



Original Investigation

Effects of Maximal Anaerobic Exercise on Neutrophil Oxidants/Antioxidants in among the Sportsmen Trained at Various Levels

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Abstract

The influence of very high intensity exercise on antioxidant capacity and oxidative burst activity of neutrophils in sportsmen with various levels of training status were examined. Blood samples from sportsmen with high (n=12, 5 hours training program for a week), moderate (n=9, 2-5 hours training program for a week), and low physical activity (n=11, less than 2 hours training program for a week) were collected to determine superoxide anion production, superoxide dismutase, catalase and glutathione peroxidase activity and TBARS in neutrophils before and after an "anaerobic loading coordination test". The pre-exercise values of SOD activity were significantly higher in high and moderate physical activity groups in comparison with the low physical activity group ($p<0,05$). The TBARS levels of the low physical activity group was higher than the moderate and high physical activity groups during both pre and post-exercise periods ($p<0, 05$). A training program of 2-5 hours per week may be a beneficial strategy to up-regulate the antioxidant defense system in neutrophils.

Keywords: Superoxide anion, TBARS, neutrophils, maximal aerobic exercise

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Introduction

Exercise is generally recognized as a reproducible stressor which influences neuro-endocrine and hormonal response changes that affect functional alteration in human peripheral blood. There are few data about the effects of short-term very high intensity exercise, especially in humans. Short-term very high intensity exercise may be a potent factor of oxidative stress [1]. Very high intensity exercise is known to mobilize the peripheral leukocytes [2] and the magnitude of this reaction reflects the intensity and duration of the exercise [3].

Several researchers have reported that short duration exercise resulted in a biphasic leukocyte response, in which the immediate transient response consisted of lymphocytosis, monocytosis, and neutrophilia, followed by a delayed response mainly due to neutrophilia [3]. Although, it has been reported that concentrations of neutrophil-priming substances increase or decrease after exercise [4] in response to nonspecific immune system activation [2], it has not been elucidated whether they affect neutrophil function after a short high intensity exercise or not.

The neutrophil is a phagocytic cell of non-specific host defence [5] involved in acute inflammatory reactions [3] and forms the of first-line-of-defence against to a wide range of foreign pathogens [5]. These cells are the major source of extracellular reactive oxygen species (ROS) produced in the blood. They generate superoxide anions by activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase located on the plasma membrane. The NADPH oxidase system is activated in response to various stimuli that can be provoked by strenuous exercise [6]. It is unclear whether acute exercise or training alters the neutrophil superoxide anion production. Exhaustive exercise increases the ability of the neutrophils to produce superoxide anion regardless of training [6]; however, the intense running training reduces exercise-induced superoxide anion production. They also showed that the ability of neutrophils to generate superoxide anion was markedly increased in untrained rats after endurance exercise. ROS production may be enhanced by higher circulatory cell numbers, increased spontaneous activation and increased response (ROS production per cell) to stimuli (e.g. opsonized zymosan) [6]. In addition, during anaerobic exercise, the generation of ROS and lipid peroxides are formed at the reoxygenation stage following exercise [2]. Strenuous exercise in an unconditioned individual or someone unaccustomed to exercise will induce oxidative damage and

result in tissue injury [7]. A limited number of studies about the effect of anaerobic exercise on various aspect of neutrophil function have been undertaken.

The mechanisms of ROS generation after intensive exercise are complicated and remain uncertain. It is hypothesized that anaerobic exercise would influence neutrophil antioxidant capacity and oxidative burst activity in sportsmen with various levels of training similar to the effect of aerobic exercise as reported in animal and human experimental studies. The aims of this study were investigate the effect of short-term very high intensity exercise on neutrophil antioxidant enzyme activities, superoxide anion production and levels of thiobarbituric acid-reactive substances (TBARS).

