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ARE OXIDATIVE STRESS AND ANTIOXIDANT DEFENSE STATUS ASSOCIATED WITH ENERGY EXPENDITURE IN ATHLETES OF VARIOUS SPORTS ?

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Abstract The main purpose of this study was to demonstrate the impact of different training regimes and the type of metabolism that predominates as the source of energy on the oxidative stress / anti-oxidative defence status in elite-level trained athletes. One hundred twenty-four athletes were divided into three groups: "aerobic" (karate, rowing, triathlon), "anaerobic" (wrestling and swimming) and "mixed" (volleyball, water polo, kick boxing). The following parameters were measured: oxidative stress status parameters: [(reactive oxygen metabolites (ROMs), superoxide anion (O_2^-), malondialdehyde (MDA), advanced oxidation protein products (AOPP) and lipid hydroperoxides (LOOH)] and anti-oxidative defence parameters [biological anti-oxidative potential (BAP), superoxide-dismutase (SOD), sulphydryl groups (-SH) and pro-oxidant-antioxidant balance (PAB)]. In general, significant differences were found in oxidative stress parameters between three experimental groups (Wilks' Lambda = 0.366, F_{value} = 8.185, $p < 0.001$). Comparing the athletes from the anaerobic and mixed groups, we found that the anaerobic group had significantly lower ROMs ($p = 0.019$), AOPP ($p < 0.001$), O_2^- ($p = 0.003$) and LOOH ($p < 0.001$). The aerobic group of athletes differed from the group with mixed training regime by lower AOPP, MDA, O_2^- and LOOH values ($p < 0.001$). Discriminant analysis of the three experimental groups indicated protein oxidation marker (AOPP) and pro-oxidant-antioxidant balance as the most important discriminant variables. Oxidative stress status parameters adequately discriminated 74.2 % of the athletes with different energy expenditure during their training programs. The results obtained provide evidence that there are differences in the oxidative stress / anti-oxidant defence status between athletes that have different energy expenditure during exercise and identify athletes who participate in team sports as most susceptible to oxidative stress.

Key words: Oxidative stress, pro-oxidant-antioxidant balance, free radicals, exercise

INTRODUCTION

Numerous studies have investigated the effects of exercise or estimates of physical activity on oxidative stress in athletes. The consistent conclusion from these investigations is that exercise is strongly associated with increased oxidative damage and dependent on other factors such as mode and intensity of exercise, and site of free radical production. Increased production of free radicals does not necessarily have a negative impact on athletes' health, considering that the consequences of oxidative stress include adaptation mechanism by upregulation of antioxidative defence system. However, it is difficult to predict the effect of different training regimes and bioenergetic requirements on oxidative status because of different mechanisms of free radical generation.

Aerobic exercise is accompanied by an increased VO_2, which leads to increased free radical production. However, this phenomenon is manifested only with high exercise intensity in case of which antioxidant capacity is overreached and free radical induced damage occurs [2]. Oxidative stress in anaerobic exercise is accompanied with ischaemic reperfusion of muscles and xanthine oxidase activity in addition to electron leakage that happens during aerobic training regime [14]. Also, it has been shown that anaerobic training regime has a positive effect on oxidative status and lower muscular damage was observed compared with aerobic regime [26, 28]. Considering mixed training, studies showed significant improvement in enzymatic antioxidant defense system but higher training and competitive load can induce

excessive free radical production [27]. And if different types of exercise involve different mechanisms of free radical generation, the outcome is probably a product of synergistic action [22]. To demonstrate exercise-induced oxidative stress in athletes, most researchers have used indirect markers of oxidative modifications to various molecules. Besides, there are many methods that have been developed for the comprehensive oxidative stress and antioxidative defence measuring.

We aimed to investigate the effect of training mode on the oxidative status and antioxidative defence system in athletes and to determine parameters that could discriminate them according to the level of oxidative stress.

