, Vessby B, Kallner A, et al. Supplementation with vitamins C and E inhibits the release of interleukin-6 from contracting human skeletal muscle. J Physiol. 2004; 558(Pt 2):633-45

10. Vassilakopoulos T, Karatza MH, Katsaounou P, Kollintza A, Zakynthinos S, Roussos C. Antioxidants attenuate the plasma cytokine response to exercise in humans. J Appl Physiol. 2003; 94(3):1025-32

11. Peake JM, Suzuki K, Coombes JS. The influence of antioxidant supplementation on markers of inflammation and the relationship to oxidative stress after exercise. J Nutr Biochem. 2007; 18(6):357-71

12. Childs A, Jacobs C, Kaminski T, Halliwell B, Leeuwenburgh C. Supplementation with vitamin C and N-acetyl-cysteine increases oxidative stress in humans after an acute muscle injury induced by eccentric exercise. Free Radic Biol Med. 2001; 31(6):745-53

13. 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. 2003; 89(3-4):393-400

14. Aguiló A, Tauler P, Sureda A, Cases N, Tur J, Pons A. Antioxidant diet supplementation enhances aerobic performance in amateur sportsmen. J Sports Sci. 2007; 25(11):1203-10

15. Avery NG, Kaiser JL, Sharman MJ, Scheett TP, Barnes DM, Gomez AL, et al. Effects of vitamin E supplementation on recovery from repeated bouts of resistance exercise. J Strength Cond Res. 2003; 17(4):801-9

16. Finaud J, Lac G, Filaire E. Oxidative stress: Relationship with exercise and training. Sports Med. 2006; 36(4):327-58

17. Ramel A, Wagner KH, Elmadfa I. Plasma antioxidants and lipid oxidation after submaximal resistance exercise in men. Eur J Nutr. 2004; 43(1):2-6

18. Morillas-Ruiz JM, Villegas Garcia JA, López FJ, Vidal-Guevara ML, Zafrilla P. Effects of polyphenolic antioxidants on exercise-induced oxidative stress. Clin Nutr. 2006; 25:444-53

19. Bloomer RJ, Falvo MJ, Schilling BK, Smith WA. Prior exercise and antioxidant supplementation: effect on oxidative stress and muscle injury. J Int Soc Sports Nutr. 2007:4:9

20. Davison G, Gleeson M. The effect of 2 weeks vitamin C supplementation on immunoendocrine responses to 2.5 h cycling exercise in man. Eur J Appl Physiol. 2006; 97(4):454-61

21. Rousseau AS, Hininger I, Palazzetti S, Faure H, Roussel AM, Margaritis I. Antioxidant vitamin status in high exposure to oxidative stress in competitive athletes. Br J Nutr. 2004; 92(3):461-8

22. 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. 2000; 21(2):146-50

23. Falvo MJ, Bloomer RJ. Review of exercise-induced muscle injury: Relevance for athletic populations. Res Sports Med. 2006; 14(1):65-82

24. Close GL AT, Cable T, Doran D, Holloway C, McArdle F, MacLaren DP Ascorbic acid supplementation does not attenuate post-exercise muscle soreness following muscle-damaging exercise but may delay the recovery process. Br J Nutr. 2006; 95(5):976-81

25. Viitala PE, Newhouse IJ, La Voie N, Gottardo C. The effects of antioxidant vitamin supplementation on resistance exercise induced lipid peroxidation in trained and untrained participants. Lipids Health Dis. 2004; 3:14

26. Venkatraman JT, Feng X, Pendergast D. Effects of dietary fat and endurance exercise on plasma cortisol, prostaglandin E2, interferon-gamma and lipid peroxides in runners. J Am Coll Nutr. 2001; 20(5):529-36

27. Food and Nutrition Board, Institute of Medicine. Dietary Reference Intakes: Applications in Dietary Assessment. Washington, D.C.: National Academic Press; 2000.