Material and Methods

Subjects

Thirty-two male subjects were randomly selected among the soccer players from the students of the Department of Physical Education and Sport, Faculty of Education at Inonu University by the trainer. All subjects with no history of any diseases and laboratory data without abnormal limits were considered healthy. Male subjects from 18 to 26 years old were categorized into three groups according to their training status. All groups were subjected to exercise for at least a period of three months. A high physical activity group, were individuals (n=12) who performed 5 hours training per week. Moderate physical activity sportsmen (n=9) consisted of soccer players who trained 2-5 hours per week. Low physical activity sportsmen (n=11) had less than 2 hours of regular or irregular training per week. Body mass, height, body mass index (BMI), resting heart rates and maximal heart rates in high, moderate and low physical activity groups are shown in Table 1. All participants were volunteers. After being informed of the study design and possible risks, all subjects gave their written consent to participate. The Ethics Committee of Inonu University School of Medicine has approved this study protocol (Protocol No: 2001/10).

Table 1. Group characteristics

	Low Physical Activity (n=11)	Moderate Physical Activity (n=9)	High Physical Activity (n=12)
Resting Heart Rate $\pm$ SE (min^{-1})	67.0 $\pm$ 2.7*	66.7 $\pm$ 2.8**	59.5 $\pm$ 1.4*,**
Maximal Heart Rate $\pm$ SE (min^{-1})	189.1 $\pm$ 3.0	201.4 $\pm$ 7.7	194.5 $\pm$ 3.5
Body Mass (kg)	68.1 $\pm$ 1.6*	74.9 $\pm$ 2.2	76.5 $\pm$ 3.1*
Body Height (cm)	175.0 $\pm$ 1.8*	179.6 $\pm$ 1.8	181.8 $\pm$ 2.1*
Body Mass Index	22,3 $\pm$ 0,5	23.2 $\pm$ 0.7	23.0 $\pm$ 0.5

*= $p < 0,05$ between high physical activity group and low physical activity group

**= $p < 0,05$ between high physical activity group and moderate physical activity group

Special care was given to exclude subjects who were taking any drugs or supplements such as vitamins or other antioxidants, or who were smokers and consumed alcohol in the preceding 4 weeks. The subjects fasted for 12 h before the maximal exercise and refrained from exercise 24 h before the study. Medical and laboratory examination of subjects including clinical chemistry for liver and kidney function, blood cell counting, the sedimentation rate of blood and the chest X-ray graph were done. Venous blood samples were collected from the antecubital vein of subjects before and after 10 min postexercise. Pre- and post-exercise electrocardiograms, heart rate and arterial blood pressures were recorded.

The exercise protocol consisted of an “anaerobic loading coordination” test which was very high intensity that had a period of about 45 seconds. The maximal anaerobic exercise test was performed between 08.00 and 10.00 a.m. After warm up, the subjects jumped firstly three times over balls laterally to the left, then three times over sticks anteriorly, thirdly three times over ball laterally to the right within 20 m distance and they continued to the process up to exhausted of the subject (at about 45 $\pm$ 5 s) or maximal heart rate in table 1. At this period, heart rate recording was done by M22 Polar Heart Monitor (Finland). When heart rate reached to maximal value, a sign was heard from the metronome. Subjects who failed achieved to maximal heart rate, defined as 220-age min^{-1} [8] were excluded from the study.

Preparation of peripheral blood neutrophils

Reagents:

All of the chemical substances used in the study were obtained from Sigma Co. The human neutrophils were purified from the whole blood by means of a method developed by English and Anderson [9]. Whole blood was mixed with Na- citrate, precipitated using dextran 6 % for 30 min at room temperature, and the neutrophils isolated from the leukocyte-rich supernatant by Ficoll-Hypaque (specific gravity: 1.077) centrifugation. Erythrocytes were removed by hypotonic shock. The cells were washed twice and resuspended in the Hank's balanced salt solution (pH= 7.40, CaCl₂, and phenol red free) at the appropriate concentration. The number of cells was determined using a manual haemocytometer. The resulting cell suspension routinely contained live neutrophils more than 95 % by trypan blue exclusion of total 1×10^7 /ml neutrophils. The cell suspensions were kept in deep freezer prior to the determination of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSHPx) activities and thiobarbituric acid-reactive substances (TBARS) concentrations. The cell suspensions were ultrasonically treated for a period of 30-90 s (60 W in a Bandelin UW70), cooled on wet ice. The disrupted material was centrifuged at 3660x g for 50 min at 5 ° C. The supernatants were used as raw source for enzyme measurements.