MATHERIALS AND METHODS

SAMPLES

The study included 124 athletes (Men: 17 karate professionals, 12 wrestlers, 8 kick boxers, 8 rowers, 6 triathlon; Women: 48 volleyball players, 11 water polo players, 6 karate professionals, 8 swimmers) (Table 1). According to training regimes and energy expenditure (bioenergetics), the athletes were divided into three categories: “aerobic” (karate, rowing, triathlon), “anaerobic” (wrestling and swimming) and “mixed” athletes (volleyball, water polo, kick boxing). All participants underwent routine health checks and gave written informed consent to participate in the study. All participants completed a questionnaire assessing their weekly training workload. All the athletes were highly skilled professional competitors with international experience. Any individual with suspect pathological findings during physical examination, recent history of disease or injuries, altitude exposure or intake of iron supplements or other medications were excluded.

Two days prior to taking part in the study all participants refrained from strenuous physical training. All study procedures were in accordance with the Helsinki declaration and were approved by the Faculty of Pharmacy Ethics Committee for Clinical Trials (University of Belgrade, Belgrade, Serbia).

Table 1. Characteristics of athletes and sports.

Oxidative stress parameters	Aerobic (n=37)	Anaerobic (n=20)	Mixed (n=67)	Total (n=124)
Age (yrs)	24.3 ± 3.7	22.0 ± 6.2	20.7 ± 2.9	22.03 ± 4.16
Training experience (yrs)	11.9 ± 6.1	10.4 ± 4.7	8.35 ± 3.4	9.75 ± 4.8
Training volume per week	10.2 ± 3.1	9.4 ± 2.6	12.6 ± 4.3	10.73 ± 3.3

INSTRUMENTATION AND PROCEDURE

Blood sampling took place under standard conditions between 7 and 8 am after a 12-hour overnight rest. Venous blood was collected into evacuated tubes (Vacutainer, Becton Dickinson, CITY, USA) from the antecubital vein with minimal stasis. Blood samples were transported and stored in the laboratory where analyses were performed strictly following international guidelines [5]. Plasma and serum were separated by centrifugation and multiple aliquots of each sample were stored at -80ºC until analysis. The following parameters were measured: oxidative stress status parameters [(reactive oxygen metabolites (ROMs), superoxide anion (O_2^-), malondialdehyde (MDA), advanced oxidation protein products (AOPP) and lipid hydroperoxides (LOOH)] and anti-oxidative defence parameters [biological anti-oxidative potential (BAP), superoxide-dismutase (SOD), sulphydryl groups (-SH) and pro-oxidant-antioxidant balance (PAB)].

A ROS analytical system (FRAS 4, H&D, Parma, Italy) incorporating a spectrophotometric device reader and a thermostatically-regulated mini-centrifuge was used to measure the ROMs and BAP following instructions supplied by the manufacturer (Diacorn, Parma, Italy). The ROMs test was performed using capillary blood and expressed in CARR units (U), where one CARR U is equivalent to 0.08 mg x dL^{-1} of an aqueous solution of hydrogen peroxide. The intra-assay and inter-assay CVs were 2.9% and 4.0% for ROMs test. The BAP test is based on the ability of a coloured solution containing ferric ions bound to a chromogenic substrate (a thiocyanate derivate) to decolour when its ferric ions are reduced to ferrous after adding herarinised plasma. Solution discolouration was detected spectrophotometrically at 505 nm and was directly proportional to the concentration of all substances able to reduce ferric ion (expressed in µmol /L, the intra-assay CV was 3.8% and the inter-assay CV was 4.5%). Plasma malondialdehyde (MDA, µmol/L) was measured using the thiobarbituric acid-reactive substances (TBARS) assay employing the molar absorption coefficient of 1.56×10^5 M^{-1} cm^{-1} at 535 nm, as previously described (the intra-assay CV was 5.1% and the inter-assay CV was 5.7 [15]. Plasma superoxide dismutase activity (SOD, U/L) was measured according to a previously published method [21]. One unit of SOD activity is defined as the activity that inhibits the auto-oxidation of adrenalin by 50% (the intra-assay CV was 5.2% and the inter-assay CV was 6.3%). The rate of nitroblue tetrazolium reduction was used to measure the level of superoxide anion [4]. The concentration of sulphydryl groups (SH groups, mmol/L) in plasma was determined using 0.2 mmol/L