28. Ainsworth BE, Haskell WL, Whitt MC, Irwin ML, Swartz AM, Strath SJ, et al. Compendium of physical activities: an update of activity codes and MET intensities. Med Sci Sports Exerc. 2000; 32(9 Suppl):S498-504

29. Thompson J, Manore M. Predicted and Measured Resting Metabolic Rate of Male and Female Endurance Athletes. J Am Diet Assoc. 1996; 96(1):30-34

30. Frisancho R. Anthropometric standards for the assessment of growth and nutritional status. Michigan: University of Michigan Press; 1990.

31. Norton K WN, Carteer L, Kerr D, Gore C, Marfell-Jones M. Measurement techniques in anthropometry. In: Norton K OT, editor. Anthropometrica: A Textbook of Body Measurement for Sports and Health Courses. Sydney: University of New South Wales Press; 1996. p. 25-76.

32. Siri W. Body composition from fluid spaces and density: analysis of methods. In: Brozek J, Henschel A, editores. Techniques for Measuring Body Composition. Washington, D.C: National Academy of Sciences-National Research Council; 1961. p. 223-44.

33. Dill DB, Costill DL. Calculation of percentage changes in volumes of blood, plasma, and red cells in dehydration. J Appl Physiol. 1974; 37(2):247-8

34. Kostic D, White WS, Olson JA. Intestinal absorption, serum clearance, and interactions between lutein and β-carotene when administered to human adults in separate or combined oral doses. Am J Clin Nutr. 1995; 62(3):604-10

35. Micozzi MS, Brown ED, Edwards BK, Bieri JG, Taylor PR, Khachik F, et al. Plasma carotenoid response to chronic intake of selected foods and β- carotene supplements in men. Am J Clin Nutr. 1992; 55(6):1120-25

36. Schröder H, Navarro E, Mora J, Galiano D, Tramullas A. Effects of alpha-tocopherol, beta-carotene and ascorbic acid on oxidative, hormonal and enzymatic exercise stress markers in habitual training activity of professional basketball players. Eur J Nutr. 2001; 40(4):178-84

37. Tauler P, Aguiló A, Gimeno I, Fuentespina E, Tur JA, Pons A. Response of blood cell antioxidant enzyme defences to antioxidant diet supplementation and to intense exercise. Eur J Nutr. 2006; 45(4):187-95

38. Schippinger G, Wonisch W, Abuja PM, Fankhauser F, Winklhofer-Roob BM, Halwachs G. Lipid peroxidation and antioxidant status in professional American football players during competition. Eur J Clin Invest. 2002; 32(9):686-92

39. Davison G, Gleeson M, Phillips S. Antioxidant supplementation and immunoendocrine responses to prolonged exercise. Med Sci Sports Exerc. 2007; 39(4):645-52

40. Traber MG. Relationship of vitamin E metabolism and oxidation in exercising human subjects. Br J Nutr. 2006; 96 Suppl 1:S34-7

41. Zembron-Lacny A, Szyszka K, Sobanska B, Pakula R. Prooxidant-antioxidant equilibrium in rowers: Effect of a single dose of vitamin E. J Sports Med Phys Fitness. 2006; 46(2):257-64

42. Nieman DC, Henson DA, McAnulty SR, McAnulty L, Swick NS, Utter AC, et al. Influence of vitamin C supplementation on oxidative and immune changes after an ultramarathon. J Appl Physiol. 2002; 92(5):1970-7

43. Nieman DC, Henson DA, McAnulty SR, McAnulty LS, Morrow JD, Ahmed A, et al. Vitamin E and immunity after the Kona Triathlon World Championship. Med Sci Sports Exerc. 2004; 36(8):1328-35

44. McAnulty SR, McAnulty LS, Nieman DC, Morrow JD, Shooter LA, Holmes S, et al. Effect of alpha-tocopherol supplementation on plasma homocysteine and oxidative stress in highly trained athletes before and after exhaustive exercise. J Nutr Biochem. 2005; 16(9):530-7

