Comparison of blood pro/antioxidant levels before and after acute exercise in athletes and non-athletes

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Abstract. The aims of our study were to assess the redox state of adolescent athletes and non-athletes both at rest and after acute exposure to physical load and to find relations between parameters of redox state and morphofunctional characteristics of subjects. 58 young handball players and 37 non-athletes were subjected to body composition analysis, measuring of maximal oxygen consumption and blood sampling immediately before and after a maximal progressive exercise test. At rest, athletes had significantly higher superoxide dismutase (SOD) and catalase (CAT) activity, higher levels of reduced glutathione (GSH) and nitric oxide (NO) and lower levels of lipid peroxidation (TBARS) compared with non-athletes. A maximal exercise test induced statistically significant rise of superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2) and NO levels in non-athletes, while TBARS levels decreased. Athletes experienced the fall in NO levels and the fall in CAT activity. After exercise, athletes had significantly lower levels of O_2^- compared with non-athletes. Two way repeated measures ANOVA showed that the response of O_2^-, NO and TBARS to the exercise test was dependent on the sports engagement (training experience) of subjects. Significant correlations between morphofunctional and redox parameters were found. These results suggest that physical fitness affects redox homeostasis.

Key words: Sports training — Exercise — Oxidative stress — Nitrites — Antioxidant defence

Abbreviations: ADS, antioxidant defense system; CAT, catalase; GSH, reduced glutathione; NBT, nitroblue tetrazolium; NO_2^-, nitrite; O_2^-, superoxide anion radical; PRS, phenol red solution; RBCs, red blood cells; RONS, reactive oxygen and nitrogen species; SOD, superoxide dismutase; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; TCA, trichloroacetic acid; VO_2max, maximal oxygen consumption.

Introduction

Oxidative stress is a condition in which the delicate balance that exists between production of prooxidants and their subsequent amelioration *via* the antioxidant defense system (ADS) becomes skewed in favor of prooxidants (Fisher-Wellman and Bloomer 2009). Prooxidants, i.e. reactive oxygen and nitrogen

species (RONS), are constantly being generated in the body to a small extent and, since they have a potential to react with a variety of chemical species, they have multiple functions in cell signaling and enzymology (Cooper et al. 2002; Juranek and Bezek 2005; Jacob and Winyard 2009; Powers et al. 2009). On the other hand, excessive RONS production, that appears to be induced by both psychological and physical stress, may lead towards oxidative damage and numerous pathological processes (Møller et al. 1996; Tiidus 1998; Block et al. 2002; Szocs 2004; Dalle-Donne et al. 2006; Valko et al. 2007).

From work over the past three decades, it is clear that exercise of sufficient volume, intensity and duration can lead to an

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increase in RONS production, which may lead to the oxidation of several biological molecules (lipids, proteins, nucleic acids) (Fisher-Wellman and Bloomer 2009). Whether or not this condition is indicative of a harmful stimulus, however, remains a topic of debate (Vollaard et al. 2005). Although exercise-induced RONS production represents a potential detriment to physiological functions, a repeated exposure of the system to increased RONS production from chronic exercise training leads towards positive changes in the body including upregulation of many cell processes and gene expression (Allen and Tresini 2000; Dröge 2002; Ji 2002). According to the principle of hormesis, and basic principle of exercise – stress-adaptation, the rise in RONS production represents stimuli for an upregulation in endogenous antioxidant defenses (Finaud et al. 2006; Radak et al. 2008; Fisher-Wellman and Bloomer 2009). This provides adaptive protection from RONS during subsequent training sessions, as well as during non-exercise related conditions (Fisher-Wellman and Bloomer 2009).

It was previously shown that well trained subjects have more efficient ADS compared with sedentary subjects, however, those studies compared only the basal (resting) levels of pro/antioxidants in blood of athletes and non-athletes (Ørtenblad et al. 1997; Balakrishnan and Anuradh 1998; Brites et al. 1999; Evelson et al. 2002; Cazzola et al. 2003; Gougoura et al. 2007; Yamaner 2010). Thus, the primary aim of our study referred to the comparison of the effects of acute maximal intensity exercise on pro/antioxidant response in athletes and non-athletes. We hypothesized that athletes would have not only higher activity of antioxidants in rest, but that the extent of redox disturbance induced by a maximal progressive exercise test would be lower in athletes compared with non-athletes. The secondary aim of our study was to explore the correlations between morphofunctional characteristics of subjects and parameters of redox homeostasis both before and after the exercise test.