5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) reported by Ellmann (the intra-assay CV was 3.5% and the inter-assay CV was 5.5%) [9]. The pro-oxidant-antioxidant balance (PAB) was measured using 3,3′,5,5′-tetramethylbenzidine according to a previously published method [1] and expressed in arbitrary HK units (the intra-assay CV was 4.1% and the inter-assay CV was 6.9%). Advanced oxidation protein products were spectrophotometrically detected at 340 nm and expressed as chloramine-T equivalents (μmol x L^{-1}) (the intra-assay CV was 3.4% and the inter-assay CV was 5.4%) [29]. Measurements were performed in duplicate and the results were averaged. Quality control was provided by using quality control samples (pooled plasma).

Statistical Analysis

Descriptive statistics were determined for each variable recorded. Data are presented as Means ± SD. To determine whether there was a statistically significant difference between athletes with different energy expenditure, we used the general linear model analysis of variance and Bonferroni post hoc criteria. Multiple discriminant analysis was employed to classify the three study groups. In the first step an F test (Wilks' Lambda) was used to test if the discriminant model as a whole was significant. In the second step the co-variance matrices, coefficients of canonical correlation and the standardized canonical discriminant function coefficients were used to classify the dependent variable. The standardised canonical discriminant function coefficients were used to compare the relative importance of the independent variables. The result was considered significant when P<0.05. All analyses were performed using Statgraphics 4.2 software (STSC, Inc. & Statistical Graphics Corporation 1985–1989) and CBstat 4.3.2 version software (K. Linnet, Risskov, Denmark).

RESULTS

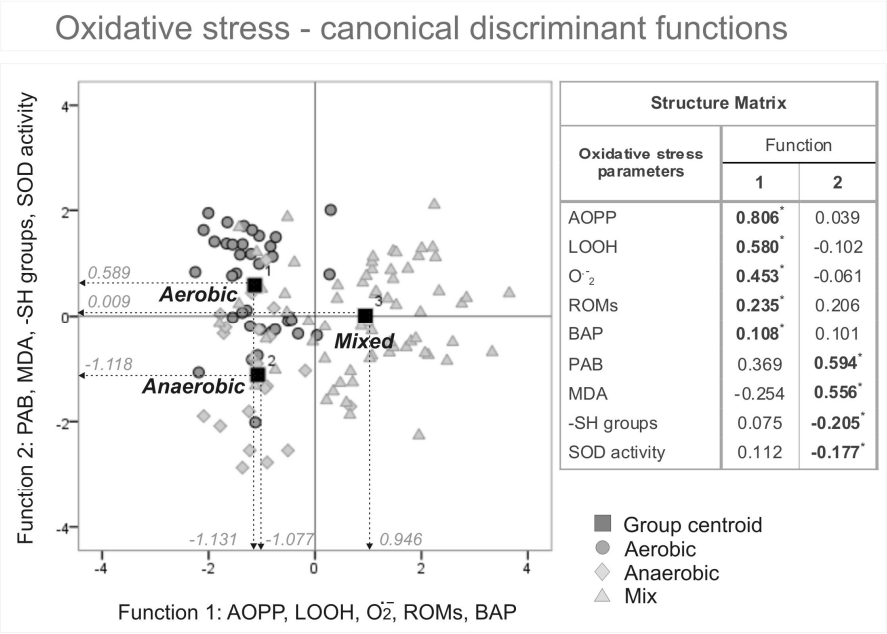
In general, significant differences were found in oxidative stress parameters between the groups of athletes that were investigated in this study (Wilks' Lambda = 0.366, F_{value} = 8.185, p < 0.001) (Table 2). Using multiple comparisons (Bonferroni post hoc criteria), we found that aerobic athletes had higher PAB (p=0.001) and MDA values (p = 0.002) compared to the group of anaerobic athletes. Comparing the anaerobic with the mixed group of athletes, we found that the anaerobic group had significantly lower ROMS (p = 0.019), AOPP (p < 0.001), O_2^- (p = 0.003) and LOOH (p < 0.001). The aerobic group of athletes differed from the group with mixed training regime by lower AOPP, MDA, O_2^- and LOOH values (p<0.001).