Assay method of superoxide anion production

Superoxide dismutase (SOD) contained 4.100-units/mg proteins. A stock solution containing 2 mg SOD/ml water was prepared weekly and stored at 4° C. SOD solution was dissolved in 0.9 % saline to make a 1 mM concentration. Formyl-MLP was stored at -84° C in aliquots of a 0,4386 mg/ml stock solution in dimethyl sulfoxide (DMSO). fMLP solution was dissolved in 0.9 % saline to make a 10^{-6} M concentration..

We used a cytochrome c assay for monitoring O₂⁻, a method in which O₂⁻ reduces ferric cytochrome c to its ferrous form, a reduction that can be sensitively monitored at 550 nm. A substantial portion of superoxide anion is released extracellularly; its measurement serves as a sensitive and convenient way of monitoring respiratory burst activity in phagocytic cells. The extent to which O₂⁻ is responsible for the cytochrome c reduction can be determined by measuring the amount of this reduction that is sensitive to elimination by superoxide dismutase (SOD) [10].

O₂⁻ generation:

After the immediate isolation of the neutrophils, 3 ml 1X10⁷ cells/ml were added to each of two polypropylene tubes which was signed as A and B. In addition, 1.2 ml human serum, obtained from a healthy voluntary subject, was also added to tube B. The contents of each tube were mixed and placed in the 37° C and 10 % CO₂ incubator for 30 min. A and B tubes were lightly been mixed for periods of 10 min. Then each tube was sealed with a cap and placed in 37° C water incubator, and solution of 1.2 ml cytochrome c (from horse heart mitochondria (Type IV) 1.19 mM) was added on tube A and tube B cells solutions. The following reagents were added to each four test tubes (I, II, III, IV), as indicated in Table-2.

Table 2. Tubes during superoxide assay and their contents.

Tube Number of B	Saline	fMLP	SOD	Cells suspension
I	100 µl	-	-	400 µl
II	50 µl	50 µl	-	400 µl
III	50 µl	-	50 µl	400 µl
IV	-	50 µl	50 µl	400 µl

After the contents of each tube had been mixed thoroughly by vortex and then placed in the 37° C incubator, 400-µl-cell suspensions from tube B were added to the each test tube as shown in table 2. Immediately after the contents of each test tube had been mixed with 500 µl N-ethylmaleimide (NEM) solution (0.5 mM) and each of these test tubes had also been added and mixed for 2 min to complete cytochrome c reducing reaction. Then, the tube A and I, II, III,, and IV were centrifuged at 550-x g for 5 min at room temperature. The reaction solutions were decanted directly into cuvettes to determine absorption value at 550 nm by using a Shimadzu UV 1601 spectrophotometer. Calculations of superoxide anion production were determined according to the equation;

$$\% \text{ Inhibition} = \text{OD (example-I, II, III, IV tube)} / \text{OD (Blank-A tube)}$$

Determination of superoxide dismutase activity

Total (Cu–Zn and Mn) SOD (EC 1.15.1.1) activity was determined according to the method of Sun et al., [11]. The principle of the method is based on inhibition of nitro blue tetrazolium (NBT) reduction by the xanthine–xanthine oxidase system as a

superoxide generator. One unit of SOD was defined as the amount of enzyme causing 50 % inhibition in the NBT reduction rate. The SOD activity was expressed as U/ 1×10^7 /ml neutrophils.

