THE REDOX STATE OF YOUNG FEMALE HANDBALL PLAYERS FOLLOWING ACUTE EXERCISE AND A ONE-MONTH PRECOMPETITIVE TRAINING PERIOD

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REDOKS STATUS MLADIH RUKOMETAŠICA NAKON JEDNOKRATNOG VEŽBANJA I JEDNOMESEČNOG PREDTAKMIČARSKOG PRIPREMNOG PERIODA

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ABSTRACT

Although the relationship between exercise and oxidative stress has been intensively investigated for over 3 decades, there remains a lack of empirical data on exercise-induced oxidative stress in athletes engaged in sporting games, specifically among the population of elite female athletes. Blood samples were taken from female handball players on the Serbian U20 national team at the beginning and end of a one-month preparatory training period, as well as immediately before and after acute treadmill exercise. Levels of superoxide anion radical, hydrogen peroxide, nitric oxide and lipid peroxidation were measured in plasma samples , while levels of reduced glutathione and the activity of superoxide dismutase and catalase were measured in erythrocytes. Both experimental protocols demonstrated significant increases in plasma levels of hydrogen peroxide and decreases in superoxide dismutase activity in erythrocytes. Despite the increase in plasma levels of hydrogen peroxide after both the treadmill exercise and the one-month training period, the levels of the two antioxidants responsible for eliminating H_2O_2 hydrogen peroxide were not significantly different, as may be expected. Moreover, the marker of lipid peroxidation, TBARS, was not significantly increased. These findings suggest that the first line of antioxidative defence was effective in the prevention of oxidative stress among young female handball players.

Keywords: *oxidative stress, redox balance, handball, training, treadmill*

SAŽETAK

Iako se veza između vežbanja i oksidativnog stresa intenzivno istražuje već više od 3 decenije, još uvek postoji nedovoljno naučnih informacija o vežbanjem-izazvanom oksidativnom stresu kod sportista koji se bave sportskim igrama, a naročito u populaciji žena vrhunskih sportistkinja. Rukometašicama reprezentacije Srbije do 20 godina uzeti su uzorci venske krvi na početku i kraju jednomesečnog pripremnog perioda, kao i neposredno pre i nakon akutnog vežbanja. Nivoi superoksid anjon radikala, vodonik peroksida, azot monoksida i lipidne peroksidacije mereni su u plazmi, dok su nivoi redukovanog glutationa, i aktivnost superoksid dismutaze i katalaze mereni u eritrocitima. I akutno vežbanje i jednomesečni trenažni period doveli su do značajnog porasta nivoa vodonik peroksida u plazmi i snižene aktivnosti superoksid dismutase u eritrocitima. Bez obzira na povećanje nivoa vodonik peroksida i nakon jednokratnog vežbanja i nakon trenažnog procesa, ni u jednom slučaju nije došlo do promene nivoa ostalih antioksidanata, kao što bi se moglo očekivati. Takođe, povećan nivo vodonik peroksida nije imao za posledicu povećanje nivoa lipidne peroksidacije ni u jednom slučaju. To ukazuje na mogućnost da je prva linija antioksidativne odbrane bila dovoljna da zaštiti organizam mladih rukometašica od oksidativnog stresa.

Ključne reči: *oksidativni stres, redoks ravnoteža, rukomet, trening, tredmil*

ABBREVIATIONS

ADS - antioxidative defence system;
CAT - catalase;
GSH - reduced glutathione;
RBCs - red blood cells;
RONS - reactive oxygen and nitrogen species;
SOD - superoxide dismutase;
TBARS - thiobarbituric acid reactive substances;
VO_2max - maximal oxygen consumption.