Table 2. Multiple discriminant analysis. Oxidative stress / anti-oxidative defence parameters in the blood of three groups of athletes according to Wilks' Lambda, F and P values.

Oxidative stress parameters	Aerobic (n=37)	Anaerobic (n=20)	Mixed (n=67)	Total (n=124)	Wilks' Lambda	F	P
ROMs, carr	291 ± 67	262 ± 31*	322 ± 101	303 ± 87	0. 932	4.41	0.014
BAP, μmol/L	2381 ± 269	2331 ± 361	2431 ± 301	2400 ± 302	0 .985	0.95	0.391
PAB, HK units	455 ± 104††	332 ±124**	507 ± 133	463 ± 137	0.795	15.59	<0.001
SOD U/L	95.8 ± 30.7	108 ± 47	109 ± 44.8	105 ± 41	0.9 77	1.41	0.248
-SH, mmol/L	0.501 ± 0.095	0.534 ± 0.070	0.527 ± 0.09 9	0.520 ± 0.094	0.981	1.16	0.317
MDA, μmol/L	1.046 ± 0.184††	0.873 ± 0.149	0.893 ± 0.185‡‡	0.935 ± 0.193	0.858	10.05	<0.001
O_2^-, μmol/minL	206 ± 187	253 ± 150**	563 ± 439‡‡	406 ± 301	0.818	13.48	<0.001
AOPP, μmol/L	9.6 ± 6.3	9.2 ± 5.8**	38.6 ± 23.1‡‡	25 ± 19	0.588	42.45	<0.001
LOOH, μmol/L	101 ± 32	108 ± 36**	148 ± 40‡‡	127 ± 43	0.732	22.14	<0.001

†p<0.05, ††p<0.01, aerobic vs. anaerobic; *p<0.05, **p<0.01, anaerobic vs. mixed; ‡p<0.05, ‡‡p<0.01, aerobic vs. mixed. Data are presented as means ± SD.

Discriminant analysis of oxidative stress parameters between the three groups of athletes (aerobic, anaerobic and mixed) indicated statistically significant differences at both discriminant functions: Function 1 [AOPP, LOOH, O_2^-, ROMs and BAP] (Wilks' Lambda = 0.366, χ^2 =117,454, p < 0.001) and Function 2 [PAB, MDA, -SH groups and SOD activity] (Wilks' Lambda = 0.762, χ^2 = 31.84, p < 0.001) (Figure 1).



Structure Matrix		
Oxidative stress parameters	Function	
	1	2
AOPP	**0.806***	0.039
LOOH	**0.580***	-0.102
O^{-}_{2}	**0.453***	-0.061
ROMs	**0.235***	0.206
BAP	**0.108***	0.101
PAB	0.369	**0.594***
MDA	-0.254	**0.556***
-SH groups	0.075	**-0.205***
SOD activity	0.112	**-0.177***

Figure 1. Two-dimensional plot of the centroid belonging to each of the three study groups and Z score differences for Function 1 and Function 2 and pooled within-groups correlations between discriminating variables and standardized canonical discriminant functions. Differences between the three groups of athletes in their oxidative stress and anti-oxidative defence status parameters are evident. Variables in Function 1 (AOPP, LOOH, O_2^-, ROMs and BAP) and Function 2 (PAB, MDA, -SH groups and SOD activity) were ordered by absolute size of correlation within function.

When interpreting each function, the larger the standardised regression coefficient, the greater the contribution to the total function score. The first discriminant function primarily characterised the differences in oxidative biomarkers between the three groups of athletes according to their training regime. This discriminant function highlighted the difference between the aerobic and anaerobic athletes on the one side and the mixed on the other, and described them via parameters of oxidative damage, primarily AOPP (standardised canonical discriminant function coefficient = 0.806) as the most important discriminant variable (Wilks' Lambda = 0.588, F_{ratio} = 42.45 and $p < 0.001$). The second discriminant function highlighted the difference between the aerobic and anaerobic athletes and described them via pro-oxidant-antioxidant balance (standardised canonical discriminant function coefficient = 0.594) as the most important discriminant variable (Wilks' Lambda = 0.795, F_{ratio} = 15.59 and $p < 0.001$). The measured variables defined the three groups of athletes. Centroids for each group were calculated for Function 1 (Aerobic: -1.131, Anaerobic: -1.077 and Mixed: 0.946) and Function 2 (Aerobic: 0.589, Anaerobic: -1.118 and Mixed: 0.009) and are depicted graphically in Figure 1 and Table 3.