Materials and Methods

Participants

A group of 58 young handball players (age 16–19 years) and 37 age-matched adolescents who did not perform regular physical activity took part in this research. Athletes were engaged in regular handball (mixed – aerobic-anaerobic) training 5 times a week for 90 min.

Four weeks prior to blood sampling the participants were instructed to abstain from any vitamin or antioxidant dietary supplementation. None of the participants reported any eating disorder, had no ongoing or previous (last half-year) injuries, they were not on any medication known to affect oxidative stress and were non-smokers. They were asked not to engage in any heavy physical activity for 24 h before the test and not to consume alcohol for 48 h before the test. To exclude the influence of different dietary intake on nitrite level, all participants were on the same dietary protocol 5 days before the study.

All participants gave a written informed consent (participants under 18 gave the parent´s consent). The study was approved by the Ethical committee of Medical Faculty, University of Kragujevac.

Protocol

The research protocol started at 8 a.m. After participants had filled in the standard sports medicine questionnaire, but before breakfast, a blood sample was taken from their antecubital vein. Then, measurement of body composition was performed using an apparatus for bioelectrical impedance analysis In Body 720 (Biospace, Korea) whose validity was previously confirmed (Lim et al. 2009). Measurement was performed according to manufacturers instructions. Body weight was measured with an accuracy within 0.1 kg and body fat with an accuracy of 0.1%. Body height was measured by means of an anthropometer (GPM, Switzerland) and the results of the measurements were accurate within 0.1 cm.

After anthropometrical measurement and blood sampling, subjects were subjected to a maximal progressive exercise test on a bicycle ergometer AX1 (Kettler, Germany). Subjects were familiarized to testing procedure. The saddle height was adjusted for each subject and the load was set to 2 W/kg. The load was increased by 50 W every 3 min until the end of the exercise test. Subjects were instructed to ride at 60 rpm. Oxygen uptake was determined using an automated cardiopulmonary exercise system (FitMate Pro, Cosmed, Italy) which was calibrated prior to data collection. The validity, reliability, and accuracy of the FitMate gas analyzer were previously reported (Nieman et al. 2006, 2007). We hypothesized that the maximal oxygen consumption (VO_2max) was reached when the oxygen consumption reached its plateau (when an increase in workload cannot induce an increase in oxygen consumption) (Howley et al. 1995). The participants stated their subjective feeling of exhaustion by using Borg's CR10 exhaustion scale of at least 8 (Borg 1982).

Biochemical assays

Blood samples were taken from an antecubital vein into Vacutainer test tube containing sodium citrate anticoagulant. Blood samples were analyzed immediatelly. Blood was centrifuged to separate plasma and red blood cells (RBCs). Biochemical parameters were measured spectrophotometrically.

Superoxide anion radical determination

The level of superoxide anion radical (O_2^-) was measured using NBT (nitroblue tetrazolium) reaction in TRIS-buffer

combined with plasma samples and read at 530 nm (Auclair and Voisin 1985).

Hydrogen peroxide determination

The protocol for measurement of hydrogen peroxide (H_2O_2) is based on oxidation of phenol red in the presence of horseradish peroxidase (POD; Pick and Keisari 1980). 200 μl sample with 800 μl PRS (phenol red solution) and 10 μl POD were combined (1:20). The level of H_2O_2 was measured at 610 nm.

Nitric oxide determination

Nitric oxide (NO) decomposes rapidly to form stable metabolite nitrite/nitrate products. Nitrite (NO_2^-) was determined as an index of nitric oxide production with Griess reagent (Green et al. 1982). 0.1 ml 3 N PCA (perchloric acid), 0.4 ml 20 mM EDTA (ethylenediaminetetraacetic acid) and 0.2 ml plasma were put on ice for 15 min, then centrifuged 15 min at 6000 rpm. After pouring off the supernatant, 220 μl K_2CO_3 was added. Nitrites were measured at 550 nm. Distilled water was used as a blank probe.