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INTRODUCTION

During exercise, the several-fold rise of energetic needs and oxygen consumption may induce increased production of reactive oxygen and nitrogen species (RONS) and possible disruption of the redox state, i.e., oxidative stress [1]. The first study of the relationship between exercise and oxidative stress was performed in 1978 by Dillard and colleagues [2] and demonstrated that acute aerobic exercise increases lipid peroxidation, which may be mediated by vitamin E consumption. Although intensively researched during the following decades, there is a dearth of scientific data describing exercise-induced oxidative stress, especially among athletes partaking in sporting games such as handball [3]. Recently published studies showed that handball players have significantly higher superoxide dismutase activity [3, 4], higher levels of reduced glutathione and nitric oxide, and lower levels of lipid peroxidation compared to non-athletes [3], while catalase activity was in one study higher [4] and in another lower [3] than in controls. Another study showed that nonspecific intensive activity, such as maximal progressive tests on a cycle ergometer, induced oxidative stress in young handball players [5]. Conversely, after standard handball training, there were no significant changes to pro/antioxidants except for the activity of superoxide dismutase, which suggests that the first line of antioxidant defence was sufficient in preventing oxidative stress [5]. It was also shown that participation in a handball match [6], as well as creatine supplementation in combination with specific endurance training [7], induced the disturbance of the redox state in handball players. Monitoring the redox states of players during the handball season showed increased parameters of oxidative stress in plasma and decreased parameters in erythrocytes during periods of intensive training and competitions [8].

Furthermore, despite hundreds of papers on the effects of exercise on the redox state, there is a lack of studies investigating this relationship in women. The reason for the disproportionate number of studies on exercise-induced oxidative stress in men and women may be due to the complexity of research and interpretation of the redox state due to the hormonal characteristics of women, which may influence the results. Recent studies [9, 10] concluded that elite female football players have efficient and well-regulated antioxidative defence systems (ADS), both endogenous and exogenous, because increased levels of oxidised glutathione in plasma samples taken immediately after a match did not influence the level of lipid peroxidation. Another study of female football players confirmed that adequate nutrition improves ADS by affecting the activity of primary antioxidative enzymes, such as superoxide dismutase and glutathione peroxidase [11]. However, intensified training and matches during the competitive season resulted in increased lipid peroxidation and protein oxidation index among elite female water polo players [12].

The aim of this study was to assess the redox state of young, elite female handball players after both acute exercise sessions and intensive training periods.

MATERIALS AND METHODS

Subjects

This research was carried out among a group of 20 female handball players on the young Serbian national team, which took 4th place in the European U20 handball championship in 2011 and 4th place on the World U20 handball championship in 2012.

All participants were healthy, used no medications or supplements before the beginning of the study, and were non-smokers. Participants were asked to abstain from heavy physical activity 24 h before the test and not to consume alcohol 48 h before the test.

All participants, and their parents if they were younger than 18 years of age, provided written informed consent. The study was performed in accordance with the Helsinki Declaration and was approved by the ethical committee of The Faculty of Medical Sciences, University of Kragujevac.

Protocol

The study was performed during the preparatory period for the 2012 World U20 handball championship. At the beginning of the preparatory period, all players were evaluated in The Republic Institute for Sports by medical examination and motoric and psychological testing. Acute effects of exercise on oxidative stress markers were assessed by taking a blood sample immediately before and after maximal progressive exercise tests on a treadmill, which were performed to assess the maximal oxygen consumption of players. The effects of the one-month training program were assessed by taking a morning (basal) blood sample at the beginning and the end of the one-month preparatory period.

Anthropometrical measurement

Body composition was measured using an apparatus for bioelectrical impedance analysis, the *In Body 720* (Biospace, Korea), whose validity has been previously confirmed [13]. Measurement was performed according to the manufacturer's instructions. Body weight was measured with an accuracy within 0.1 kg, and body fat was measured with an accuracy of 0.1%. Body height was measured by means of an anthropometer (GPM, Switzerland), and the measurements were accurate within 0.1 cm.

Exercise testing

Maximal progressive exercise testing was performed on a treadmill (T 200, Cosmed, Italy), according to the modified Ellestad Memorial Hospital B protocol [14] presented in Table 1. The maximal oxygen consumption (VO_2max) was assessed through usage of an automated metabolic cart (Quark b2, Cosmed, Italy). The participants stated their subjective feeling of exhaustion by using Borg's CR10 exhaustion scale of at least 8 [15]. We hypothesised that

Load		Duration of exercise	Speed km/h	Grade %
0	Rest	0	0	0 %
I	Stage I	180 sec.	2.7	10 %
II	Stage II	180 sec.	4.8	10 %
III	Stage III	180 sec.	6.4	10 %
IV	Stage IV	180 sec.	8.0	10 %
V	Stage V	Until exhaustion	8.0	15 %
R1	Recovery I	90 sec.	6.4	10 %
R2	Recovery II	90 sec.	4.8	10 %

Table 1. Ellestad B protocol - modified version.

the VO_2max was reached when the oxygen consumption plateaued (the point at which increasing workload cannot affect an increase in oxygen consumption) [16].