Determination of catalase activity

Catalase (CAT, EC 1.11.1.6) activity was determined according the method of Aebi [12]. The principle of the assay is based on determination of the rate constant k (s^{-1}) or the H_2O_2 decomposition rate at 240 nm. Results were expressed as 'k' (rate constant) per 1×10^7 /ml neutrophils.

Determination of glutathione peroxidase activity

Glutathione peroxidase (GSH-Px, EC 1.6.4.2) activity was measured by the method of Paglia and Valentine[13]. The enzymatic reaction in the tube containing reduced NADPH, glutathione, sodium azide and glutathione reductase was initiated by addition of H_2O_2 , and the change in absorbance at 340 nm was monitored by a spectrophotometer. Activity was given in units per 1×10^7 /ml neutrophils.

Determination of thiobarbituric acid-reactive substance concentration

The neutrophil TBARS concentration was determined used the method of Esterbauer and Cheesemans [14], which is based on a reaction with thiobarbituric acid (TBA) at 90–100 °C. In the TBA test reaction, malondialdehyde (MDA) or MDA-like substances and TBA react to produce a pink pigment with absorption maximum at 532 nm. The reaction was performed at pH 2–3 and 90 °C for 15 min. The sample was mixed with two volumes of cold 10 % (w/v) trichloroacetic acid to precipitate the protein. The precipitate was pelleted by centrifugation and an aliquot of the supernatant was reacted with an equal volume of 0.67% (w/v) TBA in a boiling water-bath for 10 min. After cooling, the absorbance was read at 532 nm. Results were expressed as nmol / 1×10^7 /ml neutrophils.

Statistical Analysis

Data were analyzed by using a commercially available statistics software package (SPSS Ver. 10.0 for Windows). Distribution of the groups was analyzed with one sample Kolmogorov-Smirnov test. All groups were normally distributed and parametric statistical methods were used to analyze the data. One-way ANOVA test was performed to analyze differences between groups and Post Hoc multiple

comparisons were made with least significant difference (LSD). Paired t tests were used to analyze the differences between pre and post-exercise periods within the groups. Relationships among pre- and post-exercise values of the neutrophils superoxide anion production, and SOD, CAT, GSHPx activities and TBARS levels were performed by Pearson's correlation test. Results are presented as means $\pm$ SE. p values <0.05 have been regarded as statistically significant.

Table 3. The activities of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), thiobarbituric acid-reactive substance (TBARS) level and superoxide anion production in neutrophils induced by fMLP response to maximal anaerobic exercise among sportsmen trained at various levels.

	Low Physical Activity (n=11)		Moderate Physical Activity (n=9)		High Physical Activity (n=12)	
	Pre-exercise	Post-exercise	Pre-exercise	Post-exercise	Pre-exercise	Post-exercise
Superoxide anion (% / 10^7 neutrophils per ml)	74.2 $\pm$ 3.8	76.4 $\pm$ 4.3	78.6 $\pm$ 5.6	86.5 $\pm$ 3.8	81.2 $\pm$ 5.3	78.3 $\pm$ 4.1
SOD (U/ 10^7 neutrophils per ml)	1.2 $\pm$ 0.3	1.6 $\pm$ 0.3	2.5 $\pm$ 0.3*	2.3 $\pm$ 0.3	2.5 $\pm$ 0.2*	2.4 $\pm$ 0.5
CAT (k/ 10^7 neutrophils per ml)	304.0 $\pm$ 55.4	338.6 $\pm$ 47.0	453.8 $\pm$ 65.8	423.5 $\pm$ 77.0	460.0 $\pm$ 74.1	386.1 $\pm$ 63.0
GSH-Px (U/ 10^7 neutrophils per ml)	70.1 $\pm$ 10.5	61.4 $\pm$ 6.0	83.0 $\pm$ 14.0	61.6 $\pm$ 10.7	92.3 $\pm$ 9.3	81.1 $\pm$ 12.7
TBARS (nmol / 10^7 neutrophils per ml)	0.6 $\pm$ 0.2**	0.6 $\pm$ 0.2*	0.2 $\pm$ 0.0**	0.1 $\pm$ 0.0*	0.1 $\pm$ 0.0**	0.1 $\pm$ 0.0*

* p <0.05 in comparison with Low Physical Activity group post exercise.

