Oxidative Stress Biomarker bei unterschiedlichen Belastungsintensitäten

von

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Inhaltsverzeichnis

VORWORT

Das Studium der Medizinischen Wissenschaft brachte wichtige und praxisrelevante Bereicherungen für meine Fachkompetenz. Durch die Freiheit der selbstständigen Studienplangestaltung liegt es in der eigenen Hand das Nützliche und Sinvolle für jeden einzelnen auszusuchen. Das schafft Motivation, macht Spaß, bedingt aber auch eine gewissenhafte Bewusstmachung der eigenen Karriereziele. Ich wurde in den letzten Semestern auch von „Publikationsglück“ begleitet, was die ursprünglich geplante Einreichung von Originalarbeiten als Dissertation möglich machte. Ich darf daher sagen, dass ich jede Minute dieses Studiums genoss.

Ich möchte dieses Vorwort auch nutzen um meinen beiden Betreuern Prof. Günther Schwaberger und Prof. Joachim Greilberger herzlichst zu danken. Ihr wart immer für mich zur Stelle und es ist eine Freude mit Euch zusammen arbeiten zu dürfen. Günther wünsche ich an dieser Stelle alles Gute für den neuen Lebensabschnitt. Ich hoffe trotzdem, dass der Kontakt zu Dir in Deinem Ruhestand aufrecht erhalten bleibt. Mit Joachim werden mich hoffentlich noch viele gemeinsame Arbeitsjahre verbinden.

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Two weeks of antioxidant supplementation can increase lipid peroxidation in trained men

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RUNNING TITLE: lipid peroxidation, exercise and antioxidant supplementation

Abstract

Aim: To assess the effects of an encapsulated antioxidant concentrate (EAC) and exercise on lipid peroxidation (LIPOX) and plasma antioxidant enzyme glutathione peroxidase (pl-GPx). **Methods**: 8 trained male cyclists (VO_{2max} >55mL/kg · min^{-1}) participated in this randomized, placebo-controlled, double-blinded, cross-over study to conduct 4 cycle ergometer bouts: 2 moderate exercise bouts over 90 minutes at 45% and 2 strenuous exercise bouts at 75% of individual VO_{2max} for 30 minutes. The first two exercise tests – one moderate and one strenuous test – were conducted after 4 weeks wash out and after 12 and 14 days of EAC (107 IU vitamin E, 450 mg vitamin C, 36 mg ß-carotene, 100 µg selenium) or placebo treatment. Following another 4 weeks wash out subjects were given the opposite capsule treatment and repeated the two exercise tests. Physical exercise training was equal across the whole study period and nutrition was standardized by a menu plan the week prior to the tests. Blood was collected before exercise (BE), immediately post exercise (IE), 30 minutes (30M) and 60 minutes (60M) after each test. Plasma samples were analyzed for LIPOX marker malondialdehyde (MDA) and antioxidant enzyme pl-GPx. **Results**: MDA concentrations were significantly increased after EAC supplementation at rest BE and after moderate exercise ($p<0.05$). MDA concentrations showed no differences between treatments after strenuous exercise ($p>0.1$). Pl-GPx concentrations decreased at all time points of measurement after EAC treatment ($p<0.05$). **Conclusions**: The EAC induced an increase of LIPOX as indicated by MDA and decreased pl-GPx concentrations pre and post exercise.

KEY WORDS: malondialdehyde, glutathione peroxidase, athletes, strenuous exercise

Introduction

Dietary intake of vitamins C, E and ß-carotene is an exogenous contribution to support the body´s antioxidant system. Together with several endogenous antioxidant compounds, e.g. the enzymatic antioxidants glutathione peroxidases (GPx) or superoxide dismutases (SOD), these vitamins help to prevent the body against oxidative stress in its cells and tissues (Hamilton 2007).

Physical exercise enhances oxygen consumption (VO_2) by the organism, particularly by heart and skeletal muscle. The increase in VO_2 is associated with a rise in the production of reactive oxygen and nitrogen species (RONS). Even in trained skeletal muscles, exercise of high intensity and certain duration can overwhelm the antioxidant system with RONS generation, leading to a condition called oxidative stress (Powers et al. 2004).

Frequent targets of RONS attacks are poly unsaturated fatty acids from cell membranes. They are oxidized through a series of reactions that are collectively called lipid peroxidation (LIPOX) (Radak et al. 2001).

Antioxidant supplementation containing vitamins C and E, sometimes in combination with ß-carotene and/or selenium, can decrease pre and post exercise concentrations of LIPOX marker malondialdehyde (MDA) (Goldfarb et al. 2005, Kanter et al. 1993). On the other hand it is also reported that these vitamin supplementations had no significant effects on pre and post exercise MDA concentrations (Bryant et al. 2003, Bloomer et al. 2006, Schroeder et al. 2001).

In this study we investigated a trained group of male cyclists which considered to use a specific encapsulated antioxidant concentrate (EAC) in their daily life, containing the vitamins C, E and ß-carotene + selenium. Besides the scientific interest, it was of practical relevance to observe the effect of this supplement on LIPOX, in combination with cycling exercises of different

intensities. We proofed bioavailability of the supplements´ vitamins and, additionally, we estimated the influence of this EAC on concentrations of plasma glutathione peroxidase (pl-GPx). This enzyme is a member of the selenocysteine-containing GPx family. It is regarded as an important antioxidant enzyme in plasma and as a valuable marker for vascular oxidative stress (Rush and Sandiford 2003).

METHODS

Subjects: 8 healthy trained men (maximum oxygen uptake, VO_{2max} >55mL/kg · min^{-1}), non smokers, volunteered to participate in this study. Each subject was informed to perform no hard physical training 3 days prior to the exercise tests, to document diet, sleep time and well-being the week prior to each test. Exclusion criteria were use of tobacco products, chronic or excessive alcohol consumption, recent surgery or illness, use of drugs, or dietary supplements at least 4 week prior to the study. After explanation of all experimental procedures all subjects signed informed consent prior to participation in this study. The protocol of the study was approved by the local Ethics Committee at the Medical University of Graz, Austria.

Study design: This study was double-blinded, randomized, and placebo-controlled with a cross-over design. VO_{2max} testing, anthropometric measurements and a blood chemistry panel were carried out before start of investigation, followed by a 4 week wash out. On day 1 all baseline blood measurements were conducted and subjects received placebo capsules or an EAC. After 12 days treatment with placebo or EAC the first 45% VO_{2max} exercise test was carried out. On day 14 the first 75% VO_{2max} exercise test was conducted. After another 4 week washout the procedure was repeated using the opposite capsule treatment for each participant. A study timetable is provided in Supplementary File 1. Before all exercise tests subjects were checked by a physician for health status as precondition to perform the tests.

Dietary treatment and analyses: All exercise tests were performed 3 hours after a standardized breakfast (Supplementary File 2). The week before the exercise tests subjects received a 7 day menu plan with recipes to guarantee same food (-preparation), nutrient and fluid intake the last 5-7 days prior to each exercise test. The menu plan provided carbohydrate rich food like pasta, rice, potatoes, bread, at least 5 servings of fruit and vegetables daily, 2 servings of meat or fish per week, and plenty of water, fruit juice intake and polyunsaturated fatty acids. The participants were taught and instructed by a dietician to meet all particular items of the menu plan. Food intake was analyzed using "opti diet" software (version 3.12., GOEmbH, Germany).

Body composition determination: Before and after wash out and treatment periods, and before all exercise tests lean body mass, body fat content and distribution were estimated by a computerized optical device Lipometer (Moeller Messtechnik, Austria).

Antioxidant and placebo treatment: EAC or placebo capsules were given for 2 week, always after a 4 week wash out. Daily EAC treatment (2 capsules of "Oxytex Quatro-Quencher", Wörwag Pharma, Germany) contained 450 mg vitamin C (L-ascorbic acid), 107 IU vitamin E (RRR-α-tocopherol), 36 mg ß-carotene (all-trans-ß-carotene) and 100 µg selenium (given as 100 mg selenium yeast). Placebo capsules had identical appearance and were filled with microcrystalline cellulose. Subjects were instructed to consume 1 capsule with breakfast and the other one with dinner. Compliance was assessed by the returned capsule count and by questionnaires.

Physical activity records: The subject´s training program was standardized during the whole study period, beginning 4 weeks prior to day 1. Each week was documented in subjects training program: 3-4 times a week endurance training, mainly mountainbiking, for at least 90 minutes

with intensities at or between 45% and 75% VO_{2max}. 2 - 3 times a week gymnastics for at least 60 minutes with exception the week prior to the exercise tests.

Incremental exercise tests: All subjects performed an incremental cycle ergometer test (Schiller "ERG 900S" Ergometer, Switzerland) at 80 rpm to determine individual VO_{2max}. After a 3 minute rest phase sitting inactive on the ergometer, work rate started at 40 W for 3 minutes and was increased 20 W every minute until voluntary exhaustion. A standard electrocardiogram was recorded during the entire test, which was supervised by a physician.

Respiratory gas exchange (RGE): RGE variables were measured throughout all exercise tests using a breath-by-breath mode with data being stored in 10s intervals. During the tests, subjects breathed through a facemask. RGE-data were continuously obtained by means of a portable open-air spiroergometry system (MetaMax I, Cortex Biophysik,, Germany). Heart rate (HR) was monitored throughout the tests using a commercially available heart rate monitor (Polar Vantage NV, Polar Electro, Finland).

45% and 75% VO_{2max} exercise tests: 2 different exercise intensities were performed at 80 rpm: at 45% of individual VO_{2max} with a duration of 90 minutes, and at 75% of individual VO_{2max} with a duration of 30 minutes. Subjects performed each of the two 45% VO_{2max} tests after 12 days of placebo or EAC treatment and each of the two 75% VO_{2max} tests after 14 days of placebo or EAC treatment. Similar to the VO_{2max} test, subjects started with the same procedure: rest phase, 40 watt at the 1st step, thereafter work rate was increased by 20 watt every minute until the workload at 45% or 75% of VO_{2max} was reached as calculated from the incremental VO_{2max} test. Workload was adjusted every 5 min to maintain the target %VO_{2max} intensity.

Blood collection and sample preparation: 12 mL of venous EDTA- and heparinized blood from a forearm vein was collected by an indwelling cannula (17G/1.4mm: “TriCath In”) before

exercise (BE), immediately post exercise (IE), 30 minutes (30M) and 60 minutes (60M) after each test. After centrifugation at 3000 rpm for 10 minutes at 4°C, EDTA- and heparinized plasma were removed. EDTA samples were frozen at -70°C until analysis of MDA and at -196°C until analysis of vitamins E and ß-carotene. The EDTA-plasma for determination of vitamin C was mixed with 9% metaphosphoric acid and centrifuged again by 3000 rpm for 4 minutes. The supernatant was placed into cryo-tubes and coated with nitrogen. Samples were frozen in liquid nitrogen at -196°C until analysis. For lactate determination 20 µL blood was drawn from the earlobe. Concentrations were measured at rest, and every 5 minutes at 45% or 75% of VO_{2max} via Eppendorf fully enzymatic analysis ("EBIO plus" lactate analyzer, Eppendorf, Germany).

Determination of malondialdehyde: 500 µL EDTA-plasma was used for HPLC-determination as previously described by Khoschsorur et al. (2000). Briefly: The MDA-thiobarbituric acid-adduct was separated via HPLC and detection followed fluorimetrically at 550 nm. Concentrations were determined by calibration curve of a 1,1,3,3-tetramethoxypropane standard solution (0.1-0.2 µM).

Determination of pl-GPx: Heparinized plasma was frozen at -70°C until analysis. We used a sandwich ELISA assay (Biomedica, Vienna, Austria) for plasma specific glutathione peroxidase (pl-GPx). The kit employs a microtiter plate precoated with affinity purified polyclonal anti-human pl-GPx. After washing, bound pl-GPx is detected spectrophotometrically (405 nm) using a biotinylated polyclonal antibody to pl-GPx, with amplification via streptavidin coupled to alkaline phosphatase.

Determination of vitamins: Alpha-tocopherol, ß-carotene and vitamin C: EDTA-samples were used for determination of vitamins E and ß-carotene via HPLC by UV-detection as described by

Jakob and Elmadfa (14). Vitamin C was analyzed electrochemically as described by Maxwell et al (15).

Blood chemistry panel: Standard blood chemistry panels were made before and after wash out periods and treatments, using EDTA plasma from peripheral blood. Analysis was performed using routine clinical chemistry methods and the clinical chemistry analyzer "Eurolyser" (Dia Team, Diagnostica und Arzneimittel Großhandel GmbH, Linz, Austria). Additionally, creatine kinase, uric acid, total protein and C-reactive protein were measured before and after all exercise tests by "Eurolyser". This goes to haemoglobin and iron concentrations as well which were assessed by using the Advia clinical analyzer (Bayer, Leverkusen, Germany). To avoid circulatory hemodynamic artefacts caused by exercise dehydration we corrected all blood values by total plasma protein and haemoglobin concentrations.

Statistical Analysis: MDA, pl-GPx and vitamin data were analyzed using a 2 (treatment = grouping factor) x 2 (type of exercise = grouping factor) x 4 (time = within factor) repeated measurements analysis of variance (ANOVA). If appropriate, for selected time points, additional analyses were conducted by t-test. Subject characteristics, lactate, and standard blood variables were also compared by repeated measures ANOVA. Statistical analysis was performed by SPSS software, version 12.0. All data presented as mean ± SD. Statistical significance was set at $p<0.05$.

RESULTS

Subjects characteristics, exercise and nutrition data: No significant changes were noted for weight, body fat, lean body mass and watt performance over the whole study period ($p>0.05$). Also no chances were noted for lactate and creatine kinase kinetics during the intensity-equal exercise tests ($p>0.05$). Table 1 provides the characteristics of the 8 subjects. Clinical blood

chemistry parameters showed no remarkable concentrations or changes within the study period (data not shown). Supplement compliance was > 95% and no indigestibility or intolerance was reported. Before each exercise series food intake was matched. Analysis of the 7 d menu plan is presented in Supplementary File 3.