Training program

The preparatory training program lasted 1 month, during which 31 trainings were held and 4 matches were played. Details about the training program are presented in Table 2.

Nutrition

During the study period, all athletes consumed a standardised menu consisting of 3 main meals and two snacks. For athletes needing to lose weight, a nutrition plan was made according to dietary recommendations made by the Joslin Diabetes Research Center at the Harvard Medical School for the treatment of obesity, metabolic syndrome, and diabetes in 2005 [17]. Although there is some controversy as to whether a low glycemic load diet leads to improved weight loss [18], there is no question that a low glycemic load diet will generate a lower inflammatory burden [19]. Sport nutrition clinical studies clearly demonstrate that low-carbohydrate diets elicit greater decreases in body weight and fat than energy-equivalent low-fat diets, especially over a short duration [20]. Such a proposed anti-inflammatory diet consisted of approximately 1500 calories per day (approximately 50 grams of monounsaturated fat, 100 grams of low-fat protein, and 150 grams of low glycemic load carbohydrates This anti-inflammatory diet has a 1 : 2 : 3 ratio of fat to protein to carbohydrates based on individual weight. The caloric ratio is approximately 30% fat, 30% protein, and 40% carbohydrates. Athletes maintaining their weight consumed the same diet, with a daily caloric intake of 30 Kcal/kg of their body mass. The daily intake of essential macronutrients for those on a weight loss diet is shown in Table 3.

Athletes also took supplements consisting of 200 mg magnesium citrate twice daily; 1000 mg vitamin C 1x a day; 30 mg CoQ10 with 200 IU d-alpha tocopherol twice daily; and vitamin B-50 complex 1x a day. In each training session and every game, athletes drank 500 ml of water

Microcycle	Morning training		Afternoon training	
No. 1 (7 days)	No. of trainings:	6	No. of trainings:	6
	Aim:	STR-COND	Aim:	TE-TA STR & SPE
	Gym:	6 exercises, 3-4 series 3 tr: 40-50% 1RM 2 tr: 80-90% 1RM 1 tr: 90-100% 1 RM	Sports hall:	3 tr: TE-TA + STR 2 tr: TE-TA + SPE 1 tr: training match
	Sports hall:	3 tr: SPE + SPEND 3 tr: END		
Tournament 1	**2 matches**			
No. 2 (8 days)	No. of trainings:	5	No. of trainings:	7
	Aim:	STR-COND	Aim:	TE-TA STR & SPE
	Gym:	6 exercises, 3-4 series 3 tr: 70-80% 1RM 1 tr: 80-90% 1RM 1 tr: 90-100% 1 RM	Sports hall:	2 tr: TE-TA + STR + AG 2 tr: TE-TA + SPE 2 tr: training match
	Sports hall:	2 tr: SPE + SPEND with ball 2 tr: END + TE-TA 1 tr: TE-TA		
Tournament 2	**2 matches**			
No. 3 (5 days)	No. of trainings:	4	No. of trainings:	3
	Aim:	TE-TA STR & SPE	Aim:	TE-TA STR & SPE
	Sports hall:	2 tr: TE-TA + STR + SPE 2 tr: TE-TA	Sports hall:	1 tr: TE-TA + STR + AG 2 tr: TE-TA + SPE + STR

Table 2. One-month training program (TE-TA: technique and tactics. STR-COND: strength and conditioning, tr: trainings, SPE: speed, STR: strength, END: endurance, SPEND: speed endurance, AG: agility)

Meals	Calories (kcal)	Proteins (gr)	Carbs (gr)	Fats (gr)
Breakfast	455	35	45	15
Morning snack	182	14	18	6
Lunch	364	28	36	12
Afternoon snack	182	14	8	6
Dinner	364	28	36	12
Sum	1547	119	143	51

Table 3. The total daily intake of calories and macronutrients amounts distributed over the meals in one day (subjects on weight loss program).