Index of lipid peroxidation

The degree of lipid peroxidation in plasma was estimated by measuring of thiobarbituric acid reactive substances (TBARS) using 0.4 ml 1% TBA (thiobarbituric acid) in 0.05 NaOH mixed with 0.8 ml of plasma, incubated at 100°C for 15 min and measured at 530 nm. Distilled water was used as a blank probe. TBA extract was obtained by combining 0.8 ml plasma and 0.4 ml TCA (trichloroacetic acid), then samples were put on ice for 10 minutes, and centrifuged for 15 min at 6000 rpm. This method was described previously (Ohkawa et al. 1979).

Determination of antioxidant enzymes

Isolated RBCs were washed three times with 3 volumes of ice-cold 0.9 mmol/l NaCl and hemolysates containing about 50 g Hb/l (prepared according to McCord and Fridovich 1969), were used for the determination of catalase (CAT) activity. CAT activity was determined according to Beutler (1982). Lysates were diluted with distilled water (1:7 v/v) and treated with chloroform-ethanol (0.6:1 v/v) to remove haemoglobin (Tsuchihashi 1923). Then 50 μl CAT buffer, 100 μl sample and 1 ml 10 mM H_2O_2 were added to the samples. Detection was performed at 360 nm. Distilled water was used as a blank probe. Superoxide dismutase (SOD) activity was determined by the epinephrine method of Misra and Fridovich (1972). 100 μl lysate and 1 ml carbonate buffer were mixed, and then 100 μl of epinephrine was added. Detection was performed at 470 nm.

Determination of glutathione

The level of reduced glutathione (GSH) was determined based on GSH oxidation with 5.5-dithio-bis-6.2-nitrobenzoic acid, using Beutler method (Beutler 1975); the concentration is expressed as nanomoles *per* milliliter of RBCs.

Statistics

The statistical analysis was performed with SPSS program 19.0 for Windows. Results are expressed as means ± standard error of the mean (S.E.M.) Data distribution was checked with the Shapiro-Wilk test and depending on its results the appropriate parametric or nonparametric test was used. The differences between two groups (athletes and controls) were assessed using T-test or Mann Whitney test, while the differences between the values of means from two related samples (before and after the maximal exercise test) were assessed by Paired t-test. To check if exercise-induced changes of biochemical parameters are dependent on sports engagement (training experience), Two-way repeated measures ANOVA was used („exercise test" was set as within subjects factor and „training experience" as between subjects factor). Correlation between various variables was found using Bivariate correlation (Pearson's coefficient of correlation).

Results

Demographic and anthropometric characteristics of participants are shown in Table 1. Athletes had statistically lower

Table 1. Age, training experience and morphological characteristics of the investigated groups

	Age (years)	Training experience (years)	Height (cm)	Weight (kg)*	BMI	Fat %	Muscle %	n
Controls	17.5 ± 0.3	–	185.5 ± 3.1	81.6 ± 6.1	23.6 ± 1.3	17.7 ± 3.4	39.6 ± 1.4	19
Athletes	17.3 ± 0.2[a]	7.8 ± 0.3	183.8 ± 0.8[b]	80.9 ± 1.4[c]	23.9 ± 0.3[d]	12.0 ± 0.6[e]	50.2 ± 0.3[f]	58

[a] $p = 0.882$; [b] $p = 0.485$; [c] $p = 0.433$; [d] $p = 0.823$; [e] $p = 0.009$; [f] $p = 0.000$ (significant: $p < 0.01$); * Mann-Whitney test; others, T-test. Results are presented as mean ± S.E.M.

Table 2. Functional characteristics of the investigated groups

	VO$_2$max (ml/kg/min)	Time on test (min)	Workload (W)	n
Controls	39.7 ± 1.3	9.42 ± 0.42	250.5 ± 6.98	19
Athletes	44.6 ± 0.9[a]	10.57 ± 0.24[b]	280.93 ± 7.63[c]	58

[a] $p = 0.034$; [b] $p = 0.041$; [c] $p = 0.030$ (significant $p < 0.05$); T-test used. Results are presented as mean ± S.E.M.

percentage of fat and higher percentage of muscle compared with non-athletes.

Athletes also had significantly higher aerobic power compared to non-athletes (Table 2).

At rest, athletes had significantly higher levels of NO (NO$_2^-$) (Fig. 1C), lower levels of TBARS (Fig. 1D), higher activity of SOD (Fig. 2A), higher activity of CAT (Fig. 2B) and higher levels of GSH (Fig. 2C) compared with non-athletes.