VO_2 data: There was no treatment effect on the observed oxygen uptakes for both intensities at 45 % and at 75% VO_{2max} ($p>0.05$). At the 75% VO_{2max} tests subjects performed at 74.8 ± 2.4% of individual VO_{2max}. At the 45% VO_{2max} tests subjects performed at 45.1 ± 2.2% of individual VO_{2max}.

Malondialdehyde (MDA) : BE concentrations increased from 0.78µmol/L ± 0.07µmol/L at baseline and after placebo treatment, to 1.16µmol/L ± 0.11µmol/L ($p<0.05$) after EAC treatment (Fig. 1A-B). After the 45% VO_{2max} tests (Fig. 1A), MDA concentrations increased at IE, 30M and 60M from 0.76µmol/L ± 0.10µmol/L after placebo treatment to 1.21µmol/L ± 0.14µmol/L after EAC supplementation ($p<0.05$). There was an exercise x time x treatment effect at the strenuous 75% VO_{2max} test ($p<0.05$, Fig. 1B): With EAC treatment, MDA concentrations decreased from BE to IE to similar levels as measured after placebo treatment. These IE concentrations remained stable at 30M and 60Mwith both treatments (0.83µmol/L ± 0.13µmol/L).

Glutathione peroxidase in plasma (pl-GPx): Pl-GPx concentrations decreased at all time points of measurement after EAC supplementation: from 1.16mg/L ± 0.18mg/L at baseline and after placebo treatment to 0.46mg/L ± 0.16mg/L after EAC supplementation ($p<0.05$). The types of exercise had no influence on pl-GPx at any time point of measurement (Fig. 2A-B).

Vitamin C: There were no effects of EAC or placebo treatment observed on vitamin C concentrations at the 45% VO_{2max} tests (data not shown). There was a time and an exercise x

time effect at the strenuous 75% VO_{2max} test with EAC treatment ($p<0.05$, Fig. 3): we observed increased values from BE to IE (from 9.1mg/L ± 1.4mg/L to 13.9 mg/L ± 1.9mg/L), returning again to baseline concentrations at 30M.

Vitamin E and ß-carotene: There was a significant effect of EAC supplementation in both vitamins showing increased concentrations at all measured time points. α-tocopherol concentrations increased from 7.9mg/L ± 2.3mg/L to 13.6 ± 3.0mg/dL ($p<0.05$, data not shown). ß-carotene concentrations increased from 31µg/L ± 22µg/L to 89µg/L ± 18µg/L ($p<0.05$, data not shown). The types of exercise had no influence on these vitamins.

DISCUSSION

Data from this study show that, 1) EAC supplementation led to an increase of MDA concentrations at rest BE, indicating an increase of LIPOX in trained men. 2) With EAC treatment, strenuous aerobic exercise for 30 minutes at 75% of individual VO_{2max} led to a decrease of post exercise MDA concentrations. 3) With EAC treatment and at 75% VO_{2max} exercise intensity for 30 minutes, increase in plasma vitamin C concentrations was observed IE, returning to BE values at 30M, and 4) EAC supplementation attenuated pl-GPx concentrations in plasma at all measured time points.

Malondialdehyde (MDA) is a widely used and commonly regarded marker to estimate LIPOX (Khoschsorur et al. 2000, Toroser et al. 2007, Ishii et al. 2008, Deepa et al. 2008). The main finding in our study was the significantly higher MDA concentrations after 2 weeks of EAC treatment at rest BE and after 45% of individual VO_{2max} (Fig. 1A-B). These results are in contrast to other exercise studies investigating LIPOX and MDA in which trained men were supplemented with vitamins E + C (Bloomer et al. 2006, Bryant et al. 2003). Bloomer et al. (2006) found no effect on MDA after 2 week of vitamin C (1000 mg/day) and E (400 IU/day)

treatment. Bryant et al. (2003) found a non-significant increase in pre exercise MDA concentrations after 3 weeks application of 1000 mg/day vitamin C + 200 IU/day vitamin E. Both studies did not use ß-carotene. Therefore, we tended to assume that the lack to a significant MDA increase might be the additional ß-carotene content of 36mg/day provided by our applied EAC.

But this hypothesis is attenuated by the findings of Kanter et al. (1993), who observed significantly decreased MDA concentrations after supplementation with a vitamin mixture consisting of 1000 mg/day vitamin C, ~880 IU/day vitamin E and 30 mg/day ß-carotene. Consequently, the increase of MDA in our study cannot be referred to ß-carotene alone.

The treatments of the compared studies were all higher in daily concentrations of vitamin C (1000mg) and vitamin E (200-880 IU) compared to ours (vitamin C 450 mg, vitamin E 107 IU). The Kanter study was equivalent to the Bloomer and Bryant studies in vitamin C (1000 mg daily) but highest in vitamin E (880 IU/day vs. 200-400 IU/day) and used additional ß-carotene (30 mg/day), the last was similar to our study (32 mg ß-carotene/day). Further, the Kanter treatment lasted 6 weeks, Bloomers´ 2 weeks, Bryants´ 3 weeks, ours 2 weeks. Therefore, the influence of a vitamin C, E, and ß-carotene mixture on LIPOX must depend on the applied proportions of concentrations of these vitamins, and on duration of treatment as well.

In our study, the plasma concentrations of vitamin C - a possible scavenger of generated vitamin E and carotene radicals and those may contribute to LIPOX (Buettner 1993) – showed no increase after EAC supplementation. On the other hand, plasma vitamin E and ß-carotene concentrations increased significantly, ß-carotene even above normal values (Stacewicz-Sapuntzakis et al 1987): from 31µg/L in mean up to 90µg/L.

We conclude that a lack of vitamin C availability in plasma at rest and after 45% VO_{2max} intensity, combined with the significantly increased ß-carotene concentrations, was responsible for insufficient regeneration of vitamin E and particularly of ß-carotene. Hence, this disproportion of these 3 vitamins in plasma led to enhanced LIPOX as indicated by increased MDA concentrations.

It still remains the question why there was a lack of vitamin C availability in plasma under resting and mild exercise conditions? We suggest that the concentration of vitamin C in the EAC and/or it´s bioavailability was not adequate to increase plasma concentrations and/or a surplus in plasma was stored up into the adrenal gland (see next paragraph).

Another finding was the significant decrease of MDA values from BE to IE after EAC treatment at the 75% VO_{2max} test, and the increase of vitamin C concentrations at the same time. In contrast, no effect of exercise on both substances was found at the other 3 tests. We provide a hypothesis discussing a connection between MDA and vitamin C to explain this phenomenon: Vitamin C increased from BE to IE after EAC treatment at the 75% VO_{2max} test (Fig. 3). Although this change was still within the normal range from 8-14mg/L (Baessler et al. 2002), this significant effect indicates a mobilisation of vitamin C during strenuous aerobic exercise. The adrenal gland is the major source of vitamin C efflux into the circulation during exercise and the rapid rise in plasma vitamin C is associated with the release of cortisol during exercise (Pedersen et al. 1998). We assume that vitamin C has mainly been delivered by the adrenal gland which can save vitamin C in concentrations more than 150 times compared to plasma (Gleeson et al. 1987). The EAC treatment provided a surplus in plasma and this was stored up into the gland. The vitamin C supply to plasma at 75% VO_{2max} intensity braked LIPOX processes and decreased post exercise MDA concentrations. At 30M, vitamin C concentrations returned to BE

values because the vitamin was primarily consumed for vitamin E and ß-carotene regeneration and further, in recovery, no more vitamin C affecting cortisol efflux was needed.

Glutathione peroxidase (GPx) is a selenocysteine-containing antioxidant enzyme which scavenges hydrogen peroxide and organic hydroperoxides (Maddipati et al. 1987). Of the 5 known GPx isoforms, only one is found in extracellular space, which is primarily synthesised in the kidneys proximal tubules and hepatic cells (Avissar et al. 1994 and 1989, Chu et al. 1992). Decreased pl-GPx concentrations and activities are found in cerbrovascular and hemodyalis patients and are accociated with decreased bioavailability of vascular nitric oxide, enhanced platelet activation, and extracellular oxidative stress (Voetsch et al. 2007).

In this investigation we found decreased pl-GPx concentrations after 2 weeks EAC treatment (Fig. 2A-B) although 100µg selenium (Se) was supplemented daily. Compared to reported pl-GPx concentrations from Australia and Canada (Jacobson et al 2006, Rush & Sandiford 2003), baseline values of our subjects were lower: ~1mg/L compared to 3 – 30mg/L. Our detected concentrations were closer to those reported from Latvian people (Hagmar et al. 1998). 7-day food selenium intake of our subjects was adequate (Supplementary File 3), but unfortunately we did not measure plasma Se at baseline to estimate possible deficiency prior to the experimental procedure.

In untrained men and women, Rush and Sandiford (2003) detected higher pl-GPx concentrations than we did in our trained subjects. These researchers reasoned that the level of fitness might have an influence on pl-GPx concentrations. To our best knowledge, no study exists in which different levels of fitness were compared with pl-GPx concentrations. Therefore, hypothesis is still critical that level of fitness might influence pl-GPx concentrations until further research provides adequate data. However, due to the cross-over design, the 7-day menu plan prior to the

exercise tests and the 3 day recovery time prior to each test, nutritional influences (between baseline and exercise tests) or effects of heavy exercise (prior to the testing date) on pl-GPx can be excluded. Consequently, evidence remains that the EAC treatment led to a kind of down regulation of expression for this enzyme and/or to decreased secretion from hepatic or renal cells.

Although the number of 8 subjects in this study is small our results demonstrate substance as we standardized strictly nutritional intake, training regimen, the exercise protocol and level of fitness. We conclude that the used antioxidant supplementation induced an increase of LIPOX accompanied with effects on pl-GPx to lower concentrations. Therefore, we did not recommend to our probationers to apply this supplement for daily use.

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Table 1

Characteristics of the 8 study subjects

Variable	
Age; yr	28 ± 2.2 yrs
Height; cm	178.2 ± 5.4
Weight; kg	72.6 ± 4.1
Total body fat; %	11.3 ± 2.3
Lean body mass; kg	64.7 ± 3.6
VO_{2max}; mL/kg/min	60.4 ± 4.5
Max. workload; W	360 ± 20
Lactate max; mmol/L	11.9 ± 2.7
Heart rate max	189 ± 8
Ventilatory threshold (VT2); % of VO_{2max}	78.6 ± 3.1
Lactate turn point 2 (LTP2); % of VO_{2max}	77.8 ± 2.8
Lactate during exercise; mMol/L	
at 45% of VO_{2max}	1.72 ± 0.41
at 75% of VO_{2max}	3.63* ± 2.34
Creatine kinase; U/L	
at 45% of VO_{2max}	127 ± 24
at 75% of VO_{2max}	251* ± 58

Values are means ± SD. Comparison of repeated measures data resulted in $P>0.1$ (repeated measures ANOVA). Lactate and CK concentrations showed differences between the two exercise intensities (*$P < 0.05$, repeated measures ANOVA).

Figure Legends

Figure 1A-B

Malondialdehyde (MDA) concentrations in µmol/L plasma, three hours after a standardized breakfast, at 45% (duration: 90 min) and 75% (duration: 30 min) of individual VO_{2max} exercise intensity. Supplementation either with encapsulated antioxidant concentrate (EAC) or placebo (N=8). Data are presented in means ± SD. Statistical analyses via repeated measures ANOVA. * indicates statistical significance.

Four time points of blood collection:

BE = before exercise

IE = immediately after the exercise test

30M = 30 minutes after the exercise test

30H = 30 hours after the exercise test

Figure 1A: Effect of EAC supplementation (dark columns) to increased MDA concentrations pre- and postexercise ($p<0.05$).

Figure 1B: Effect of exercise x time x treatment at the strenuous 75% VO_{2max} test after EAC treatment ($p<0.05$): at BE, MDA was increased after 2 weeks EAC supplementation followed by decreased post exercise concentrations, similar to placebo values.

Figure 2A-B

Plasma glutathion peroxidase (pl-GPx) concentrations in mg/L plasma. See note for Figure 1. Significant effect of EAC supplementation (dark columns) led to decreased enzyme concentrations at all measured time points ($p<0.05$).

Figure 3

Vitamin C concentrations in mg/L plasma. See note for Figure 1. There was a time and an exercise x time effect at the strenuous 75% VO_{2max} test at the end of the EAC treatment period (dark columns; $p<0.05$): we observed increased values from BE to IE, reaching BE and placebo values again at 30M.