Characteristic	X±SD
Age (years)	19.14±1.10
Height (cm)	170.14±6.48
Weight (kg)	71.74±9.95
Body mass index	23.37±2.50
Fat (%)	19.07±5.36
Muscle (%)	45.55±2.97
Training and competition experience (years)	8.90±2.25
Hours per week of training (h)	12.26±3.12
Maximal oxygen consumption (ml/kg/min)	43.07±4.78

Table 4. Characteristics of the investigated group.

with 20 g dextrose and 10 g of whey protein isolate. After each training session, athletes drank a protein shake (30 g of protein plus 5 g of glutamine in 500 ml of water). A piece of fruit was eaten after each training session (400 g).

Biochemical assays

Blood samples were drawn from an antecubital vein into Vacutainer test tubes containing sodium citrate anticoagulant. Blood samples were analysed immediately. Blood samples were centrifuged to separate plasma and red blood cells (RBCs) . Biochemical parameters were measured spectrophotometrically.

Superoxide anion radical determination

Levels of superoxide anion radical (O_2^-) were measured using nitro blue tetrazolium reaction in TRIS-buffer combined with plasma samples and read at 530 nm [21]. Levels of O_2^- are presented in nmol/ml of plasma.

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 [22]. Samples of 200 µl s were combined with 800 µl phenol red solution and 10 µl horseradish peroxidase (1:20). Plasma levels of H_2O_2 were measured at 610 nm. Levels of H_2O_2 are presented in nmol/ml of plasma.

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 [23], 0.1 ml 3 N perchloric acid, 0.4 ml 20 mM ethylenediaminetetraacetic acid and 0.2 ml plasma were put on ice for 15 min, then centrifuged for 15 min at 6000 rpm. After pouring off the supernatant, 220 µl of K_2CO_3 was added. Nitrites were measured at 550 nm. Distilled water was used as a blank probe. Levels of NO_2^- are presented in nmol/ml of plasma.

Index of lipid peroxidation (thiobarbituric acid reactive substances, TBARS)

The degree of lipid peroxidation in plasma was estimated by measuring the thiobarbituric acid reactive substances (TBARS) using 1 % thiobarbituric acid in 0.05 M NaOH, incubated with plasma at 100 °C for 15 min and read at 530 nm. Distilled water was used as a blank probe. Thiobarbituric acid extract was obtained by combining 0.8 ml plasma and 0.4 ml trichloroacetic acid. Samples were put on ice for 10 minutes and then centrifuged for 15 min at 6000 rpm. This method has been previously described in the literature [24]. Levels of TBARS are presented in µmol/ml of plasma.

Determination of antioxidant enzymes

Isolated RBCs washed three times with 3 volumes of ice-cold 0.9 mmol/l NaCl and haemolysates containing approximately 50 g Hb/l (prepared according to McCord and Fridovich [25]) were used for the determination of catalase (CAT) activity. Catalase activity was determined according to Beutler [26]. Lysates were diluted with distilled water (1:7 v/v) and treated with chloroform-ethanol (0.6:1 v/v) to remove haemoglobin [27]. Then, 50 µl catalase buf-

Parameter	Before exercise test (X±SD)	After exercise test (X±SD)	Significance
O_2^- (nmol/ml)	3.31±2.45	4.18±2.18	P=0.153
H_2O_2 (nmol/ml)	0.96±0.53	2.41±1.40	P=0.001
NO_2 (nmol/ml)	6.06±3.55	6.57±2.74	P=0.740
TBARS (µmol/ml)	4.59±2.26	5.36±1.73	P=0.084
SOD (U/g Hb x 10^3)	3231.58±2510.51	1936.84±1723.27	P=0.031
CAT (U/g Hb x 10^3)	28.20±28.21	25.23±9.57	P=0.463
GSH (nmol/ml of RBCs)	757.30±466.52	745.35±473.10	P=0.936

Table 5. Levels of pro/antioxidants (X±SD) in athletes' blood before and after exercise test.

fer, 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 [28]. One-hundred µl lysate and 1 ml carbonate buffer were mixed, and then 100 µl of epinephrine was added. Detection was performed at 470 nm. The activities of SOD and CAT in RBCs are presented in units per gram of haemoglobin x10^3 (U/g Hbx10^3)

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 the Beutler method [29]. The concentration of glutathione is expressed as nanomoles per millilitre of RBCs.