The maximal exercise test induced a statistically significant rise of O$_2^-$ (Fig. 1A), H$_2$O$_2$ (Fig. 1B) and NO (NO$_2^-$) (Fig. 1C) levels in non-athletes and fall of TBARS levels (Fig. 1D), while athletes experienced the decrease in NO (NO$_2^-$) levels (Fig. 1C) and CAT activity (Fig. 2B) after exercise test.

The differences in levels of pro/antioxidants in blood of athletes and non-athletes after exercise were seen only in case of O$_2^-$. Athletes had significantly lower O$_2^-$ levels compared with non-athletes (Fig. 1A).
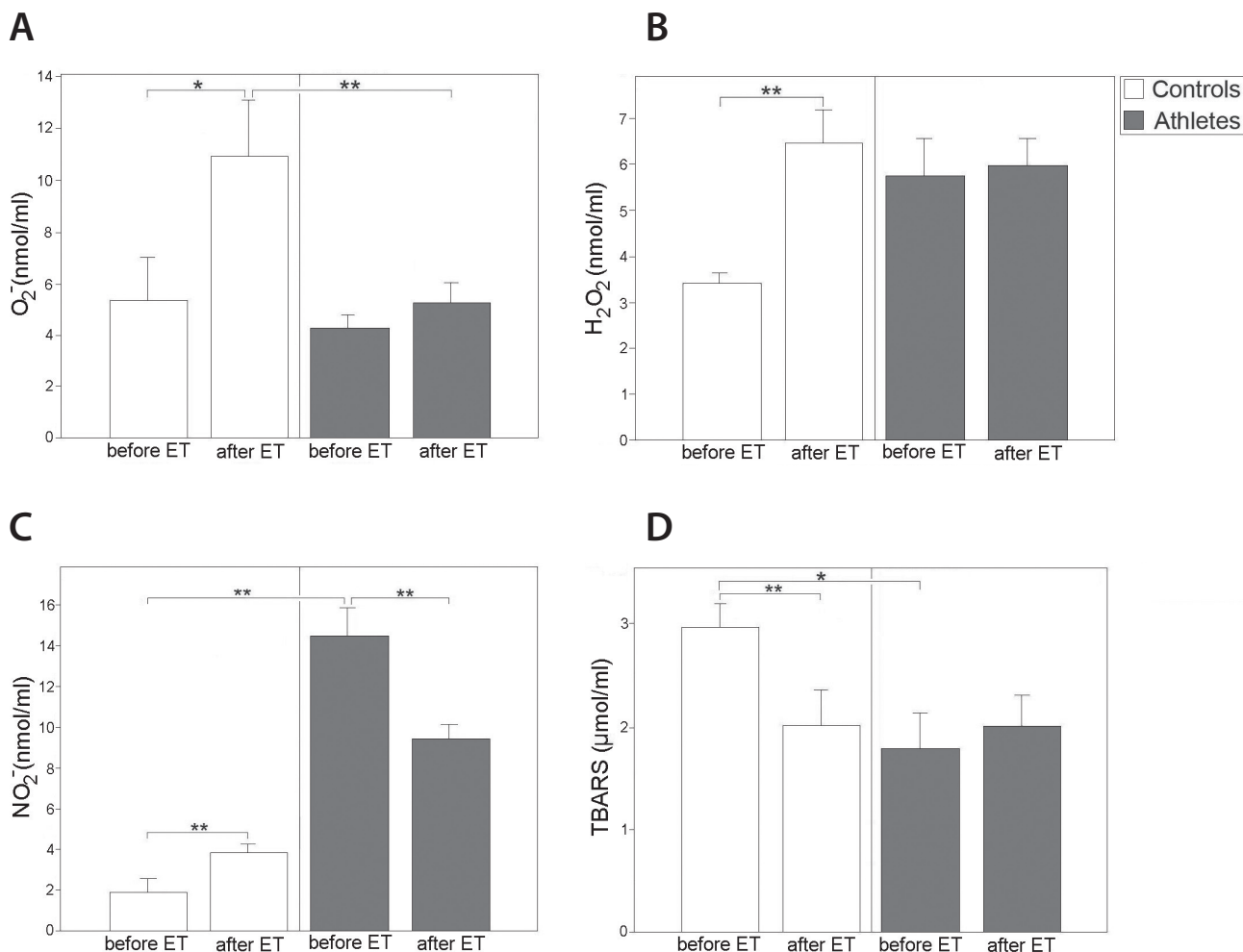

Figure 1. Values of prooxidative parameters: superoxide anion radical O$_2^-$ (**A**), hydrogen peroxide H$_2$O$_2$ (**B**), nitrites NO$_2^-$ (**C**) and index of lipid peroxidation TBARS (D) in athletes and controls before and after the exercise test.* $p < 0.05$; ** $p < 0.01$; ET, exercise test. Data were expressed as mean ± S.E.M.