Figure 1A

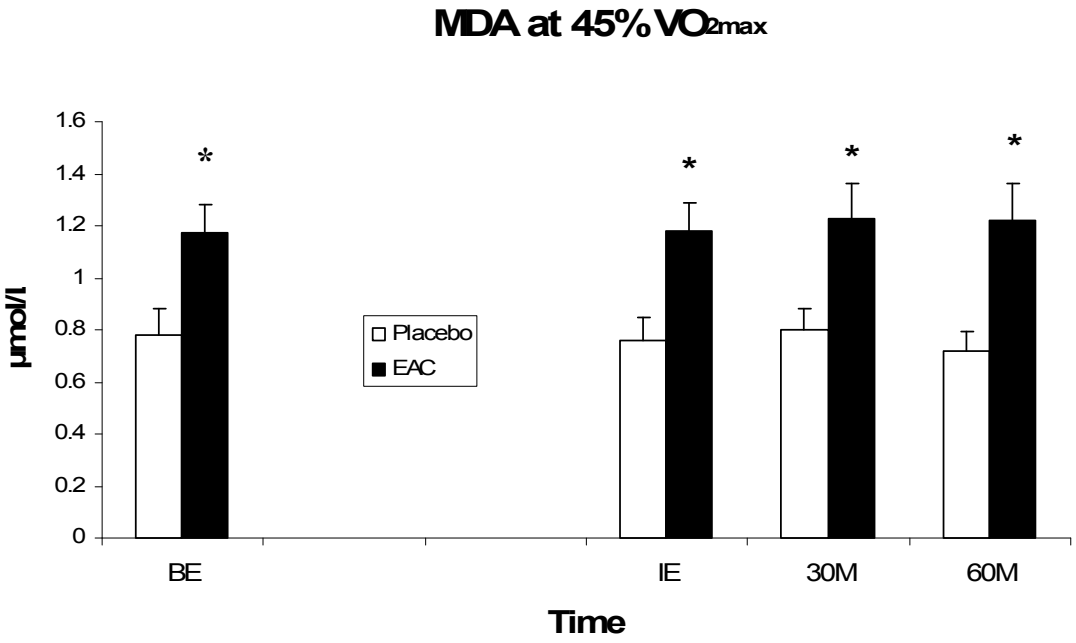

MDA at 45% VO2max
µmol/l
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Placebo
EAC
*
*
*
*
BE
IE
30M
60M
Time

Figure 1B

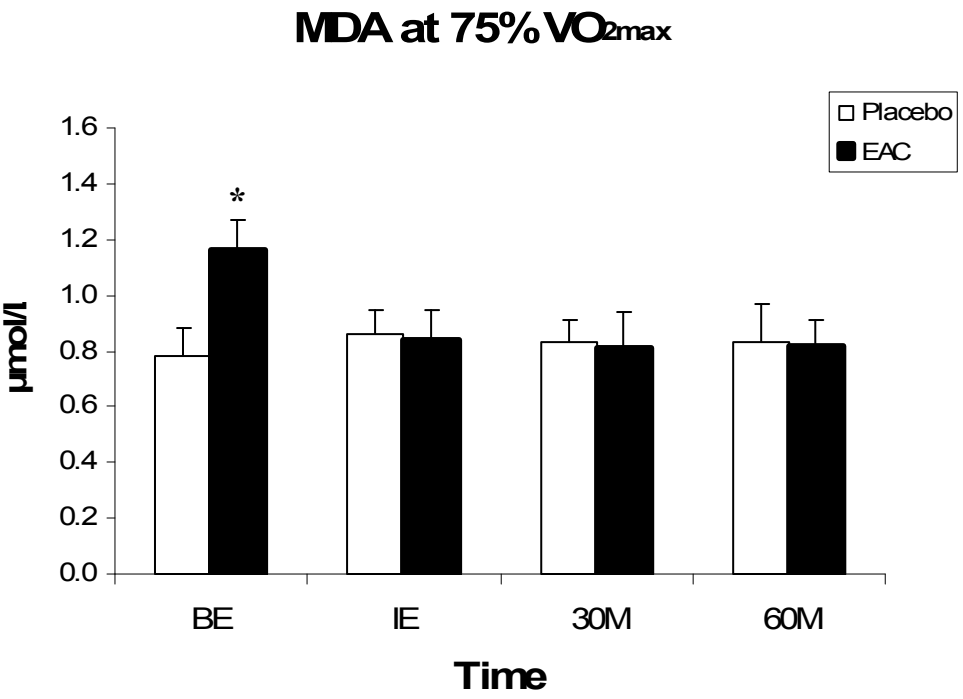

MDA at 75% VO2max
Placebo
EAC
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
µmol/l
*
BE
IE
30M
60M
Time

Figure 2A

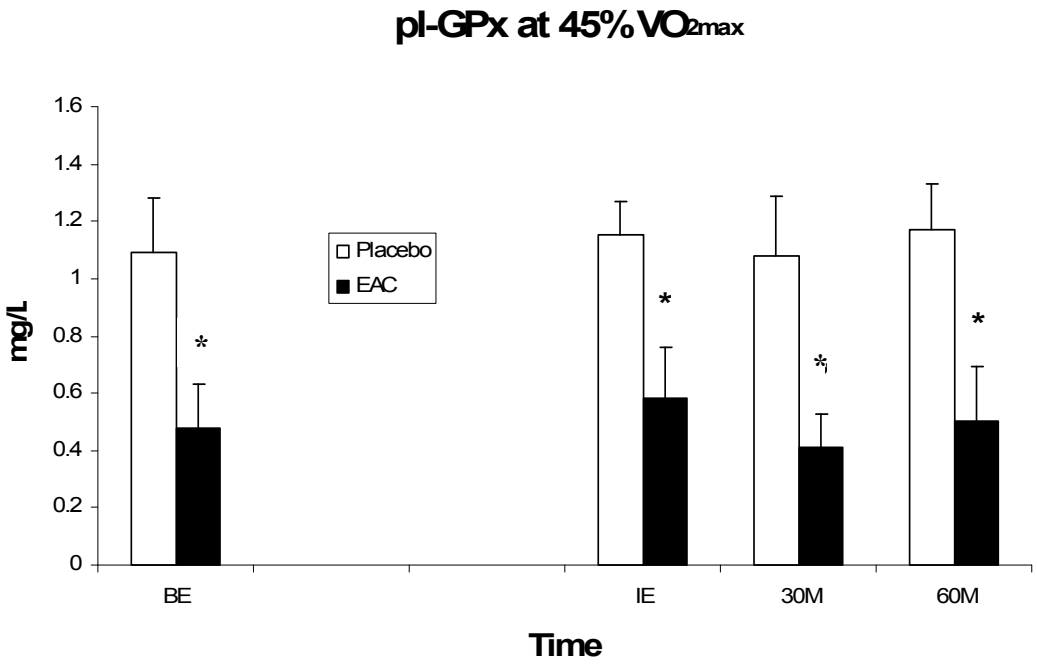

pl-GPx at 45% VO2max
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
mg/L
Placebo
EAC
*
*
*
*
BE
IE
30M
60M
Time

Figure 2B

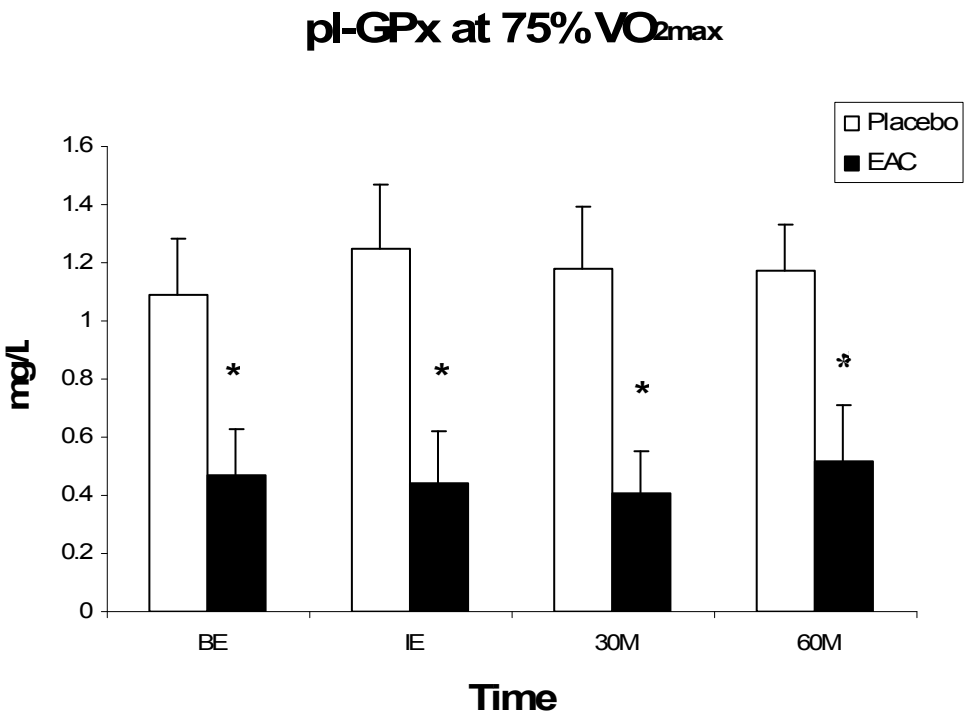
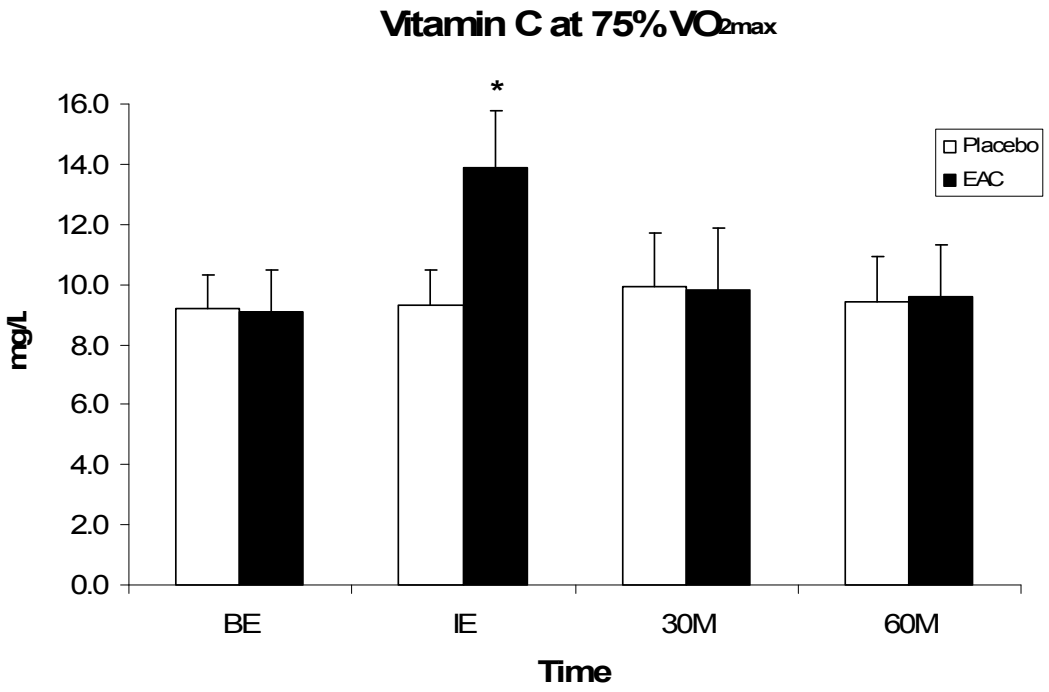

pl-GPx at 75% VO2max
Placebo
EAC
mg/L
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
*
BE
IE
30M
60M
Time

Figure 3


Vitamin C at 75% VO2max
*
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
mg/L
Placebo
EAC
BE
IE
30M
60M
Time

Supplementary File 1

Study Timetable

Study weeks and days	Action
- 4 weeks	• informed consent • incremental exercise test; VO_{2max} testing • body composition and physician check • 1st blood chemistry panel
- 4 weeks to day 1	• 1st wash out from any supplements
- 4 weeks to day 56	• physical activity records
day 1	• 1st baseline blood measurements at rest: MDA, GPx, vitamins • 2nd blood chemistry panel • body composition check • 1st dispense of placebo and EAC capsules
days 1 to 14	• placebo or EAC treatment
day 7	• hand out and instruction to the 7 d menu plan with recipes
days 8 to 14	• food and fluid intake guided by menu plan • no intense training (max. heart rate: 140/min)
day 12	• body composition and physician check • 1st 45% VO_{2max} test with a duration of 90 min • MDA, GPx, vitamins and lactate measurements
day 14	• body compostition and physician check • 1st 75% VO_{2max} exercise tests over 30 min • MDA, GPx , vitamins and lactate measurements • 3rd blood chemistry panel
days 15 to 42	• 2nd wash out from possible supplementation
day 43	• 2nd baseline blood measurements at rest: MDA, GPx, vitamins • 4th blood chemistry panel • body composition check • 2nd dispense of placebo and EAC capsules
days 43 to 56	• placebo or EAC treatment
days 50 to 56	• food and fluid intake guided by the menu plan as provided on day 7 • no intense training (max. heart rate: 140/min)
day 54	• body composition and physician check • 2nd 45% VO_{2max} test with a duration of 90 min • MDA, GPx, vitamins and lactate measurements
day 56	• body composition and physician check • 2nd 75% VO_{2max} exercise tests over 30 min • MDA, GPx, vitamins and lactate measurements • 5th blood chemistry panel

Supplementary File 2

Composition of the standardized breakfast consumed 3 hours prior to the exercise tests

Food	kJ	Protein (g)	Fat (g)	Carbohydrates (g)
Coffee with milk (low fat) or				
Tea with lemon and honey (10g)	180	0-2	0-2	4-10
3 slices wheat or rye bread	1390	8	1	75
Butter 20g	652	-	16	-
Marmalade/jam 30g	343	-	-	19
One slice low fat ham	331	6	6	-
One piece of cheese	490	16	5	-
250mL fruit juice	836	2	-	46
250mL water	-	-	-	-
Total	4222	32-34	28-30	144-150
Meal energy %		13%	27%	60%

Supplementary File 3

Analysis of the 7 d menu plan compared to recommended daily allowance (RDA) by the German, Austrian, and Swiss Nutrition Societies (2000).

Nutrient	RDA	menu plan	% RDA
kJ	13011	12935	99
Protein; g/kg body weight	0.8	1.37	17 1
Carbohydrate; % of kJ	>50	61	
Fat; % of kJ	30	25.5	85
Cholesterol; mg	300	277	92
Water; L	1.41	3.8	270
Vitamin C; mg	100	99	99
Vitamin E; mg	14	13.3	95
ß-Carotene; mg	6	5.7	95
Folate; µg	400	409	102
Vitamin B_{12}; µg	3	4	133
Sodium, mg	550	1050	192
Calcium; mg	1000	1101	110
Magnesium; mg	350	423	121
Iron; mg	10	14.1	141
Copper; mg	1-1.5	1.6	107
Zinc; mg	10	12.8	128
Manganese; mg	2-5	2.9	~100
Selenium; µg	30-70	67	~100

Acceptance letter

13-Jan-2009

Dear Dr. Lamprecht:

It is a pleasure to accept your manuscript entitled "Two weeks of antioxidant supplementation can increase lipid peroxidation in trained men" in its current form for publication in the International Journal of Sport Nutrition & Exercise Metabolism. The comments of the reviewer(s) who reviewed your manuscript are included at the foot of this letter.