Statistics

The statistical analysis was performed with SPSS 20.0 for Windows. The results are expressed as the means ± standard deviation of the mean. The data distribution was checked with the Shapiro-Wilk test, and depending on the results, appropriate parametric or nonparametric tests were used. The differences between the values of means from two related samples (before and after the maximal exercise test, before and after the one-month training period) were assessed by Paired t-tests or Wilcoxon tests, where appropriate. The differences were considered to be significant when the P value was lower than 0.05 and highly significant when the P value was lower than 0.01.

RESULTS

Morphofunctional characteristics and data about training experience of the investigated sample are presented in Table 4. Subjects had long training and competitive experience, well body composition, and cardiorespiratory fitness that may be classified as good (when compared with age-matched healthy females) or satisfactory for playing predominantly anaerobic sports such as handball.

Results of the biochemical analysis regarding the acute effects of exercise are presented in Table 3, while chronic effects of exercise are presented in Figures 1 and 2.

Acute exposure to the maximal progressive load test on a treadmill significantly increased levels of hydrogen peroxide and decreased activity of superoxide dismutase (Table 3).

As presented in Figure 1, the only pro-oxidant that significantly changed after the training period was hydrogen peroxide (from 0.96±0.53 to 1.69±1.01 nmol/ml; P=0.005). Among antioxidant activity, presented in Figure 2, activity of superoxide dismutase was significantly lower at the end compared to the beginning of the study (3231.58±2510.51 versus 1458.97±1550.56 U/g Hb x 103; P=0.004).

DISCUSSION

The effect of exercise on the redox state of an individual depends on many factors, such as the type of training, training load, individual characteristics including age, and coexisting factors of risk and physical condition [30].

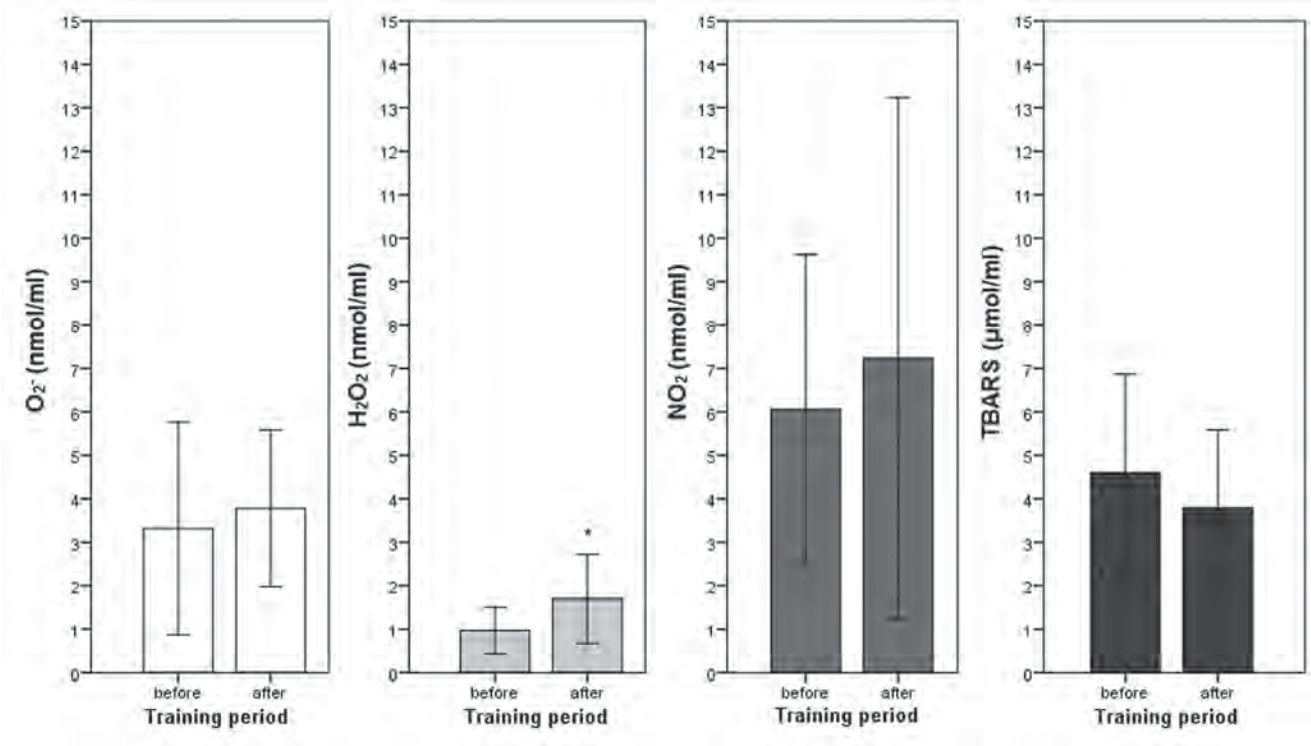

Figure 1. Levels of pro-oxidants (X±SD) in athletes' blood before and after the training period.