** p <0.05 in comparison with Low Physical Activity group pre exercise.

Results

Pre-exercise values of SOD activity were significantly higher in high and moderate physical activity groups in comparison with low physical activity group (p <0.05) as shown in the Table 3. Low physical activity group TBARS level was higher than in the moderate and high physical activity groups during both pre and post-exercise samples (p <0.05). The other parameters did not show significant differences between groups during neither pre- nor post-exercise periods.

In the moderate physical activity group, there was a negative correlation between;

- post-exercise neutrophils CAT activity and post-exercise in neutrophil superoxide anion production (r=-0.667, p <0.05).
- post-exercise neutrophil CAT activity and the difference in neutrophil superoxide anion production - pre-and post-exercise (r=-0.720, p <0.029).

In the low physical activity group a negative correlation was found between post-exercise neutrophil superoxide anion production and post-exercise neutrophil TBARS level ($r=-0.624$, $p<0.05$).

In the low physical activity group we also found a positive correlation between;

- pre-exercise neutrophil GSH-Px activity and the difference in neutrophil superoxide anion production pre- and post-exercise ($r=-0.696$, $p<0.025$).
- post-exercise neutrophil GSH-Px activity and the difference in neutrophil superoxide anion production pre- and post-exercise ($r=-0.633$, $p<0.037$).

Discussions

This study was undertaken to investigate the effects of training status on maximal all-out exercise-induced alterations in the oxidative activity and antioxidant enzyme activities of circulating neutrophils. Recently, in many studies, neutrophil responses to exercise including mobilization, phagocytosis, degranulation, oxidative burst [3,15] and antioxidant enzymes activities have been examined [16-17]. However, studies on the influence of maximal bouts of strenuous exercise and training status on the function of neutrophils are limited. Several investigations of sportsmen suggest that very high intensity exercise during training and a competitive period may change the neutrophil functions [3,5,15-16, 18-20].

Our results showed that the superoxide anion production response of the neutrophils to fMLP was unchanged after the anaerobic loading coordination test under the same measurement conditions and was similar among groups with different training status as indicated in Table-3. The findings of studies that have examined neutrophil superoxide anion production in sportsmen are not unanimous. In some studies, the generation of ROS has been increased [16,18-19], while in others it has been decreased or has not changed [3, 5, 20] observed a decline in the relative proportions of reactive oxygen intermediates in fMLP-stimulated neutrophils after exercise. In general, high-intensity exercise suppresses most neutrophil functions both acutely and chronically whereas the effects of moderate exercise are conflicting [5,20]; Pyne et al. has demonstrated a decrease in neutrophil oxidative activity after a single bout of exercise [5]. When our results are compared with other researchers' findings, seven main points caught our attention: the kind of training performed, the duration of

training, the mode of exercise performed, the gender of subjects, the intensity of physical exercise, number of cases in the study groups, and time for withdrawing the specimens after the exercise. Finally, in a lot of studies, it has been showed that superoxide anion production by neutrophils changes depend on mode, intensity and duration of exercise, gender and age of sportsmen, and in vitro experimental procedure [3,5,15,19-20].