Thank you for your fine contribution. On behalf of the Editors of the International Journal of Sport Nutrition & Exercise Metabolism, we look forward to your continued contributions to the journal.

Sincerely,

Dr. Ron Maughan

Editor, International Journal of Sport Nutrition & Exercise Metabolism

r.j.maughan@lboro.ac.uk

Confirmation

(from the Int J Sport Nutr & Exerc Metab)

International Journal of Sport Nutrition & Exercise Metabolism - Manuscript ID IJSNEM_2008_0125.R2

28-Jan-2009

Dear Dr. Lamprecht:

Your manuscript titled "Two weeks of antioxidant supplementation can increase lipid peroxidation in trained men" has been successfully updated online in preparation for posting in Online Preview in the International Journal of Sport Nutrition & Exercise Metabolism.

Please mention the above manuscript ID in all future correspondence or when calling the office for questions. If there are any changes in your street address or e-mail address, please log in to Manuscript Central at http://mc.manuscriptcentral.com/hk_ijsnem and edit your user information as appropriate.

You can also view the status of your manuscript at any time by checking your Author Center after logging in to http://mc.manuscriptcentral.com/hk_ijsnem .

Thank you for uploading your manuscript to the International Journal of Sport Nutrition & Exercise Metabolism.

Sincerely,

International Journal of Sport Nutrition & Exercise Metabolism Managing Editor, Human Kinetics

Protein modification responds to exercise intensity and antioxidant supplementation

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Running title: Exercise and antioxidant effect on proteins

ABSTRACT

Purpose: To assess the effects of different exercise intensities and antioxidant supplementation on plasma protein modification. **Methods**: Trained men (N = 41) from a homogenous population were randomly assigned to perform cycle ergometer exercise either at 70% or 80% of individual VO_{2max}. Each intensity group was randomly assigned to received either juice powder concentrate (JPC 70%, N = 11; JPC 80%, N = 10) or placebo (Plac 70%, N = 10; Plac 80%, N = 10) capsules for 28 wk. Four controlled exercise bouts and blood collections were conducted at baseline, and study wk 4, 16 and 28. Blood samples were drawn before (BE), immediately after (IE), then 30 min (30M) and 30 h (30H) post exercise. These samples were analyzed to estimate concentrations of carbonyl groups on plasma proteins (CP) and the redox state of human serum albumin (HSA). **Results:** In the placebo group, CP concentrations increased at 80 % of VO_{2max} IE and 30M, returning to pre-exercise concentrations by 30H ($P < 0.05$). At both 16 and 28 wk, the placebo groups had significantly higher BE- and 30H CP concentrations than the JPC groups ($P < 0.05$). The reduced fraction of HSA, human mercapt albumin (HMA), decreased at all four exercise tests at both exercise intensities IE and 30M, returning to pre-exercise values by 30H ($P < 0.05$). Supplementation had no influence on HSA. **Conclusions:** These results indicate that CP concentrations increase with 80 % VO_{2max} intensity. The JPC group had lower baseline CP levels after 16 and 28 weeks and no exercise induced CP increase. HSA is reversibly shifted to a more oxidized state by recent intense exercise.

Key Words: albumin redox state, carbonylated proteins, defined exertion, dietary supplements

INTRODUCTION

Paragraph 1 Oxidative stress related exercise studies and reviews report that physical exercise of sufficient intensity and duration can result in increased generation of reactive oxygen and nitrogen species (3, 33). These are eliminated by the body´s antioxidant system, including both endogenous compounds and exogenous nutritional substances, such as those found in plant foods. If the antioxidant defense is inadequate, oxidative processes dominate, resulting in "oxidative stress" which can lead to damaged lipids, proteins, carbohydrates and DNA (6, 27, 33). In athletes, oxidative stress results in muscular oxidative damage (11), muscle soreness (30), loss of skeletal muscle force production (16), or impaired immunity (8, 25, 26).

Paragraph 2 Several researchers report exogenous antioxidant supplementation attenuates oxidative stress biomarkers such as carbonyl groups on protein (CP), malondialdehyde (MDA) or 8-hydroxydeoxyguanosine (3, 9, 18, 34). However, there is also some evidence that high dose antioxidant supplementation may increase lipid peroxidation after exhaustive exercise (15, 24).

Paragraph 3 The redox state of human serum albumin (HSA) is a potential approach to investigate the extracellular redox state in exercise. This is the main protein in extracellular fluids and its redox state appears to be influenced by physical exercise (13). This protein contains a single cysteine residue not involved in a disulfide bond. In HSA, cysteine-34 can exist in several forms: the reduced form with a free thiol group (human mercaptalbumin, HMA); in a reversibly oxidized form cysteine-34 forms a disulfide with low molecular weight thiol compounds such as cysteine (human nonmercaptalbumin 1, HNA1); or, a further oxidized form, as a sulfenic or sulfonic acid state (human nonmercaptalbumin 2, HNA2). The main fraction of serum albumin, HMA, is thought to participate in maintaining of an appropriate redox potential in blood or interstitial fluid. While the oxidation step from HMA to HNA1 is readily reversible, the oxidation to HNA2 is less so (5). To our knowledge this is

the first study of the possible influence of defined exercise intensities or antioxidant treatment on HSA redox states.

Paragraph 4 Any effects of defined aerobic exercises during a long term antioxidant supplementation on CP concentration or HSA redox state is not established. This study investigates these effects in a homogenous, trained and compliant cohort of police Special Forces. We hypothesized that the extent of exertion and/or use of a commercially available encapsulated juice powder concentrate (JPC) might influence the concentrations of CP or the redox state of HSA in plasma of trained men. We evaluated the influence of defined cycle exercise bouts at 70 and 80 % of individual maximum oxygen uptake (VO_{2max}) on these parameters, and repeated the exercise test after 4, 16 and 28 wk of JPC or placebo use.

METHODS

Paragraph 5 **Subjects.** All subjects provided written informed consent prior to participation in this investigation. This study was conducted in compliance with the guidelines of the Declaration of Helsinki for Research on Human Subjects and was approved by the Human Ethics Committee at the Medical University of Graz, Austria. The study cohort consisted of 41 healthy men, non smokers, police Special Forces ("Cobra"), who volunteered to participate. All subjects completed a medical history, diet analysis and physical activity questionnaire prior to the beginning of the investigation to determine eligibility. Exclusion criteria included use of tobacco products, chronic or excessive alcohol consumption, recent surgery or illness, and use of pharmaceuticals, drugs, or dietary supplements, within the 4 wk prior to the study baseline visit. Medications (i.e. antibiotics) or injuries during the study period were documented and the duration and time point of these events determined continued inclusion or exclusion from the study. Further determinants for eligibility included that subjects had trained aerobically at least three days per week for a minimum of a year prior to participation and had a minimum level of aerobic fitness as assessed with maximal testing (VO_{2max} > 45mL·kg^{-1}·min^{-1}). Body fat content and distribution were estimated by the

computerized optical device (Lipometer, Möller Messtechnik, Graz, Austria) to determine subcutaneous adipose tissue thickness (22).

Paragraph 6 **Study design.** The study was randomized, double-blind, and placebo-controlled. Anthropometric measurements, first diet analysis, a first standard blood chemistry panel and determination of VO_{2max} were carried out 4 wk prior to the start of the experimental period. Subjects were randomly assigned, in a double-blind fashion, to either the 70% or 80% VO_{2max} group and then further divided to take either the JPC- or the placebo capsules, resulting in four study groups. At study weeks 4, 16 and 28, exercise tests were repeated. All subjects were checked by a physician before each endurance test.

Paragraph 7 **Incremental exercise tests.** All subjects performed an incremental cycle ergometer exercise test (Schiller "ERG 900S" Ergometer, Switzerland). After three minutes sitting on the Ergometer (rest), work rate started at 40 W for three minutes and was increased by 20 W every minute until voluntary exhaustion. A standard 12-lead ECG was recorded during the entire physician supervised test.

Paragraph 8 **Respiratory gas exchange**. Respiratory variables were measured throughout all tests using a facemask and breath-by-breath mode with data stored in 10-s intervals. Oxygen uptake, carbon dioxide output, minute ventilation, breathing rate and tidal volume were continuously obtained by means of a portable open-air spiroergometry system (MetaMax I, Cortex Biophysik, Leipzig, Germany). Analyzers were calibrated prior to the tests with gases of known concentration. Ventilatory threshold (VT_2) was assessed by means of computer aided linear regression break point analysis (Prosport, Leitner, Austria).

Paragraph 9 **Blood lactate** concentration was determined enzymatically (Boehringer, Mannheim, Germany). Capillary blood samples were taken at rest, at the end of each load step, after 3 min of active and after 3 min of passive recovery. The first increase of blood lactate concentration above resting level was defined as the first lactate turn point (LTP_1). The second abrupt increase of blood lactate concentration, around 4 mmol $\cdot$ L^{-1}, was defined as the

second lactate turn point (LTP_2). Lactate turn points were assessed by means of computer aided linear regression break point analysis (Prosport, Leitner, Austria).

Paragraph 10 **Heart rate (HR)** was measured throughout all tests using a commercially available heart rate monitor (Polar Vantage NV, Polar Electro, Finland). Data were measured and stored in 5 second intervals, transferred to a computer and analyzed as described previously (12).

Paragraph 11 **70% or 80% VO_{2max} exercise tests.** Exercise tests were performed at baseline, and repeated after 4, 16 and 28 wk of supplementation with assigned capsules. Similar to the maximal test, subjects completed a rest phase sitting on the ergometer for 3 min. Exercise started at 40 W with 80 rpm for 3 min, work rate was increased by 20 W every minute until the target workload at 70% or 80% of individual VO_{2max} was attained. Workload was adjusted every 5 min to maintain the target $\%VO_{2max}$ intensity. Gas exchange variables and HR were monitored continuously throughout the exercise test as described above. Blood lactate concentration was determined at rest, after every load step and after each 5 min of constant load exercise until termination of the test. After 20 min of exercise, the facemask was removed briefly to allow consumption of 250 mL of plain water. Test duration was 40 min of exercise or after reaching subject exhaustion, defined as the inability to maintain exercise at the given work load and at 80 rpm.

Paragraph 12 **Dietary assessment.** All subjects were instructed to maintain their habitual diet during the study period and to complete daily food records for 6 d prior and 1 d after the first exercise test for nutrient intake assessment. Subjects subsequently received copies of their 7-d diet records and were instructed to replicate the diet prior to the next exercise tests, to minimize any influence of dietary fluctuation on the markers being monitored. In addition, 3 h before each exercise test a standardized breakfast was fed to the subjects providing about 4222 kJ, 32-34 g protein, 144-150 g carbohydrate and 28-30 g fat, to further minimize any fluctuation due to recent diet. Food records were analyzed twice (at baseline and after 28 wk

for total calories, proteins, carbohydrates, fat, cholesterol, fiber, water, alcohol, and micronutrient content using "Opti Diet" software (GOEmbH, Linden, Germany).

Paragraph 13 **Study Capsules.** Study subjects randomized to placebo (N = 20) received capsules containing microcrystalline cellulose identical in appearance to JPC. Subjects randomized to JPC (N = 21) received capsules containing primarily berry, fruit and vegetable juice powder concentrate (Juice Plus+®, NSA, Collierville, TN, USA). The JPC capsules provided approximately 7.5 mg β-carotene, 200 mg vitamin C, 60 mg vitamin E, 600 μg folate and about 63 kJ per day. Subjects took six capsules daily for 28 wk.

Paragraph 14 **Physical activity, duties and illnesses.** Subjects performed 2-3 sessions of endurance and weight training per week, in addition to special mission specific training. Intensity, duration, and frequency of training were recorded and summarized weekly. Each subject was instructed not to perform hard physical training 4 d prior to each exercise test and within 30 h after each test, until after the last blood sample was drawn. The unit commissioner documented all mission assignments, and the unit physician documented the types of missions, duty hours, incidents of illness, injury, and other stressors (i.e. circadian imbalance).

Paragraph 15 **Blood collection and sample preparation.** We conducted four blood collections per subject at each of the four exercise tests: at the beginning of each exercise test, before exercise (BE = resting values); immediately after the end of each exercise test (IE), 30 min (30M) and 30 h after the tests (30H). At these times, 600 μL of capillary blood (EDTA coated vials, Sarstedt, Graz, Austria) was collected to determine CP, HMA, HNA1, and HNA2. Further, erythrocyte antioxidant enzyme activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) were determined. After centrifugation at 3000 x g for 10 min, plasma was removed and samples were stored frozen at -70°C until analysis.

Paragraph 16 **Analysis of CP.** Measurement of CP was done with a sensitive chemiluminescent immuno assay (Lumistar, BMG, Germany). After derivatization with

dinitrophenyl-hydrazine (DNPH), anti-DNPH-antibody (Sigma, USA) was used on 96-well microtitration plates (Nunc, Denmark), as described elsewhere (20). Assessment of plasma protein concentration used the bicinchoninic assay (BCA; Pierce, MD, USA).

Paragraph 17 **Analysis of HMA, HNA1, and HNA2.** Albumin was fractionated using high performance liquid chromatography to give three peaks, corresponding to the cysteine-34 redox state: the free thiol form (HMA), as a mixed disulfide (HNA1), or, more oxidized (HNA2), as previously described (14). Quantification was based on the peak heights of HSA fractions and data are expressed as the percentage of HMA, HNA1, and HNA2.

Because the redox state of HSA is expressed as the fraction of HMA, HNA1 and HNA2, respectively, and CP values are related to total plasma protein content, possible dehydration due to exercise did not influence these parameters.

Paragraph18 **Analysis of SOD and GPx activity.** Assessment of SOD activity used erythrocyte lysate with xanthine oxidase in the start reagent, as previously described (28) expressed in units · $mgHb^{-1}$. Determination of GPx activity from erythrocyte lysate was performed indirectly, by a coupled reaction with glutathione reductase, utilized the ZeptoMetrix Corporation (Buffalo, NY, USA) assay kit adapted to 96-well-plates, with results expressed in units · gHb^{-1}.