Two-way repeated measures ANOVA showed that sports engagement (training experience) influenced the response of O_2^- (factor „exercise test" $p = 0.001$, combination of factors „exercise test" and „training experience" $p = 0.007$), NO (factor „exercise test" $p = 0.320$, combination of factors „exercise test" and „training experience" $p = 0.030$), and TBARS (factor „exercise test" $p=0.084$, combination of factors „exercise test" and „training experience" $p = 0.027$), to the exercise test (Figs. 1A, 1C and 1D).

The following significant correlations between parameters of body composition (height, weight, body mass index, fat percentage, muscle percentage), parameter of functional capacity (VO$_2$max) and parameters of redox state (O_2^-, H_2O_2, NO (NO_2^-), TBARS, SOD, CAT, GSH) were found:

- Fat percentage correlated positively with H_2O_2 levels both before ($p < 0.01$; r = 0.335) and after the exercise test ($p < 0.05$; r = 0.309), and with TBARS after the exercise test ($p < 0.01$; r = 0.359).
- Muscle percentage correlated negatively with H_2O_2 after the exercise test ($p < 0.01$; r = −0.347) and with TBARS after the exercise test ($p < 0.05$; r = 0.323).

Following significant correlations between aerobic power and parameters of redox state were found:

- VO$_2$max correlated negatively with H_2O_2 before the exercise test ($p < 0.01$; r = −0.366) and with TBARS both before ($p < 0.05$; r = −0.328) and after the exercise test ($p < 0.05$; r = −0.279).
- VO$_2$max also correlated negatively with NO (NO_2^-) after the exercise test ($p < 0.05$; r = −0.295).
- The only statistically significant correlation between VO$_2$max and antioxidant enzymes was found between VO$_2$max and CAT activity before the exercise test, and the correlation was positive ($p < 0.05$; r = 0.326).

Discussion

Although there is some inconsistency present within the literature, it is clear that both aerobic and anaerobic exercise have the potential to result in increased free radical production, which may or may not result in acute oxidative stress (Fisher-Wellman and Bloomer 2009). The extent of redox homeostasis disturbance induced by an acute bout of exercise depends on many factors, *inter alia*, exercise mode, intensity and duration, participant's state of training, gender, age and nutritional habits (Fanò et al. 2001; Finaud et al. 2006; Goldfarb et al. 2007). Many studies have compared the antioxidant status of trained and untrained subjects at rest, but, to our knowledge, except the study of Ørtenblad and coworkers (1997) that analysed blood antioxidant status in untrained and jump-trained humans following six bouts of 30-s continuous jumping, no study has compared the ath-
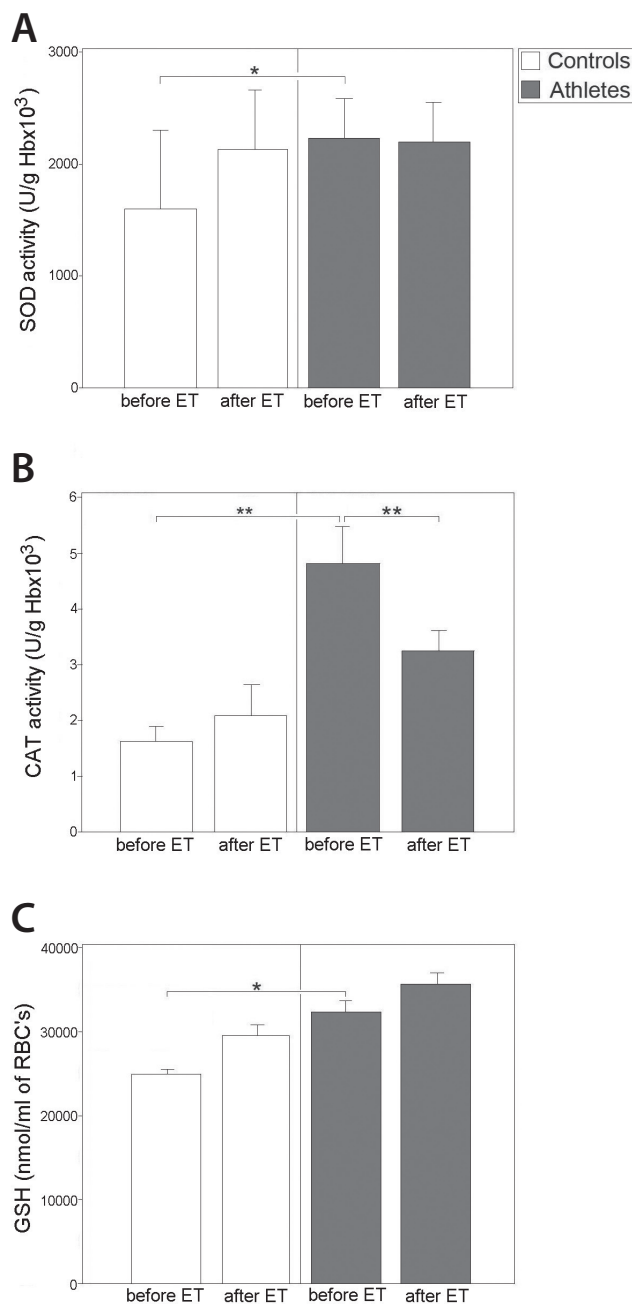