45. Williams SL, Strobel NA, Lexis LA, Coombes JS. Antioxidant requirements of endurance athletes: implications for health. Nutr Rev. 2006; 64(3):93-108

46. Edwards KM, Burns VE, Ring C, Carroll D. Individual differences in the interleukin-6 response to maximal and submaximal exercise tasks. J Sports Sci. 2006; 24(8):855-62

47. Steinberg JG, Ba A, Brégeon F, Delliaux S, Jammes Y. Cytokine and oxidative responses to maximal cycling exercise in sedentary subjects. Med Sci Sports Exerc. 2007; 39(6):964-68

48. Wozniak A, Drewa G, Wozniak B, Drewa T, Mila-Kierzenkowska C, Czajkowski R, et al. Effect of cryogenic temperatures and exercise on lipid peroxidation in kayakers. Biol Sport. 2005; 22(3):247-60

49. Meyer T, Gabriel HH, Rätz M, Müller HJ, Kindermann W. Anaerobic exercise induces moderate acute phase response. Med Sci Sports Exerc. 2001; 33(4):549-55

50. Steensberg A, Fischer CP, Keller C, Møller K, Pedersen BK. IL-6 enhances plasma IL-1ra, IL-10, and cortisol in humans. Am J Physiol Endocrinol Metab. 2003; 285(2 48-2):E433-E37

51. Reid MB. Invited Review: redox modulation of skeletal muscle contraction: what we know and what we don't. J Appl Physiol. 2001; 90(2):724-31

52. Reid MB. Free radicals and muscle fatigue: of ROS, canaries, and the IOC. Free Radic Biol Med. 2008; 44(2):169-79

53. Medved I, Brown MJ, Bjorksten AR, Murphy KT, Petersen AC, Sostaric S, et al. N-acetylcysteine enhances muscle cysteine and glutathione availability and attenuates fatigue during prolonged exercise in endurance-trained individuals. J Appl Physiol. 2004; 97(4):1477-85

54. Beaton LJ, Allan DA, Tarnopolsky MA, Tiidus PM, Phillips SM. Contraction-induced muscle damage is unaffected by vitamin E supplementation. Med Sci Sports Exerc. 2002; 34(5):798-805

55. Gomez-Cabrera MC, Martinez A, Santangelo G, Pallardo FV, Sastre J, Viña J. Oxidative stress in marathon runners: interest of antioxidant supplementation. Br J Nutr. 2006; 96 Suppl 1:S31-3

56. Khassaf M, McArdle A, Esanu C, Vasilaki A, McArdle F, Griffiths RD, et al. Effect of vitamin C supplements on antioxidant defence and stress proteins in human lymphocytes and skeletal muscle. J Physiol. 2003; 549(Pt 2):645-52

57. Gomez-Cabrera MC, Domenech E, Romagnoli M, Arduini A, Borras C, Pallardo FV, et al. Oral administration of vitamin C decreases muscle mitochondrial biogenesis and hampers training-induced adaptations in endurance performance. Am J Clin Nutr. 2008; 87(1):142-49

58. International Olympic Committee. Nutrition for Athletes: A Practical Guide to Eating for Health and Performance.; 2003. Available at: http://multimedia.olympic.org/pdf/en_report_833.pdf. Accessed April 9, 2008

59. Bjelakovic G, Nikolova D, Gluud LL, Simonetti RG, Gluud C. Mortality in randomized trials of antioxidant supplements for primary and secondary prevention: Systematic review and meta-analysis. JAMA. 2007; 297(8):842-57

4. MAIN CONCLUSIONS

The findings of our studies allow us to conclude that:

- Despite having an overall enhanced antioxidant capacity (SOD, α-tocopherol, β-carotene and α-carotene), kayakers/canoeists present augmented lipid peroxidation (TBARS) and muscle damage (CK) markers in resting conditions in comparison with sedentary controls

- A 1000m kayak race causes oxidative stress and damage in male kayakers/canoeists, as demonstrated by the decrease in TAS levels and the increase in CK activity and TBARS and IL-6 levels

- There were no major changes in the kayakers/canoeists redox status markers between the competitive periods of two consecutive seasons, being more relevant the differences between different training and competing periods of the same season

- The supplementation with an antioxidant mixture increases antioxidant capacity at rest and in response to a 1000m kayak race in highly trained kayakers/canoeists, but neither has ergogenic properties nor attenuates significantly exercise-induced lipid peroxidation and inflammation; furthermore, supplementation may hamper muscle recovery during regular training.

5. FUTURE PERSPECTIVES

Fortunately, the results of our research left us with more doubts!

Regarding exercise-induce oxidative stress, numerous *old* questions still remain to be answered definitively: have athletes higher or lower risk of oxidative damage than sedentary or physically active individuals? Do athletes performing large training loads have augmented antioxidants requirements? Should they increase their antioxidant dietary intake, particularly during periods of intensive training and competition? Are there gender differences in susceptibility to ROS oxidative damage?

However, more exciting is the shift regarding the role of ROS and the interest of antioxidants administration on exercise-induced muscle injury that we have been witnessing since we formulated our initial hypothesis. Actually, our findings also support a U-turn concerning antioxidant supplementation efficacy in this context. This is an interesting result meriting further research. It is likely that ROS generated by exercise may have an important function in muscle regeneration and its abolishment by massive antioxidant use may delay recovery. This contradicts the traditional view that the formation of ROS is a negative side effect of physical exercise that must be prevented. The ROS-associated decline in muscle function may be a desirable effect, by limiting further damage. Furthermore, ROS appears to modulate the activation of important cellular signals implicated in beneficial cellular adaptations to training, and chronically elevated antioxidants intake may have an adverse impact on the expression of these effects. This can help to understand why the antioxidant defences in skeletal muscle are lower relatively to other tissues. Perhaps the key nutrient to reduce the muscle damage is, after all, the most obvious.

On the other hand, we observed a tendency to an attenuated lipid peroxidation in response to exercise after antioxidant supplements. This led us to theorize that antioxidants may be beneficial when acutely administrated previously to an exercise known to produce a great oxidant insult, by limiting oxidative attack to unsaturated lipids, whereas the use over a longer period could hamper the phagocytes-mediated muscular regeneration process, namely after muscle injuring-exercises. The optimal levels and timing to generate the maximal effects in attenuating peroxidation without the resulting side effects needs to be further examined.

The data of investigations involving less exercise-adapted individuals, and thus more susceptible to damage when performing unaccustomed exercises, might be the reason of the

idiosyncratic use of antioxidants supplement by the athletic population. The paucity in studies on athletes in their habitual training environment deserves more attention by the research community, because it does not seem wise to extrapolate directly the outcomes of those works without questioning.

Also, we have to consider the inter-individual differences in susceptibility to oxidative stress and the wide fluctuations in redox status throughout the season. Probably the use of antioxidant supplements can be detrimental or beneficial according the competitive period, type of exercise performed, type, timing and duration of supplementation, athletes' training status, diet and smoking habits, etc. These issues need to be addressed in future investigations, using more accurate biomarkers and sophisticated methodologies.

Future work is required before firm recommendations can be made regarding the efficacy and safety of supplementing highly trained individuals with antioxidants. Certainly, the research on this topic will be effervescent in the next years, but predicting definitive conclusions about its clinical usefulness in the athletic world is gambling at this moment. The path to get there is going to be the determination of tissues' antioxidant status on an individual basis in order to identify personal requirements and formulate effective antioxidant strategies to a specific situation and condition, but we still do not know the answer.