Paragraph19 **Blood chemistry panel.** Standard blood chemistry values were determined from 5 mL EDTA plasma at subject enrollment and the end of the investigation, except CK, which was measured at each blood collection. Analysis used routine methods and the clinical chemistry analyzer "Eurolyser" (Dia Team, Diagnostica und Arzneimittel Groβhandel GmbH, Linz, Austria). Parameters monitored were: glucose, cholesterol, triglycerides, creatine kinase, albumin, C-reactive protein, uric acid, lactate and the liver enzymes lactate dehydrogenase and glutamate-pyruvate-transaminase. Assessment of hemoglobin and iron concentrations used the Advia clinical analyzer (Fa. Bayer, Leverkusen, Germany).

Paragraph 20 **Statistical Analysis.** All statistical analyses were performed using SPSS, version 12.0. Data are presented as mean $\pm$ SD. Statistical significance was set at $P < 0.05$. Baseline characteristics, performance and respiratory data, nutrient and clinical chemistry data, were compared between groups using one-way analysis of variance (ANOVA). Data obtained for CP, HMA, HNA1, HNA2 and for antioxidant enzymes were analyzed using a 2 (intensity) x 2 (treatment) x 4 (time) repeated measures ANOVA for each exercise test at baseline, and after 4, 16 and 28 weeks of supplementation. At each time point (BE, IE, 30M, 30H) HMA, HNA1, HNA2 values, CP concentrations, enzyme activities and CK values were compared between the four groups by one-way ANOVA. If there was no influence of the grouping factor treatment, the two 70% VO_{2max} groups and the two 80% VO_{2max} groups were pooled at selected time points (for HMA, HNA1 at IE and 30M) and compared by student´s t-test for unpaired samples to evaluate differences due to exercise intensity. If there was no influence of the grouping factor intensity, the two JPC groups and the two placebo groups were pooled at selected time points (for CP at BE and 30H) and compared by student´s t-test for unpaired samples to evaluate differences due to treatment. Pearson regression analysis and correlation coefficient we used to evaluate bivariate relationships for HMA, HNA1 and CP data.

RESULTS

Paragraph 21 **Characteristics of the study population.** The four groups did not differ in age, height, weight, total body fat, lean body mass, VO_{2max}, maximum workload, or duty hours during the 28 wk study period ($P > 0.1$) (Table 1). From baseline to wk 28 the amount of duty hours per month increased from ~1150 to ~1650 (approximately 40%) in all groups.

Paragraph 22 **Analysis of the 7-d food records** for daily kJ, macro- and micronutrient intake found no statistically significant differences between groups for any measured nutrient variable ($P > 0.1$, data not shown). Average food group servings were similar in all groups, although inadequate with regard to fruit and non-starchy vegetable intake averaging 3.2

servings per day (P > 0.1, data not shown). Baseline clinical blood chemistry parameters were equivalent in the groups and did not change throughout the study period. Capsule compliance was > 85% in all four groups.

Paragraph 23 **Exercise Data.** VT_2 and LTP_2 were occured at 79% ± 3.5 and 78.1% ± 3.6 of VO_{2max} respectively, and were not significantly different between exercise groups (P > 0.1, Table 1). 70% VO_{2max} exercise performance was 230 ± 15 Watt, 39.8 ± 1.4 min of duration and at 39.7 ± 1.8 $mL \cdot min^{-1} \cdot kg^{-1}$ oxygen uptake. True intensity performed was at 71.1 % ± 2.6 of VO_{2max}. 80% VO_{2max} performance was 250 ± 20 Watt, 37.6 ± 4.3 min of duration and at 44.2 ± 2.9 $mL \cdot min^{-1} \cdot kg^{-1}$ oxygen uptake. True intensity performed was at 78.3 % ± 3.4 of VO_{2max}. Exercise in the 70% VO_{2max} groups was significantly below VT_2 and/or LTP_2 exercise intensity (~ 10%) reflecting blood lactate concentrations lower than threshold values (Table 1). Exercise in the 80% VO_{2max} groups was, on average, the same as VT_2 and/or LTP_2 resulting in lactate concentrations significantly higher than the 70% VO_{2max} group values (Table 1). No significant differences between $\%VO_{2max}$ groups were noted for duration of constant load exercise (P > 0.1).

Paragraph 24 **Concentrations of CP.** Across all CP determinations, concentrations ranged from 0.21 to 0.99 nmol · mg $protein^{-1}$. There were no differences between the four groups in CP concentrations BE and 30H at the baseline exercise test and after 4 wk (P > 0.1). After 16 and 28 wk, CP concentrations were lower in the JPC groups than in the placebo groups BE and 30H (P = 0.017 after 16 weeks, P = 0.006 after 28 weeks). At baseline, we found an intensity x time dependent effect to higher concentrations at 80% VO_{2max} intensity compared to 70% VO_{2max} intensity (P = 0.021, Fig. 1A) IE and 30M. After 4, 16 and 28 wk, this increase was diminished in the 80% JPC group. In both JPC groups and in the 70% placebo group, CP concentrations did not change over the time course of the exercise tests after 4, 16 and 28 wk (P > 0.1), only the 80% placebo group showed significantly increased CP

concentrations IE and 30M ($P < 0.05$, Fig. 1B-D). After 28 wk, the increase in CP concentrations in the 80% placebo group was more pronounced IE and 30M.

Paragraph 25 **Values of HMA.** Across all HMA determinations, percentages ranged from 61.1 % to 73.2 %. There were no differences between the four groups in HMA percentages BE and 30H at any exercise test ($P > 0.1$). As shown in Fig. 2 (A-D), there were lower HMA percentages IE and 30M, returning to pre exercise values by 30H in all four groups at all four exercise tests ($P < 0.05$). This effect was more pronounced at 80% VO_{2max} intensity compared to 70% VO_{2max} intensity at each test ($P < 0.05$). Supplementation had no influence on HMA values at any exercise test ($P > 0.1$).

Paragraph 26 **Values of HNA1.** Across all HNA1 determinations, percentages ranged from 24.4 % to 38.3 %. There were no differences between the four groups in HNA1 percentages BE and 30H at any exercise test ($P > 0.1$). As shown in Fig. 3 (A-D), there were increased HNA1 percentages IE and 30M, returning to pre exercise values by 30H in all four groups at all exercise tests ($P < 0.05$). This effect was more pronounced at 80% VO_{2max} intensity compared to 70% VO_{2max} intensity ($P < 0.05$), observable at the exercise tests at baseline, after 4 wk and after 28 wk. The exercise test after 16 wk did not show a significant difference in HNA1 change between the 70% VO_{2max} and the 80% VO_{2max} group IE and 30M ($P > 0.1$). Supplementation had no influence on HNA1 values at any exercise test ($P > 0.1$).

Paragraph 27 **Values of HNA2.** HNA2 percentages ranged from 1.6 % to 3.1 %. There were no differences between the four groups in HNA2 percentages at all time points and at any exercise test ($P > 0.1$, data not shown). Neither exercise intensity nor supplementation showed an effect on HNA2 values at any exercise test or time point of blood collection ($P > 0.1$).

Paragraph 28 **SOD and GPx activities.** SOD activities were determined with a mean value of 16.8 ± 6.2 U · $mgHb^{-1}$. GPx activities were determined with a mean value of 212 ± 46 U · gHb^{-1}. There were no differences on these enzyme activities between the four groups at all

time points and at any exercise test (P > 0.1, data not shown). Neither exercise intensity nor supplementation showed an effect on SOD and GPx values at any exercise test or time point of blood collection (P > 0.1).

Paragraph 29 **Correlation analyses**. Inverse correlation was found when HMA values were compared to CP concentrations at 80% VO_{2max} at the baseline exercise test (JPC: P = 0.018, r^2 = 0.961; Placebo: P = 0.020, r^2 = 0.931). While HMA percentages decreased IE and 30M, CP concentrations increased at these time points. Both variables returned to baseline at 30H. This inverse correlation was also observed in the 80% VO_{2max} placebo group at the three subsequent exercise tests (4, 16 and 28 wk, P < 0.05). A positive correlation was found in the JPC and the placebo group when HNA1 values were compared to CP concentrations at 80% VO_{2max} intensity at the baseline exercise tests (P = 0.011, R = 0.998): both variables increased IE and 30M, returning to pre exercise values after 30H. This correlation was also observed in the 80% VO_{2max} placebo group for the 4, 16 and 28 wk exercise tests (P < 0.05).

DISCUSSION

Paragraph 30 Our main findings were: 1) CP concentrations, as indicator of protein damage, increased at 80% VO_{2max} exercise intensity with 40 min duration; 2) The JPC treatment diminished this increase in CP at 4, 16 and 28 wk; 3) The JPC treatment decreased CP concentrations at BE and 30H at 16 and 28 wk; 4) The redox state of HSA was influenced by both exercise intensities: HMA percentage was reduced IE and 30M with recovery to near resting values by 30H post-exercise. HNA1 showed the contrary course; 5) The shift of HSA from HMA to HNA1 IE and at 30M was significantly more pronounced in the 80% VO_{2max} groups; 6) HNA2 represents a minor fraction and the percentages did not change at any VO_{2max} intensity, or test time point, indicating no irreversible oxidation of HSA at cysteine-34 under these conditions; 7) The redox state of HSA was not affected by JPC treatment; and, 8) At 80% VO_{2max} cycle exercise intensity, a correlation between the reversible shift of HSA from HMA to HNA1 and CP was found at baseline and in the placebo group.

Paragraph 31 We hypothesized that the redox state of HSA and CP concentrations after intense exercise might be influenced by workload or intensity. Comparable studies have found higher plasma CP values after aerobic exercises performed at 70% or 80% VO_{2max} (2, 3, 10, 27). In accordance with these studies 70% and 80 % VO_{2max} intensities were chosen to ensure a detectable change in CP concentrations and to test our hypothesis of intensity dependent changes in HSA redox fractions. Further, the second ventilatory turn point (VT_2) and second lactate turnpoint (LTP_2) in our subjects were estimated at 78 - 79% of individual VO_{2max} (Table 1). Therefore the 70 and 80% VO_{2max} were applied to induce a high exercise load in the subjects and to perform as close as possible to VT_2 and LTP_2 or about 10% below. Performing 40 min of exercise at the specified intensities was, on average, the longest possible duration subjects could perform at 80% VO_{2max} to ensure supreme exertion together with the same exercise duration in both intensity groups.

Paragraph 32 An increase in CP concentrations was found at IE and 30M after 40min exercise at 80 %VO_{2max}. At 70% VO_{2max} exercise of similar duration, no increase in CP concentrations was observed in contrast to a study by Bloomer et al. (2). This apparent conflict could be due to the testing used to assess peak VO_2 values. Shorter protocols (8 min) could result in higher values than longer protocols (16 min), according to Yoon et al. (35). In our protocol, duration of the incremental step test was about 15 - 18 min on average, so the estimated peak VO_2 and consequently the VO_{2max} percentages could have been lower for individual exertion than those reported by Bloomer (8-12 min). However, VT_2 has been shown not to be affected by protocol duration (35), and was at 79% of VO_{2max}, which was close to LTP_2 (Table 1). This was also close to the true intensity performed by the 80%-groups (78.3% ± 3.4). Our results confirm that oxidative protein damage can occur at exercise intensities close to LTP_2/VT_2 for 40 min in trained men and can recover within 30 h.

Paragraph 33 The distribution of HSA fractions can be used as a systemic redox marker, because albumin is the most abundant protein in plasma, is constantly exchanged between

plasma and interstitial fluid, and is responsible for the largest fraction of reactive thiol groups (13). The main fraction of serum albumin, HMA, contributes to the maintenance of a constant redox potential, thus securing a certain redox buffer capacity in extracellular fluids (7). Thomas et al. (32) postulated that the oxidation of protein sulfhydryls to mixed disulfides and their reduction back to thiols might be an effective antioxidant system in extracellular fluids. Consequently, HMA might reflect an essential part of the redox buffer capacity in body fluids, especially in plasma. Therefore HSA could become useful for oxidative stress studies in exercise, similar to other thiols like glutathione or lipoic acid (4, 19, 31). To our knowledge, this is the first study to investigate any influence of exercise with antioxidant supplementation on the redox state of serum albumin over several months in a fit population.

Paragraph 34 We found a significant shift to the disulfide form IE and 30M at all four exercise tests in all four groups. This effect was more pronounced at 80% VO_{2max}. By 30H post exercise the HMA fraction had returned to pre-exercise values regardless of exercise intensity the previous day. In contrast, HNA2 remained unchanged. These results indicate that intense exercise leads to a reversible disulfide formation on albumins cysteine-34, and that irreversible oxidation did not take place. These data also show that 30 h or less are sufficient to return to the resting redox state of serum albumin. The specific model of exercise may have resulted in the direct utilization of albumin as an antioxidant during exercise. With higher intensity, greater utilization appears. In this case albumin is more a marker of redox status rather than a marker of oxidative stress. The results are in agreement with Imai et al. (13) who found significantly decreased HMA fractions after a five-day kendo training camp.

Paragraph 35 Albumin makes up approximately 55% of the total serum protein content, and combined with nine other abundant proteins, together account for more than 90% of all serum proteins (1). Therefore, some researchers postulate that the increased concentration of CP after exercise should be mainly derived from the oxidation of albumin and other major serum proteins (19, 21). If we compare HSA responses at the different exercise intensities to CP

responses, we observe that HMA is shifted to HNA1 at both exercise intensities, whereas CP concentrations only increased at 80% VO_{2max}. This supports the idea that the oxidation of protein thiols to mixed disulfides may be an early response to oxidative stress, as postulated by others (32). The HSA redox buffer system seems to act at the beginning of the radical scavenging chain in plasma ("first line of defense"), whereas CP concentrations represent the end products of overwhelmed plasma antioxidant systems.