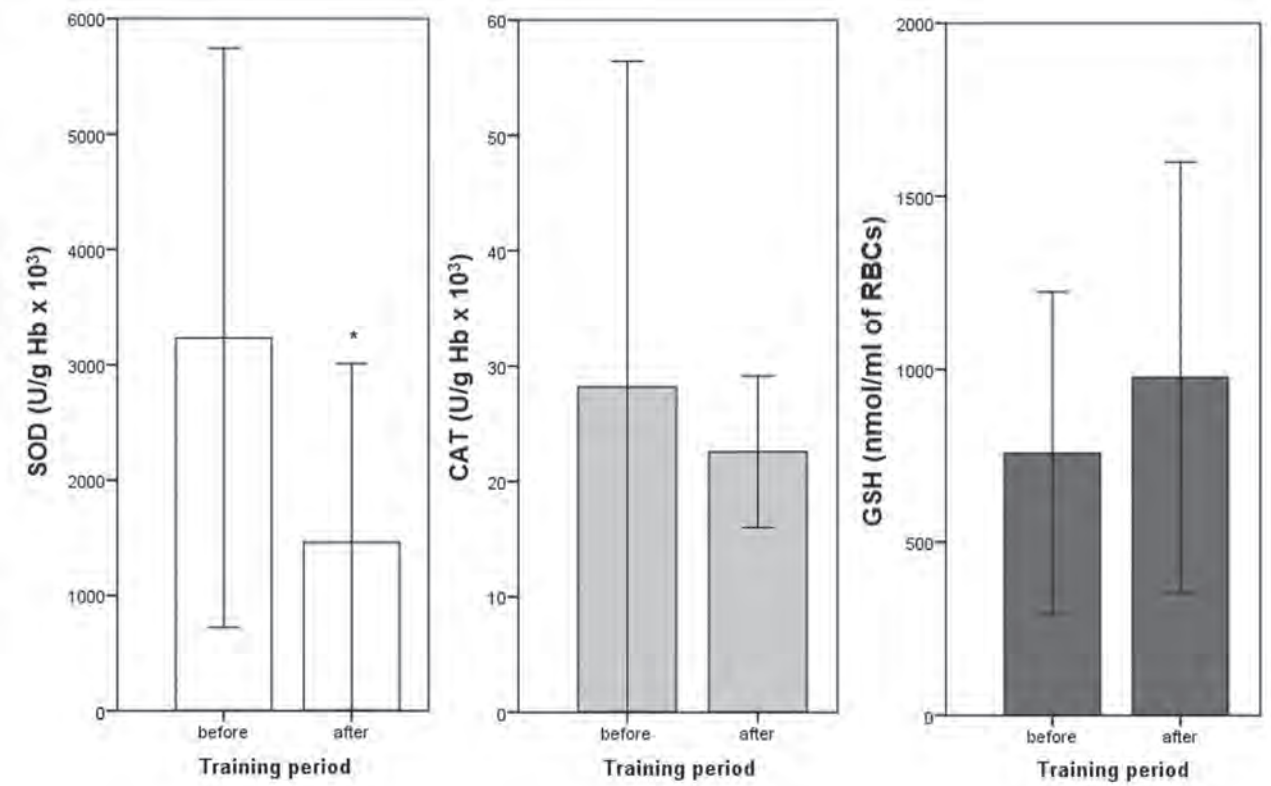

Figure 2. Levels of antioxidants (X±SD) in athletes' blood before and after the training period.