Figure 2. Values of antioxidative parameters: SOD activity (**A**), CAT activity (**B**) and GSH (**C**) in athletes and controls before and after the exercise test. * $p < 0.05$; ** $p < 0.01$; ET, exercise test; SOD, superoxide dismutase; CAT, catalase; GSH, reduced glutathione. Data were expressed as mean ± S.E.M.

letes' and non-athletes' pro/antioxidant responses to acute exercise of maximal intensity.

SOD, enzyme of the first line of defence in RBCs was most commonly found to be the one that changes under the influence of both acute and chronic exercise (Miyazaki

et al. 2001; Groussard et al. 2003; Ookwara et al. 2003) and the one that differentiates between well trained subjects and controls (Brites et al. 1999; Evelson et al. 2002; Metin et al. 2003). Our study confirmed this observance, since young handball players had significantly higher basal SOD activity compared with non-athletes. We have previously shown that SOD may be a good predictive parameter of the extent of redox homeostasis disturbance induced by an acute bout of exercise (Djordjevic et al. 2010a). In that study we found that in the whole group of investigated athletes maximal graded exercise test induced changes in 5 out of 6 investigated redox parameters (the increase of H_2O_2, NO_2^- and TBARS levels and the decrease of SOD and CAT activity), but statistical significance of those changes had its roots in the group of athletes with the lowest basal SOD activity (Djordjevic et al. 2010a). It seems that SOD activity determines the effects of exercise on redox homeostasis and that athletes with higher level of basal SOD activity are under lower risk of exercise-induced oxidative stress compared to athletes with low levels of basal SOD activity. This assumption was in part proved in this study. The exercise test induced the statistically significant increase of O_2^- levels only in non-athletes who, as already mentioned, had lower levels of SOD compared with athletes. Also, after the exercise test, non-athletes had significantly higher levels of O_2^- compared with athletes. Two-way repeated measures ANOVA showed that exercise-induced changes of O_2^- were dependent on sports engagement of subjects.

Higher levels of resting GSH found in athletes compared with non-athletes support the claim that regular exercise training leads towards desirable adaptations of the antioxidant defence system. On the one side regular exercise increases GSH levels (Kretzschmar and Müller 1993; Sen 1999), while on the other side reduced GSH levels are connected with aging and pathology of numerous liver, lung and neurodegenerative diseases (Samiec et al. 1998; Rahman and MacNee 2000; Shulz et al., 2000). GSH both directly and indirectly (as a cofactor for glutathione peroxidase (GPx)) eliminates free radicals (Masella et al. 2005). CAT and GPx are both engaged in H_2O_2 elimination, but their affinity for H_2O_2 is different and dose-dependent. Affinity of GPx for H_2O_2 is higher at low H_2O_2 levels, while CAT's affinity rises with the increase of H_2O_2 levels. Non-athletes in our study experienced a significant rise of H_2O_2 with exercise, but neither GSH levels nor CAT activity changed significantly. On the other hand, athletes did not experience the rise in H_2O_2 production, but CAT activity was decreased after exercise test. It may be that increased H_2O_2 production in non-athletes is a consequence of their less efficient ADS, while athletes' significantly higher basal GSH levels and CAT activity provided efficient elimination of excess exercise-produced H_2O_2. Subjects with a favourable blood GSH redox status at rest maintain a more favourable redox status

in response to exercise-induced oxidative stress (Laaksonen et al. 1999).