Paragraph 36 These data do not allow an estimate of the degree albumin contributes to the post exercise CP increase. This would require analysis of all abundant plasma proteins for CP content. The differences in the responses to supplementation and exercise found for albumin redox state and CP, respectively, emphasize different underlying mechanisms. The antioxidant supplemented JPC groups should have experienced the same increase in formation of reactive oxygen and nitrogen species due to exercise as the placebo groups. The HSA antioxidant system was active in all capsule groups, as represented by the shift of HMA to HNA1. However, in the JPC subjects, the antioxidant system was sufficient to prevent protein damage represented by the CP concentrations. Currently we can only hypothesize why antioxidant supplementation did not influence the HSA redox system but avoided protein damage: the provided supplement might have acted somewhere in the radical chain reaction cascade between the HSA redox system ("first line of defense") and the end products of RONS attacks on proteins indicated via CP. A lack which needs to be investigated in future.

Paragraph 37 Beside the reduction of protein oxidation IE and 30M after 80% VO_{2max} exercise, the JPC groups had attenuated CP concentrations BE and 30H after 16 and 28 wk of supplementation. This was opposite to the placebo groups, which showed higher CP concentrations BE and 30H after these time periods. The reduction of plasma CP concentrations in response to JPC treatment might be due to antioxidant bioavailability and increased antioxidant capacity in plasma as reported by others (29, 23). The increase in CP in the placebo groups and the significant higher concentrations BE and 30H, compared to the

JPC groups after 16 and 28 wk, indicates an increase in radical load at that time leading to enhanced protein oxidation. Interestingly, this increase could be prevented by supplementation. The reason for the higher radical load is speculative but could be attributed to a higher amount of duty hours and to the increase in aircraft body-guarding duties (circadian imbalance) in all groups. Duty hours increased from ~ 1150 at baseline to ~1650 hours after 28 wk; about 10 hours more duty per week for each subject, indicating an enhanced stress profile. Stress response resembles a pro-oxidative state with the potential for free radical damage to a number of cells and tissues (23).

The increase of duty hours was due to the Austrian chairmanship of the European Union from January to June 2006. This time period represents study weeks 8 to 28 in the experimental period. Changes in antioxidant enzymes have not been detected during the whole study period.

Paragraph 38 Experience from our own determinations as well as from other groups (3, 10) over the past years shows evidence that CP levels at rest within 0.2 – 0.4 nmol·mgprotein^{-1} can be regarded as normal range. Toward the end of the study the placebo group had higher CP concentrations than 0.4 nmol·mgprotein^{-1}. Detrimental consequences, e.g., on immune function or inflammation as published recently cannot be ruled out (17).

Paragraph 39 In conclusion, the results of this study indicate that HSA can act as a redox system in plasma under strenuous exercise conditions. It was affected by exercise in an intensity dependent manner, but not by exogenous antioxidants. It seems that the HSA redox system acts as an early response at the beginning of the radical scavenging chain. The HMA response to exercise will require further investigation to explore the potential of this biomarker as a valuable redox sensor e.g. to avoid protein damage induced by oxidative stress in exercise, or to steer training load and recovery. Plasma CP concentration, as indicator of oxidative protein damage, increased significantly at intensities close to LTP_2/VT_2 and this

increase was avoided in the JPC groups. The JPC groups had stable CP concentrations during increased duty hours.

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Table 1

Characteristics of the 41 study participants

Variable	JPC 70% (N=11)	JPC 80% (N=10)	Plac 70% (N=10)	Plac 80% (N=10)
Age; yr	34.3 ± 5.1	36.4 ± 3.8	33.8 ± 5.7	35.1 ± 4.2
Height; cm	183.2 ± 11.4	181.2 ± 7.4	180.8 ± 8.6	182.3 ± 9.4
Weight; kg	83.6 ± 8.1	81.7 ± 6.3	79.8 ± 5.4	82.5 ± 6.7
Total body fat; %	12.9 ± 3.8	11.9 ± 5.2	12.4 ± 4.2	13.6 ± 3.7
Lean body mass; kg	72.7 ± 6.6	71.2 ± 5.3	69.9 ± 4.7	70.8 ± 7.9
Max. workload; W	345 ± 35	340 ± 30	335 ± 25	340 ± 25
VO_2max; $mL \cdot kg^{-1} \cdot min^{-1}$	57.4 ± 7.7	56.4 ± 8.5	54.8 ± 9.3	55.8 ± 6.1
VT2; % of VO_2max	79.0 ± 3.8	77.9 ± 3.2	79.4 ± 3.1	79.7± 3.5
LTP2; % of VO_2max	78.1 ± 3.1	77.5 ± 2.9	77.8 ± 3.7	79.1 ± 4.0
Lactate during exercise, mMol · L^{-1}	3.32 ± 0.81	5.73 ± 2.44*	3.21 ± 0.97	5.95 ± 2.68*
CK; U · L^{-1}; BE	227 ± 24	220 ± 44	219 ± 38	22 1 ± 46
CK; U · L^{-1}; 30H	226 ± 31	287 ± 28*	224 ± 33	294 ± 41*
Duty hours at baseline; hours · $month^{-1}$	1253	1139	1147	1129
Duty hours after 28 wk; hours · $month^{-1}$	1768	1613	1619	1591

Values are means ± SD except for duty hours (total amounts). Comparison of groups resulted in $P > 0.1$, except for lactate and CK: *$P < 0.05$ (one-way ANOVA).

Figure Legends:

Figure 1

Carbonyl protein (CP) concentrations in nmol · mg protein^{-1} in plasma, three hours after a standardized breakfast, at 70% and 80% of individual VO_2max exercise intensity, with JPC or placebo (Plac) treatment.

Plac 70%: N = 10;

Plac 80%: N = 10;

JPC 70%: N = 11;

JPC 80%: N = 10.

Four time points of blood collection:

BE = before exercise

IE = immediately after the exercise test

30M = 30 minutes after the exercise test

30H = 30 hours after the exercise test

A = at baseline (0 wk)

B = after 4 wk of JPC or placebo treatment

C = after 16 wk of JPC or placebo treatment

D = after 28 wk of JPC or placebo treatment

***** = significant effect of intensity x time (**A**; $P < 0.05$) or intensity x treatment x time (**B, C, D**; $P < 0.05$) by repeated measures ANOVA.

= significant effect of JPC treatment ($P < 0.05$) compared to placebo groups by t-test.

Figure 2

Fraction of human mercaptalbumin (HMA) in plasma as percentage of serum albumin.

See note for Figure 1.

* = significant effect of intensity x time ($P < 0.05$) by repeated measures ANOVA.

= significant effect of intensity ($P < 0.05$) between 70% and 80% groups by t-test.

Figure 3

Fraction of reversible human non-mercaptalbumin (HNA1) in plasma as percentage of serum albumin. See note for Figure 1.

* = significant effect of intensity x time ($P < 0.05$) by repeated measures ANOVA.

= significant effect of intensity ($P < 0.05$) between 70% and 80% groups by t-test.

FIGURE 1

A

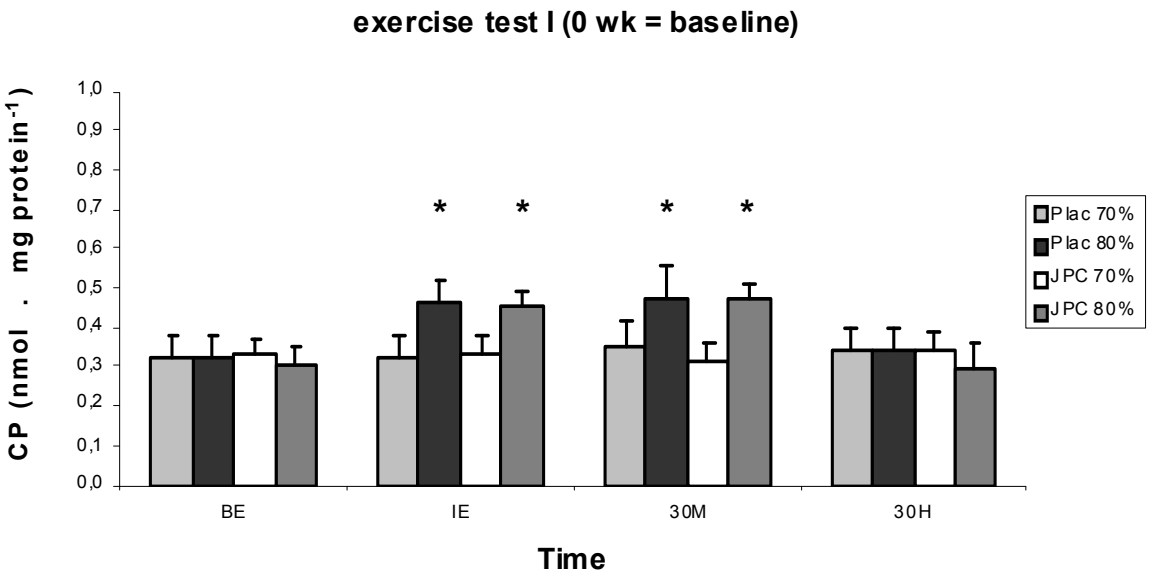

exercise test I (0 wk = baseline)
CP (nmol . mg protein-1)
1,0
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
*
*
*
*
BE
IE
30M
30H
Time
Plac 70%
Plac 80%
JPC 70%
JPC 80%

FIGURE 1

B

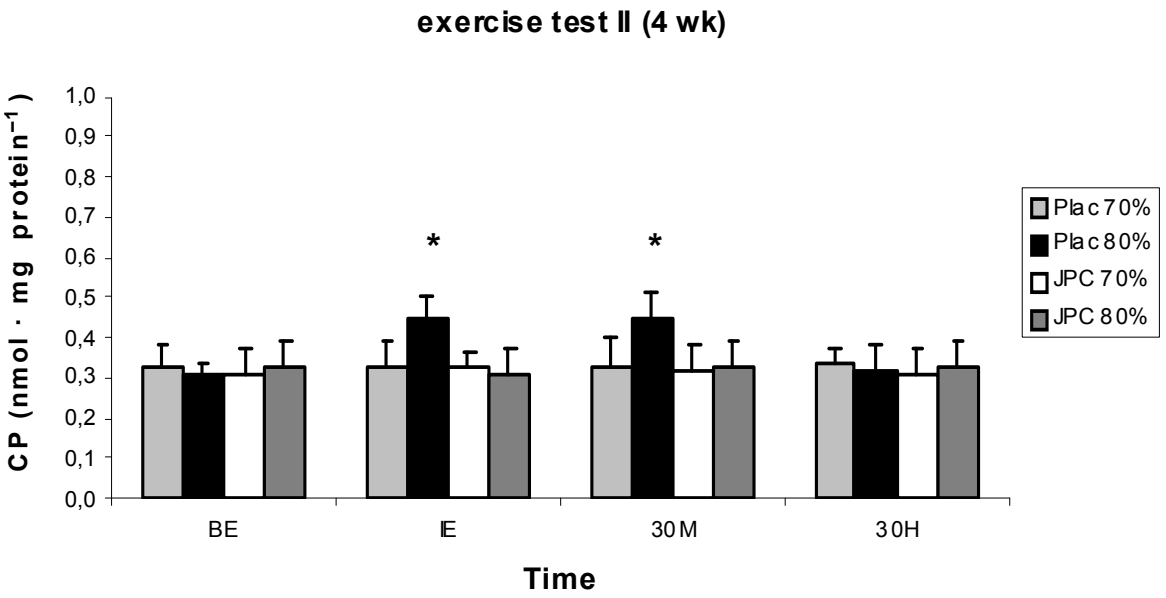

exercise test II (4 wk)
CP (nmol · mg protein⁻¹)
1,0
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
*
*
BE
IE
30M
30H
Time
Plac 70%
Plac 80%
JPC 70%
JPC 80%

FIGURE 1

C

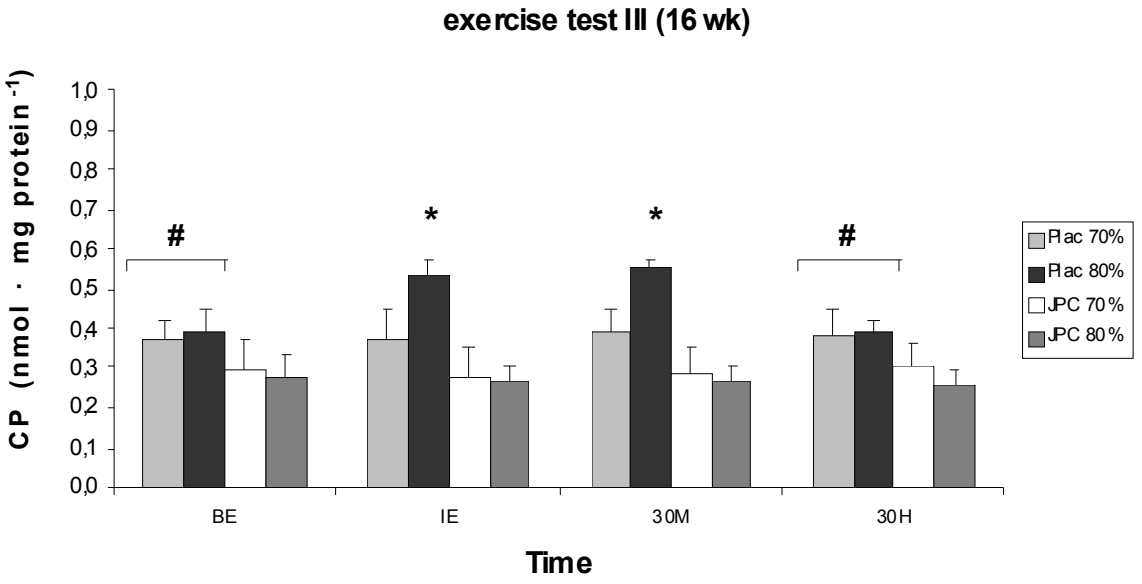

exercise test III (16 wk)
CP (nmol · mg protein-1)
1,0
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
#
*
*
#
BE
IE
30M
30H
Time
Plac 70%
Plac 80%
JPC 70%
JPC 80%