Physiological and pathophysiological effectors of extreme physical stress are considered to be ROS released from active muscle tissue itself and circulating neutrophils, because the neutrophils are strongly primed or activated by cytokines [21]. It is generally acknowledged that mobilization of neutrophils from marginated pools is mediated by increased levels of catecholamines and cortisol after exercise [3-4,6]. Stress hormones such as adrenaline, cortisol, cytokins, and growth hormone (GH), have been implicated as not only causative mediators of exercise-induced cytokine secretion but also modulators of neutrophil count and function [3-4,21]. There is evidence that the exercise-mobilized older neutrophils produce more oxygen radicals than young ones when stimulated [6]. As previously shown, resting values for oxidative activity of stimulated neutrophils in elite human athletes were lower than in untrained individuals especially when samples have been taken before the competitions [18].

Previous researchers reported that TBARS level in exercise studies increases [1,6,19,22], decreases or remains unchanged [1,4,6,23]. They suggested that these conflicting results might be attributable to methodological differences, especially in the use of nonspecific assays to detect lipid peroxidation and variations in exercise duration and intensity [6,23]. Even moderate exercise may increase ROS production, exceeding the capacity of antioxidant defense. Additionally, a previous study has shown that maximal exercise induced a larger increase in lipid peroxidation compared with moderate exercise [6]. Our results have shown that the low-level physical activity groups have higher neutrophil TBARS levels both before and after the anaerobic loading coordination test than the more active groups. Our result is supported by Groussard et al . who claim that training increases the adaptive mechanisms which regulate accumulation of lactic acid and hypoxanthine concentration, increase oxygen consumption, auto-oxidation of catecholamines, etc [1]. In addition, TBARS production in the neutrophil of sportsmen change with time

of blood sampling following exercise. Probably, low physical activity subjects have not enough adaptive mechanisms so the level of TBARS was higher in this group than in the moderate and high physical activity groups [6, 22]. For this reason, it is thought that levels of training affect the lipid peroxidation in neutrophils. The results of this study suggest that when low-trained subjects perform maximal anaerobic exercise, on alteration in neutrophil antioxidant status might not occur even though neutrophil indices of lipid peroxidation are affected.

Neutrophil SOD, CAT and GSHPx activities decrease as a consequence of the intense physical activity as indicated in Table-3. A lot of factors affect activity of antioxidant enzymes of neutrophils, such as oxidative stress induced by exercise which results in accumulation of lactic acid, oxidation of catecholamines, induction of xanthine oxidase system, stimulation of purine metabolism etc. It is suggested that exercise might cause an imbalance between free radical generation and level of antioxidant enzymes. Changes in antioxidant enzyme activity depend on duration and intensity of exercise [6]. Exercise induces neutrophil priming for oxidative activity, probably as part of the adaptation to exercise. Indirect evidence reflecting the activation of neutrophils by exercise has been pointed out after different types of exercise [17]. Neutrophil SOD activity in the low physical activity group was lower than both moderate and high physical activity groups before the test. We can explain the effect of intense exercise on antioxidant defences of neutrophils via four mechanisms: The first is the possibility of a large influx into the blood circulation of new neutrophils with lower antioxidant concentrations as a consequence of exercise; the second, as a result of the partial inactivation of the enzyme activity due to protein modifications produced by ROS or other factors. The third, the regulation of antioxidant enzyme synthesis in immune cells is modified by ROS and cytokines [17]. Fourth, following exercise antioxidant enzyme activities decrease due to extracellular secretion, although gene expression increases [16].

Conclusions

In conclusion, the metabolic adaptation of neutrophils induced by maximal all-out exercise is characterized by a decrease in intracellular antioxidant enzyme activities. The capacity of the neutrophil oxidative burst decreased after the maximal all-out exercise in moderate and high physical activity groups. The results indicate that short-

term maximal exercise induces oxidative stress, evidenced by the detection of lipid radicals and changes in the neutrophil antioxidant system. Therefore, an up-regulation of the antioxidant defense system would likely result in a reduction of exercise-induced lipid peroxidation in the neutrophil membrane in well trained sportsmen. Thus training 2-5 hours per week may be an effective strategy to up-regulate the antioxidant defense system in neutrophils.

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