Resting TBARS levels of athletes in this study were significantly lower than resting TBARS levels of non-athletes. Since blood GSH was shown to be a determinant of plasma TBARS at rest (Laaksonen et al. 1999), we hypothesize that lower resting TBARS levels in athletes compared with non-athletes are a consequence of significantly higher GSH levels in athletes' blood. Maximal exercise test induced the fall of TBARS levels in group of non-athletes, which was quite unexpected if taken into consideration the behaviour of other three prooxidative parameters. Namely, levels of O_2^-, H_2O_2 and NO (NO_2^-) increased after exercise test in group of non-athletes, but although the reactions between O_2^- and other two prooxidants may lead towards formation of hydroxyl radical, a powerful inducer of lipid peroxidation, TBARS as index of lipid peroxidation was decreased. In contrast to results of our previous study on another group of young handball players which found that athletes with higher VO_2max had higher levels of resting TBARS levels (Djordjevic et al. 2011), results of this study point to a negative correlation between VO_2max and resting TBARS levels. Negative correlations between VO_2max and TBARS both before and after the exercise test, as well as negative correlation between VO_2max and H_2O_2 resting levels and positive correlation between VO_2max and resting CAT activity support the hypothesis that aerobic power correlates positively with desirable redox state. Correlations between body composition and redox parameters found in this study also support this, since body composition can be classified as a health related physical fitness component (Thompson et al. 2009).

Athletes in our study had significantly higher basal levels of NO compared with non-athletes. It is in accordance with numerous previous studies that showed that regular physical activity increases the bioavailibity of NO (Jungersten et al. 1997; Kingwell et al. 1997; Lewis et al. 1999; Maeda et al. 2001; Maiorana et al. 2003; Mendes-Ribeiro et al. 2009) and that physically active people have greater basal NO production compared with a sedentary population (Poveda et al. 1997; Green et al. 2004; Banfi et al. 2006). Studies that investigated the effects of acute exercise on NO production yielded various results. Some studies reported NO increase with exercise, some reported no change in NO production and some reported a decrease in NO production with exercise (Jungersten et al. 1997; Poveda et al. 1997; St. Croix et al. 1999; Allen et al. 2006; Allen et al. 2009; Rassaf et al. 2007; Djordjevic et al. 2010a,b; Cubrilo et al. 2011; Jakovljevic et al. 2011). Those differences are probably due to different protocols, i.e. different characteristics of subjects (age, physical activity, health), different training and tests (type, intensity, duration of exercise tests or training), various methods of measuring RONS production, etc. The exercise test in this research induced the fall in

NO (NO_2^-) production in athletes but the rise of NO (NO_2^-) levels in non-athletes. Two-way repeated measures ANOVA showed that exercise-induced changes of NO (NO_2^-) were dependent on sports engagement of subjects. The rise of NO (NO_2^-) levels in non-athletes may be explained by effects of shear stress, while the response of athletes may be explained by endothelium preconditioning achieved by chronic exposure to shear stress during exercise trainings and i.e. structural and functional adaptations of endothelium (Kingwell 2000; Tinken et al. 2008; Bauer and Sotnikova 2010). It seems that the effects of chronic exercise on the basal NO production are more important than effects of NO production on tolerance of physical activity.

It should be noticed that there was statistically significant difference in total exercise test time and load between athletes and non-athletes, which may be the reason for observed biochemical responses, but the aim of our study was to assess the effects of a maximal exercise test, and the test was maximal for every participant. The differences in redox status post exercise are probably a function of the exercise-induced mechanical damage to muscle fibres and the subsequent inflammatory cascade in unaccustomed subjects.

The results of this study show that athletes are under lower risk of redox state disturbance both at rest and after exercise. Oxidative stress induces oxidative damage and inflammation, and it has been suggested to play a primary or secondary role in the development of more than a hundred acute and chronic human diseases. The results of our study suggest that improving physical fitness may be the way to improve the antioxidant defense and to preserve the health of an individual.

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