FIGURE 1

D

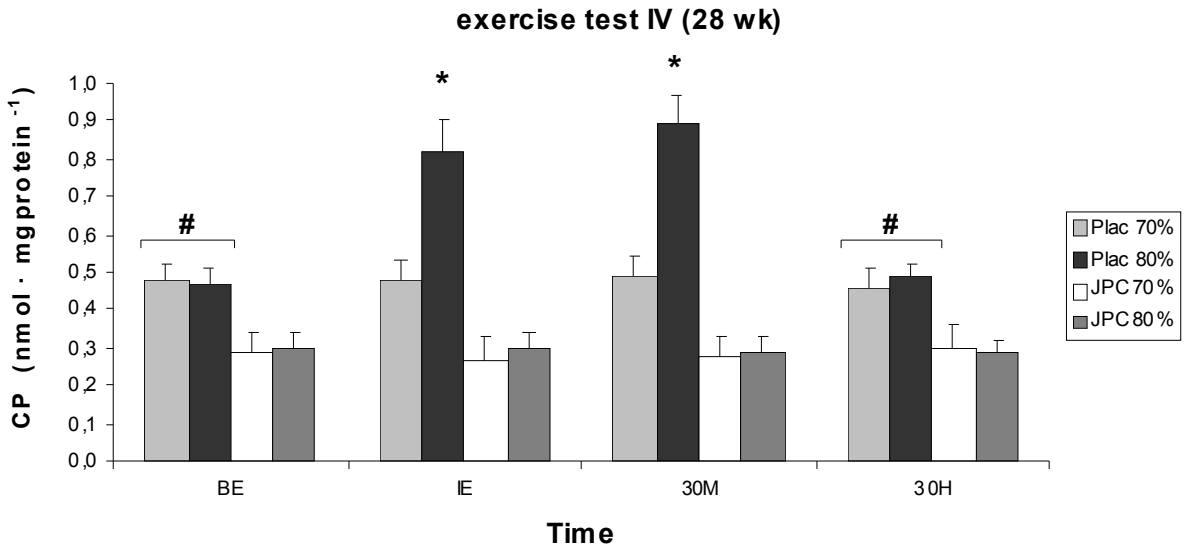

exercise test IV (28 wk)
CP (nmol · mg protein -1)
1,0
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
#
*
*
#
BE
IE
30M
30H
Time
Plac 70%
Plac 80%
JPC 70 %
JPC 80 %

FIGURE 2

A

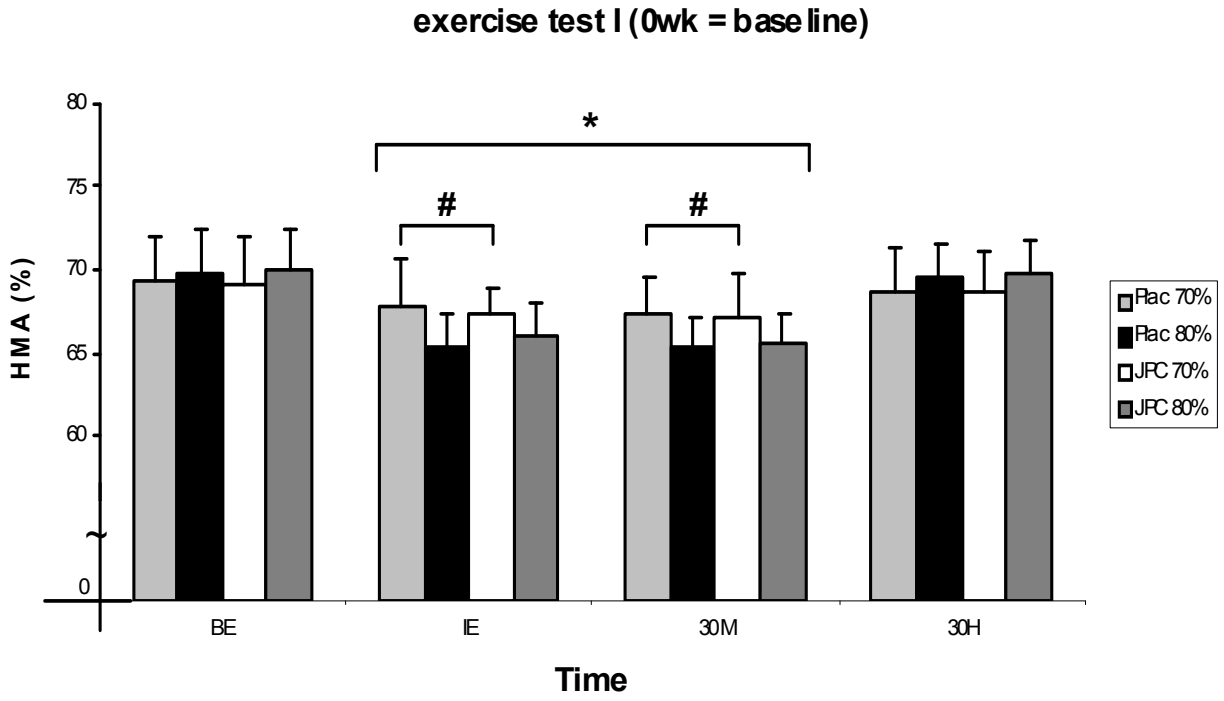

exercise test I (0wk = baseline)
HMA (%)
80
75
70
65
60
0
*
#
#
BE
IE
30M
30H
Time
Rac 70%
Rac 80%
JPC 70%
JPC 80%

FIGURE 2

B

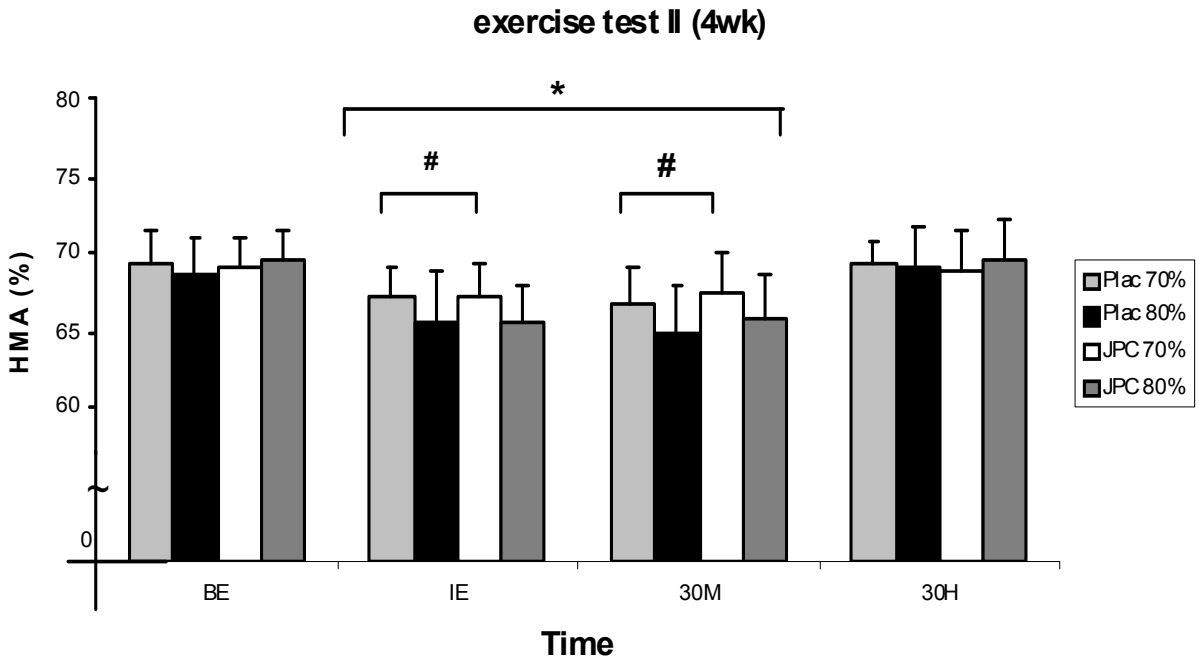

exercise test II (4wk)
*
#
#
80
75
70
65
60
0
HMA (%)
BE
IE
30M
30H
Time
Plac 70%
Plac 80%
JPC 70%
JPC 80%

FIGURE 2

C

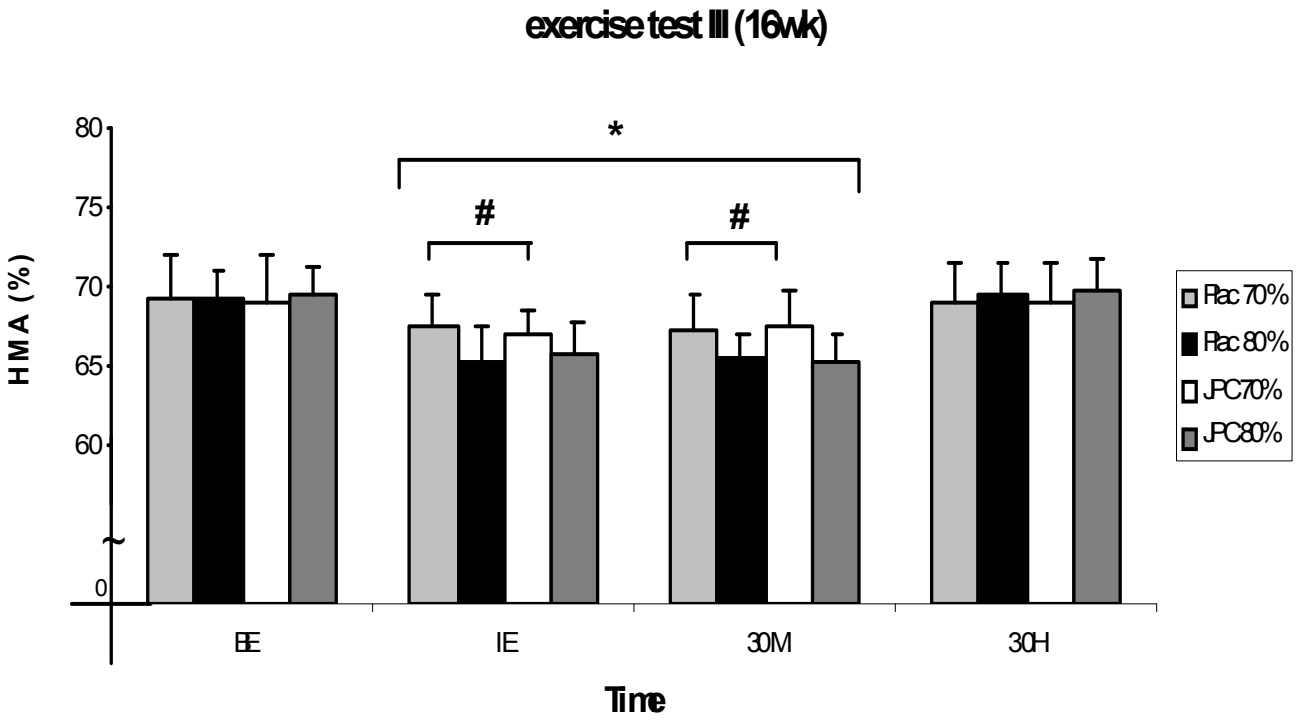

exercise test III (16wk)
HMA (%)
80
75
70
65
60
0
*
#
#
BE
IE
30M
30H
Time
Rac 70%
Rac 80%
JPC70%
JPC80%

FIGURE 2

D

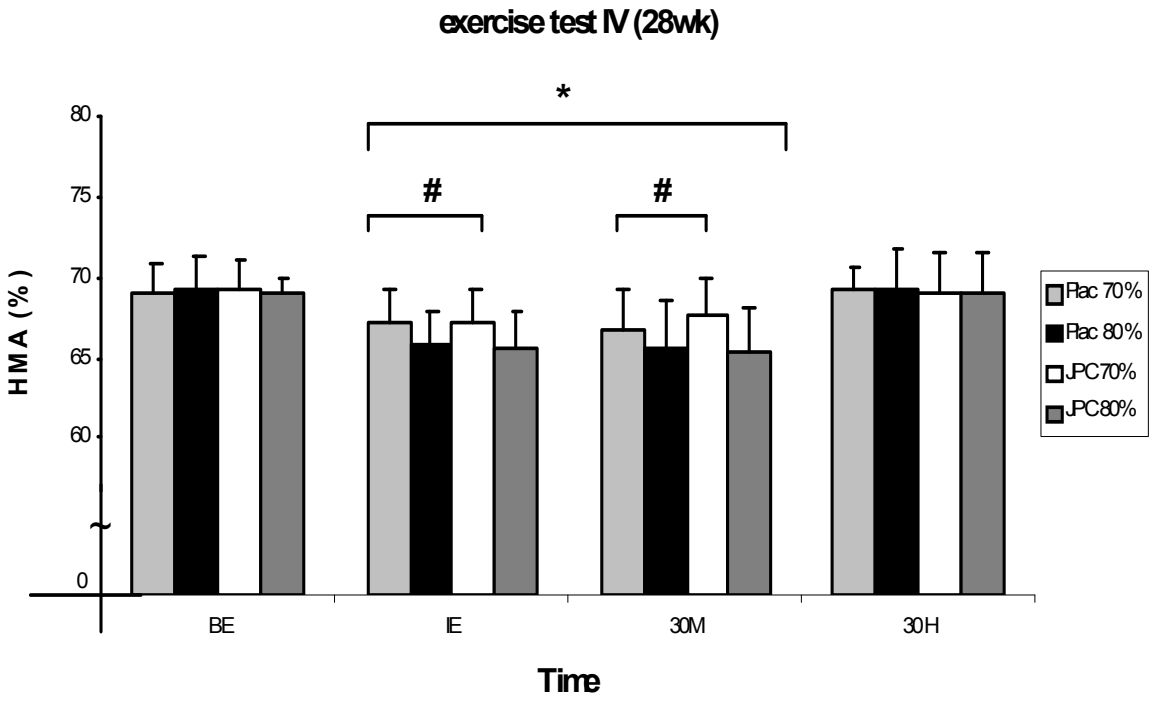

exercise test IV (28wk)
*
#
#
80
75
70
65
60
0
HMA (%)
Rac 70%
Rac 80%
JPC70%
JPC80%
BE
IE
30M
30H
Time