Table 3. Unstandardized canonical discriminant functions and Z score differences.

Functions at Group Centroids			**Z Score differences for Functions**			
Training regime	Function		Z scores for Function 1		Z scores for Function 2	
	1	2				
Aerobic	-1.131	0.589	Aerobic vs. Anaerobic	0.054	Aerobic vs. Anaerobic	1.707
Anaerobic	-1.077	-1.118	Aerobic vs. Mixed	2.077	Aerobic vs. Mixed	0.580
Mixed	0.946	0.009	Anaerobic vs. Mixed	2.023	Anaerobic vs. Mixed	1.127

The accuracy of classification of the groups based on the measured parameters and calculated indexes was 75.7% for the aerobic, 65.0% for the anaerobic and 76.1% for the mixed athletes. A total of 74.2% of the original grouped cases were correctly classified (Table 4).

Table 4. Classification of results of multiple discriminant analysis in all the athletes.

Training regime		Predicted group membership			
		Aerobic	Anaerobic	Mixed	Total
Original count	Aerobic	28	7	2	37
	Anaerobic	6	13	1	20
	Mixed	9	7	51	67
%	Aerobic	75.7	18.9	5.4	100
	Anaerobic	30	65	5	100
	Mixed	13.4	10.4	76.1	100
74.2% of original grouped cases correctly classified.					

DISCUSSION

The energy required to perform most types of exercise comes from a combination of anaerobic and aerobic sources. However, the ratio varies depending on the type of sport. The contribution of anaerobic ATP production is greater in short-term, high-intensity activities, whereas aerobic metabolism predominates during longer activities [3, 25]. Different energy expenditure (bioenergetics) is well explained in combat sport: aerobic metabolism predominates as the source of energy particularly in karate [6, 12, 17], anaerobic power and capacity are important in wrestling [23, 32] and elite kick-boxers demonstrate a high level of both aerobic and anaerobic conditioning along with an ability to produce high muscle forces [33].

As the athletes' training regimes and bioenergetic requirements were non-uniform, the differences in the measured parameters were expected. Free radical production and subsequent macromolecule oxidation occurs in response to aerobic exercise and in large part is due to a disturbance in electron transport leading to an increased leakage of superoxide radicals with the corresponding increase in oxygen consumption [7]. However, in relation to anaerobic exercise, several other pathways of reactive oxygen species generation could exist, including xanthine oxidase production, prostanoid metabolism and ischemic reperfusion conditions [11, 18, 24]. In the present study, the most significant differences were shown between the mixed and the other two groups of athletes. Significantly lower MDA and higher LOOH values in athletes who participated in team sports compared to those with aerobic exercise load, pointed to a different level of free radical-mediated lipids damage. Lipid hydroperoxides, primary oxidation products, are markers of the initial reaction of free radicals and they measure the rate of peroxidation of the membrane.

With sustained exposure to reactive oxygen species, LOOH undergoes further decomposition to reactive aldehydes, such as malondialdehyde (MDA), a secondary oxidation product [8]. Based on the MDA values, aerobic athletes seem to be more exposed to oxidative stress, but LOOH that is measured represents a steady state between lipid hydroperoxides formation and their degradation, which means they may oxidize other biomolecules. The latter is confirmed by a large amount of AOPP in team sport (mixed) athletes compared with the other two groups, although there is a possibility that the large concentration of superoxide anion partially was responsible for this kind of oxidative damage. Major molecular mechanisms leading to structural changes in proteins are metal-catalyzed protein oxidation characterized by loss of protein sulphydryl (-SH) groups and cross-linked protein products formation such as AOPP [19, 30]. The lack of significant differences in sulphydryl (-SH) group content could be the result of an adequate response of other antioxidant factors, such as glutathione and glutathione reductase.