The study contributed to the limited scientific data about exercise-induced oxidative stress in women, adolescent populations, elite athletes, and mixed (aerobic-anaerobic) sports, by assessing changes in redox state of elite young female handball players after acute exposure to an exercise test, as well as after a one-month intensive training period. The results showed significant increase in H_2O_2 levels and decrease in SOD activity both after the treadmill test and after the period of 31 trainings and 4 matches. These findings are consistent with a number of previously published studies. Superoxide dismutase enzyme represents the first line of antioxidant defence and is the enzyme most frequently affected by exercise stimulus in previous studies [31]. However, H_2O_2 is the most stable form of reactive oxygen species produced mainly by O_2^- dismutation by SOD. In vitro studies have shown that elevated H_2O_2 levels inhibit SOD activity [32]. The increase in H_2O_2 levels may explain these results; however, the levels of two other H_2O_2 eliminating antioxidants were not significantly changed, as may be expected. Additionally, the marker of lipid peroxidation, TBARS, was not significantly increased.

Although there are a few studies reporting the effects of treadmill exercise on redox state in football players or non-athletes [1, 33-37], the most adequate comparison of our results is with studies performed on young handball players [4, 5, 31], although the exercise test performed in these studies was cycling ergometer testing. However, because it has been shown that endogenous oestrogen may have a protective role in exercise-induced oxidative stress among female handball players [38] and female non-athletes [39, 40], this comparison is limited due to gender differences and the specificity of the exercise test. After maximal progressive exercise testing on the cycling ergometer, the levels of H_2O_2 among young male handball players were increased and SOD activity decreased, which is consistent with our findings. However, the activity of CAT also decreased and levels of TBARS increased [5]. Interestingly, further analysis showed that increased levels of H_2O_2 and decreased SOD activity were observed only in a group of handball players with the lowest basal SOD activity [31]. Additionally, when comparing handball players with sedentary age-matched controls, exercise testing induced the increase in H_2O_2 levels only in controls who had significantly lower basal SOD activity compared with handball players [4]. This suggests that adaptations of antioxidant defence due to chronic exercise protect athletes from oxidative stress induced not only by an exercise stimulus but also most likely in a number of non-exercise conditions. The decrease in SOD due to exercise testing on an ergometer was also confirmed by Mrowicka et al. [30], who reported that SOD activity was significantly more decreased in athletes compared to non-athletes after cycling ergometry. Another study showed that in non-athletes, SOD activity actually increased in response to any load of exercise on a cycling ergometer [41]. Because female handball players in this study had higher basal SOD activity compared to male handball players in the abovementioned studies, and considering the SOD behaviour in non-athletes during exercise test, we hypothesise that SOD changes are related to the exercise capacity of the subjects, achieved

exercise load and consequently longer duration of the exercise test. Positive correlation between VO_{2max} and H_2O_2 levels was previously established [3].

The second aim of this research was to assess changes in the redox state of our subjects after a one-month preparatory training period. The results of the one-month training period on the redox state were the same as the effects of acute exercise: H_2O_2 levels rise and SOD activity fell at the end of the one-month training period. To the best of our knowledge, only one study monitored the redox state of female handball players over a longer period of time [38]. This recent study reported that CAT activity was the highest during the most intensive period of the handball season, but only in a group of athletes with normal oestrogen levels. Furthermore, levels of lipid peroxidation were significantly lower during the middle and by the end of season, compared to the beginning [38]. In contrast, parameters of oxidative stress in plasma were significantly increased among male handball players during the most intensive periods of trainings and competition, while the opposite was observed in erythrocytes [8]. The observed decrease in plasma-levels of oxidative stress parameters was ossibly most likely due to the significantly increased activity of antioxidant enzymes in erythrocytes [8], confirming the expected adaptation of ADS as a response to programmed training.

The significant increase inof hydrogen peroxide after both treadmill exercise and the one-month training period, without a corresponding increase in the levels of H_2O_2 –eliminating antioxidants or a significant change in the levels of lipid peroxidation, suggests that the first line of antioxidative defence, in the form of superoxide dismutase, is enough to prevent oxidative stress in young female handball players. These results suggest that well-trained elite athletes have upregulated endogenous antioxidative defence systems, which protect them from exercise-induced oxidative stress and that increasing pre-competition training workload should not induce adverse biochemical changes leading to overtraining syndrome.

The limitations of this study include the lack of measurement of oestrogen levels in our subjects and the absence of a control group. However, as the training and nutrition was uniform, some conclusions may be drawn from comparing pre- and post-training values of pro/antioxidants in athletes' blood.

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