FIGURE 3

A

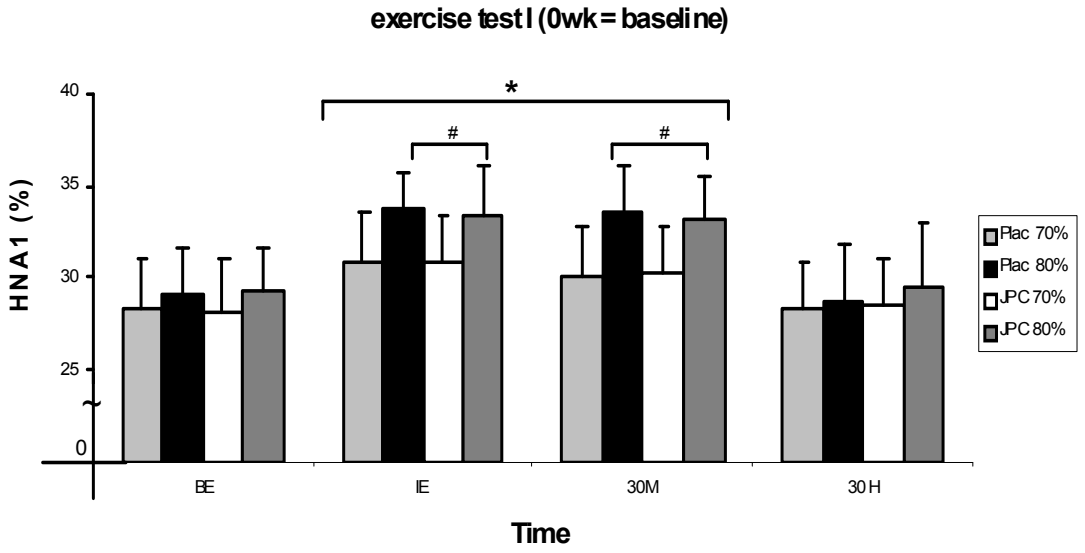

exercise test I (0wk = baseline)
HNA1 (%)
40
35
30
25
0
*
#
#
BE
IE
30M
30 H
Time
Plac 70%
Plac 80%
JPC 70%
JPC 80%

FIGURE 3

B

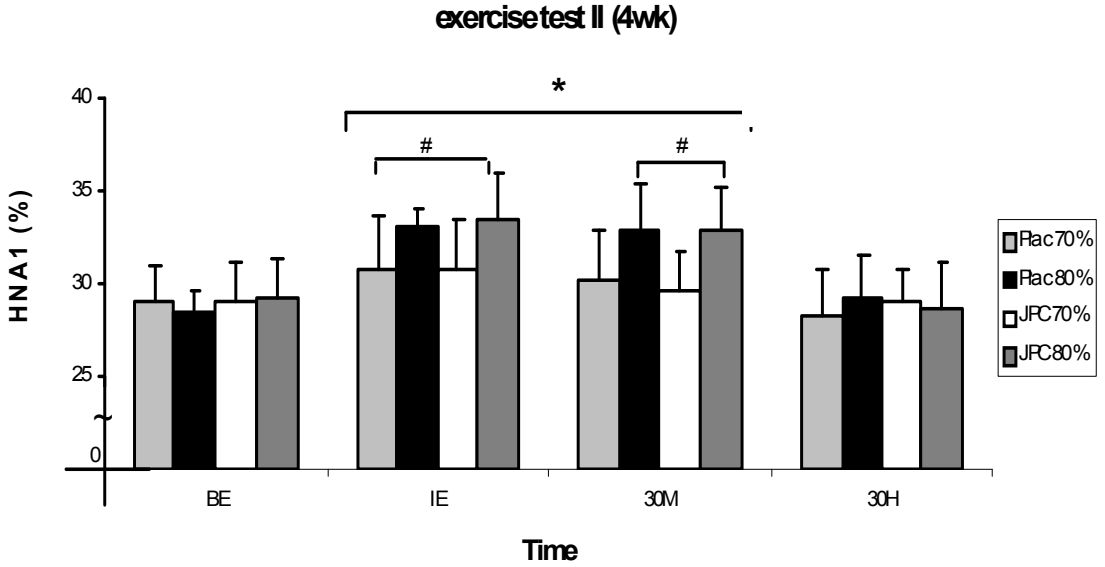

exercise test II (4wk)
*
#
#
40
35
30
25
0
HNA1 (%)
BE
IE
30M
30H
Time
Rac70%
Rac80%
JPC70%
JPC80%

FIGURE 3

C

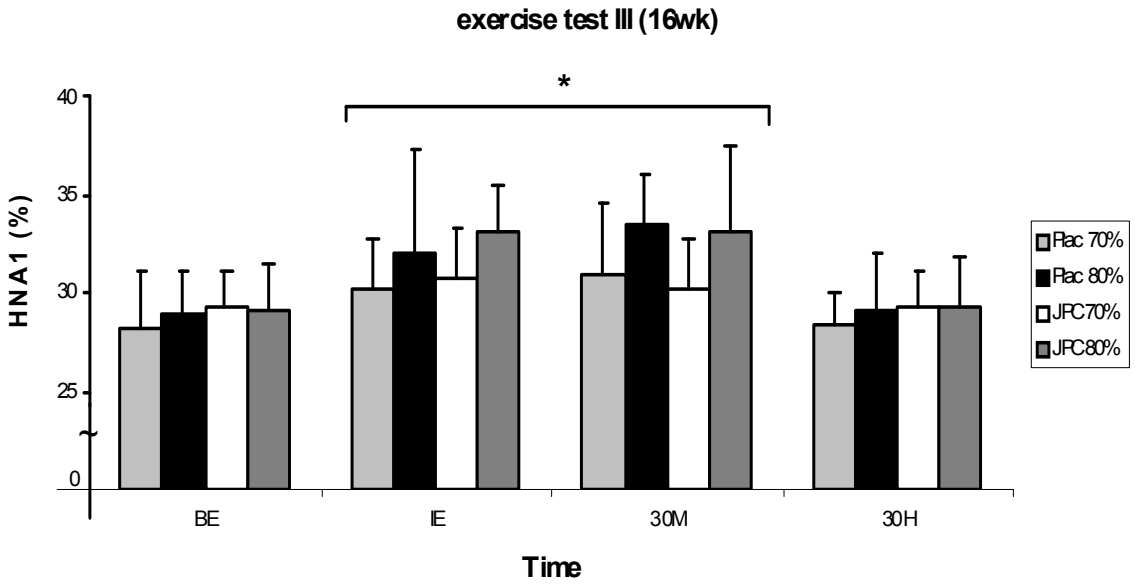
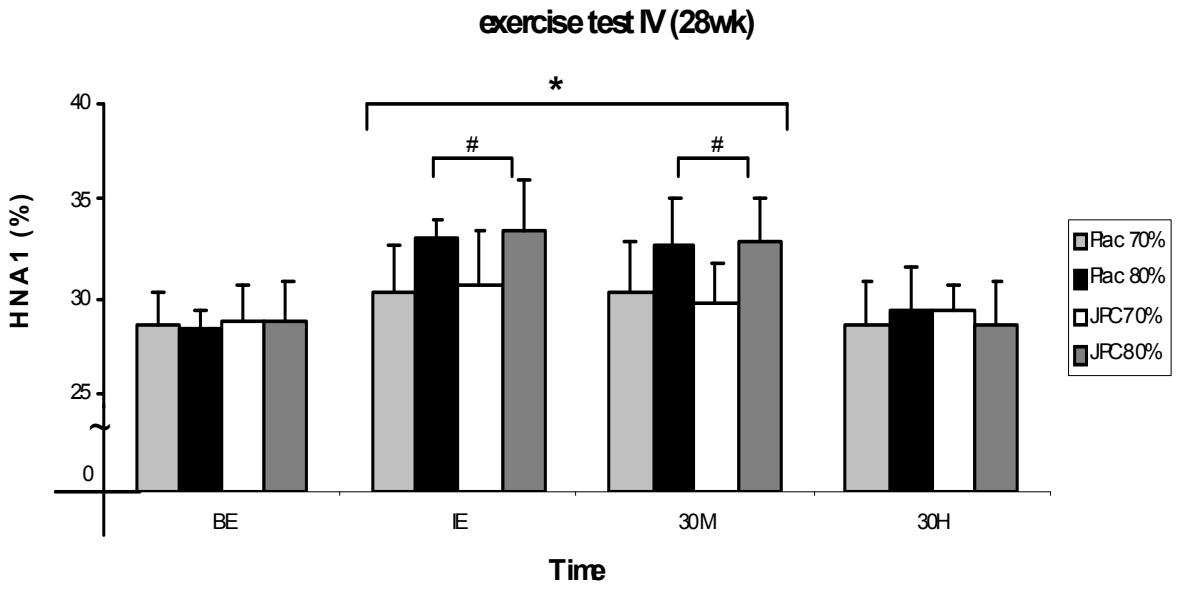

exercise test III (16wk)
*
40
35
30
25
0
HNA1 (%)
BE
IE
30M
30H
Time
Plac 70%
Plac 80%
JPC70%
JPC80%

FIGURE 3

D


exercise test IV (28wk)
*
#
#
40
35
30
25
0
HNA1 (%)
BE
IE
30M
30H
Time
Plac 70%
Plac 80%
JPC70%
JPC80%

Zusammenfassung

Aus der 1. Originalarbeit geht hervor, dass eine antioxidative Supplementation Lipidperoxidationsprozesse steigern und gleichzeitig die Konzentration eines antioxidativen Enzyms reduzieren kann. Die Ursache für diese nachteiligen Effekte der antioxidativen Supplementation wird über das Verhältnis der Konzentrationen der Antioxidantien im Präparat und über die Dosierung der Inhaltsstoffe diskutiert: Im Plasma wurden nach der 2-wöchigen Supplementationsphase bei den 8 trainierten Probanden erhöhte Konzentrationen von Vitamin E und ß-Karotin gemessen, bei gleichzeitig gleichbleibenden Konzentrationen von Vitamin C. Möglicherweise wurden durch die Supplementation Vitamin E- und ß-Karotin- Radikale in erhöhtem Ausmaß generiert, welche die verhältnismäßig kleine Dosis an Vitamin C aus dem Präparat – ein potentielles Antioxidans gegen Vitamin E- und ß-Karotin-Radikale – nicht kompensieren konnte. Folglich stiegen die Lipidperoxidationsprozesse an. Außerdem bewirkte die Supplementation eine Absenkung der Glutathionperoxidase-konzentration. Die antioxidative Supplementation bewirkte eine Art „Down-Regulation“ in der Enzymexpression und/oder eine verminderte Sekretion aus Leber- und Nierenzellen.

In der 2. Originalarbeit wird gezeigt, dass eine antioxidative Supplementation Proteinoxidationsprozesse bei intensiven Dauerbelastungen (über der anaeroben Schwelle) auf adaptationsrelevante Ausmaße vermindern kann. Belastungen unterhalb der anaeroben Schwelle verursachen keine vermehrte oxidative Proteinschädigung. Die antioxidativen Enzymaktivitäten blieben bei den 41 trainierten Männern auf konstantem Niveau, also ohne Einfluss der Belastungsintensität und der 7-monatigen antioxidativen Supplementation. Der Redoxstatus des Humanen Serum Albumins (HSA) wurde in einem intensitätsabhängigen Ausmaß beeinflusst. Aus dem Experiment geht daher hervor, dass bei sportlicher Belastung HSA als Redoxsystem im Plasma fungiert.

Diese beiden Studien zeigen, dass die Konzentrationen von Oxidativen Stress Biomarkern von vielen verschiedenen Faktoren abhängig sind: speziell von der Intensität und der Dauer der sportlichen Belastungen, der Dosierung der antioxidativen Supplementation, dem Orchester der Inhaltsstoffe der antioxidativen Supplementation (Naturbelassenheit) und dem Status der Antioxidantienzufuhr via täglicher Ernährung. Die Varietäten bei diesen Hauptdeterminanten erklären die Gegensätzlichkeit in den Ergebnissen vieler Antioxidantien-studien.

Summary

The 1st original article shows that antioxidant supplementation can increase lipid peroxidation and – at the same time – decrease the concentrations of an antioxidant enzyme. Reason for these detrimental effects is discussed via relation of the products antioxidant concentrations and via dosage of the products ingredients: Plasma vitamin E and ß-carotene concentrations were increased in these trained men after 2 weeks of antioxidant treatment. Vitamin C concentrations were not influenced. We hypothesise that vitamin E- and ß-carotene radicals were generated in higher amounts due to supplementation and the low dose of vitamin C provided by the supplement – vitamin C is a potential antioxidant against vitamin E- and ß-carotene radicals – could not compensate this. Hence, lipid peroxidation increased. Additionally supplementation led to decreased glutathione peroxidase concentrations. Antioxidant supplementation affected a kind of "down-regulation" of enzyme expression and/or reduced secretion from liver and renal cells.

The 2nd work shows that antioxidant treatment can reduce protein oxidation - provoked by intense endurance exercise (above anaerobic threshold) - to adaptation-relevant values. Intensities below anaerobic threshold did not provoke increased oxidative protein damage. Antioxidant enzyme activities were not affected in these 41 trained men, neither by exercise intensity nor by 7 month lasting antioxidant supplementation. Redox status of human serum albumin (HSA) was influenced by exercise in a dose dependent manner and the experiment revealed that HSA acts as a redoxsystem in plasma under exercise conditions.

These to studies demonstrate that the concentrations of oxidative stress biomarkers depend on several factors; specifically on intensity and duration of exercise, dose of antioxidant supplementation, the orchestra of ingredients of an antioxidant supplement (natural based),

and status of antioxidant intake from daily nutrition. The varieties of these main determinants explain the contrary results of many antioxidant studies.