Low values of parameters of oxidative damage (LOOH, AOPP, MDA and ROMs), together with the best oxidative stress/antioxidant protection ratio reflected by low PAB values, were a very positive attribute to participating in anaerobic sports. By comparing the aerobic and the anaerobic group of athletes we can conclude that secondary lipid oxidation, which was given through higher MDA values, contributed to higher PAB values in aerobic athletes. However, despite the lower pro-oxidant-antioxidant balance compared to anaerobic athletes, the aerobic group of athletes was less affected by oxidative stress-related injury than the team athletes. Based on the data obtained, we can assume that the energy expenditure (bioenergetics) that was quite different in the three examined groups, significantly affected the oxidative stress outcome. Considering that Finaud et al. defined mixed activity as an activity that involves both aerobic and anaerobic metabolism in a balanced ratio [14], we assume that several mechanisms acted simultaneously and led to measurable bimolecular damage [22]. „Mixed" training should increase the total antioxidant capacity, but the athletes in this group had the least number of training years behind them and it is possible that adaptive mechanisms did not reach the level that was substantial for adequate protection [9, 16, 20].

Discriminant analysis of the athletes according to their training regime indicated that out of all the oxidative stress/anti-oxidant defence parameters analysed the most important variables were AOPP in

Function 1 and PAB in Function 2. Parameters in Function 1, which allowed discrimination between the athletes who participated in team sports and those with aerobic and anaerobic exercise load, were generally markers of macromolecular damage and the contribution of antioxidant protection parameters were negligible. The Z Score differences for Function 1 ("Aerobic" vs. "Mixed": 2.077 and "Anaerobic" vs. "Mixed": 2.023) showed that biomarkers of oxidative damage, especially AOPP, were respectable discriminant factors. In Function 2, the greatest contribution to the total function score was provided by pro-oxidant-antioxidant balance, which enabled a clear discrimination of "aerobic" and "anaerobic' athletes (Z Score difference = 1.707). Superoxide anion is demonstrated in female volleyball athletes as an important discriminator according to years of training experience [20]. According to energy expenditure during exercise, superoxide anion compared to the AOPP was the parameter with less discriminatory potential. Although most frequently used methods for oxidative stress estimation [10, 31], measurements of free radical-mediated damages on lipids were also a weaker discriminant factor than AOPP (Table 5).

Table 5. The effects of mixed exercise on markers of oxidative stress.

Study	Sport	Increased oxidative stress marker(s)
Chang et al. [10]	Rugby	TBARS
Sureda et al. [31]	Soccer	MDA
Martinovic et al. [20]	Volleyball	Superoxide anion

The potential to discriminate athletes of AOPP and PAB is best reflected by the accuracy of classification given in Table 4. Based on the parameters in proposed discriminant functions, a correct classification of athletes was performed in 74.2% of the original grouped cases, while in 25.8 % of the cases there were similarities between them. This practically means that the measurements in these analyses provide information about the oxidative stress-related injury and pro-oxidant-antioxidant balance that could be present and associated with the different exercise mode. The limitations of our study were the absence of a nutrient intake control, due to our inability to monitor athletes all the time, and different sex comparison.

In conclusion, our study provides evidence that there are differences in the oxidative stress / anti-oxidant defence status between athletes that have different energy expenditure during exercise. Differences between the three experimental groups were mainly characterised by protein oxidation marker (AOPP) and pro-oxidant-antioxidant balance. Therefore, these discriminant factors could be useful in monitoring athletes during their training programs. Considering our results we hope to predict oxidative stress-related injury occurrence and accordingly adjust the corrective action to obtain optimal pro-oxidant-antioxidant balance.

PRACTICAL APPLICATION

Practical application of this research provides the possibility of oxidative damage prediction based on the dominant metabolic pathway. Most unfavorable oxidative stress/ antioxidative ratio in aerobic sports and the largest amount of AOPP and LOOH in team sports could be overcome by the adjustment of training and antioxidant supplementation application.

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