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Lipid Peroxidation, Erythrocyte Superoxide-Dismutase Activity and Trace Metals in Young Male Footballers

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Physical training is known to induce oxidative stress in individuals subjected to intense exercise. In this study, we investigated plasma malondialdehyde (MDA) levels and erythrocyte superoxide dismutase (SOD) activity of 25 young male footballers and a control group of similar age. Red blood cell (RBC) count, haemoglobin (Hb) and haematocrit (Hct) values, and copper (Cu) and zinc (Zn) levels were also examined. The maximal oxygen uptake (VO_{2max}) of all subjects was determined in order to establish their functional capacity. The main finding of the present study was that plasma MDA levels, one of the most commonly used markers of lipid peroxidation, of this group of footballers aged under 21 decreased slightly when compared with those of the control group ($p<0.001$). In contrast, erythrocyte SOD activity was higher in the footballer group than in the controls ($p<0.001$). Footballers who are under regular training showed an improved antioxidant activity in comparison to sedentary controls. Plasma copper concentration, RBC count and Hb concentration of the footballer group were all significantly lower than those of the control group, ($p<0.001, p<0.01, p<0.01$, respectively). Investigating the footballers' data with Spearman's correlation analyses, the correlation coefficients (r) between Zn/Cu ratio and SOD was positive (r=0.44; $p<0.05$); and between VO_{2max} and SOD (r=0.42; $p<0.05$) were both positive. On the basis of statistical analysis, we suggest that regular exercise may be beneficial in cases of oxidative damage by reducing the amount of lipid peroxidation and increasing the activity of the antioxidant enzyme SOD.

Key Words: Superoxide dismutase, malondialdehyde, copper, zinc, VO_{2max}

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INTRODUCTION

In the past decade, evidence has accumulated that unaccustomed and strenuous exercise may cause an imbalance between reactive oxygen species (ROS) and antioxidant defence, producing an oxidatively stressful environment in the body.[1,2] In addition, during exercise; the process of delivering the oxygen to working muscles may actually result in damage to polyunsaturated fatty acids in membrane structures. This has been documented by numerous investigations demonstrating increases in by-products of lipid peroxidation following exercise.[3-6]

As lipid peroxidation occurs, it reduces the membrane fluidity, permeability and excitability, as well as alters the function of membrane-bound enzymes.[7] In cells with decreased membrane fluidity, the membrane fails to maintain tonic gradients, and eventually cellular swelling and tissue inflammation occur.[8-10] The lipid peroxidation pathway is the same at rest and exercise. However, the rate at which the reactions occur is believed to be increased during exercise. Despite exercise-induced free radical changes, there is a positive aspect to the oxidative stress associated with regular exercise. An elaborate defense system providing varying degrees of cell protection against free radicals has evolved in all species. Selected components of this defense system have been reported to increase in trained tissues following regular exercise.[5,11,12] Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) provide the primary defense against ROS generated during exercise, and the

activities of these enzymes are known to increase in response to exercise in both animal and human studies.[2,13,14]

In athletes, macroelements in the ionized form contribute to heart and muscle contractions, oxidative phosphorylation and the synthesis and activation of enzymatic systems. Zinc, as well as copper and manganese (Mn) (Cu/Zn SOD, Mn SOD), protects organisms from the effects of increased ROS.[15] Among the isoenzymes of SOD, Mn SOD is a better index of physical activity than Cu/Zn SOD. However, there is a positive correlation between the activities of Mn SOD and those of Cu/Zn SOD.[16]

Copper is also involved in many aspects of energy metabolism and is an important component in the synthesis of haemoglobin (Hb), myoglobin and cytochromes.[15] For athletes, adequate amounts of these minerals are required for physical training and performance. Rodriguez, et al. reported that the levels of plasma copper and zinc concentrations in athletes were higher than those of the control group at the beginning of the season.[17] However, studies of athletes during training, as compared to nontraining control subjects, indicate the potential for increased loss of minerals in sweat and urine.[18]

It is well known that elevated oxygen radical levels may cause increased lipid peroxidation and be measured by malondialdehyde (MDA) levels. Besides, SOD as a primary detoxification enzyme has an important role to diminish lipid peroxidation by dismutating the oxygen radicals caused by induced aerobic and anaerobic metabolism. On the basis of these considerations, we studied the antioxidant defence adaptation caused by the football training process. Our aim was to determine both oxidant and antioxidant levels after long term training programmes in young footballers and to investigate the alterations in plasma Cu and Zn levels and the effects of these elements on the Cu/Zn SOD enzyme activity, as cofactors.

MATERIALS AND METHODS

Subjects

The study subjects were 25 male footballers under regular training, between the ages of 16- and 21, and with a weight range of 66-80 kg. The inclusion criteria included at least four years regular football training. The control group comprised 25 medical students with a sedentary lifestyle and lack of practice of any regular exercise activity. The footballer and control groups were not administered any special diet, and no subjects were taking any vitamins, minerals or other supplementation in dormitory and sport camps. Prior to testing, all subjects gave informed consent as approved by the University's Policy and Review Committee of Research on Human Subjects. This study was conducted in accordance with the Helsinki Doctrine on Human Experimentation.

Anthropometry

Body mass was measured on a balance beam medical scale (fairbanks) to the nearest 0.1 kg. Height was measured on a portable stadiometer to an accuracy of ± 0.5 centimeters (cm) with the subject barefoot, feet together, and head level.

Experimental protocol

The maximal oxygen uptake (VO_{2max}) of all subjects was measured in order to establish their functional capacity. In our laboratory, all tests were carried out by the same physician (GM). Exercise tests of footballers were performed on days of resting interval, within 4 days after the end of the first 5-month-period of the competitive season. Each subject underwent a comprehensive physical examination performed by a physician. Twelve-lead electrocardiogram (ECG) recording and blood pressure measurement were also performed at resting position. Pulmonary function tests were also performed before exercise test. All tests were performed in an air-conditioned laboratory at 19℃ and 40% relative humidity, to minimize thermal stress. Subjects abstained from strenuous exercise, alcohol, tobacco, and caffeine for at least 24 hours prior to the exercise trial.

Exercise testing

All the subjects exercised on a motorized

treadmill ergometer with Bruce-type protocol, which consisted of 3-minute stages of progressively increasing speed or grade. The Bruce treadmill test is a commonly used exercise test to assess exercise capacity and the electrocardiographic response to exercise stress in adults.[19] The test protocol is suitable for maximally stressing patients, healthy individuals or trained athletes. Prior to testing the subjects were acclimated to the treadmill with a 2-min walk/run. Subjects were instructed to walk or run without the aid of the handrail. The test was stopped at the maximal exercise level. It was considered maximal if the subject achieved any two of three test criteria. The criteria for reaching the maximum included a respiratory exchange ratio of 1.10 or higher, a maximal heart rate within ± 10 beats min-1 of the age-predicted maximum and the plateau of oxygen uptake with increasing work load. During maximal exercise test, a full 10-electrode, 12-lead ECG was monitored. In all cases, a Quinton 5000 recorder and lead system (Quinton Instrument company, Seattle, WA, USA) were utilized to monitor and record ECG. Heart rate was monitored electrocardiographically. Blood pressure was measured using standard cuff manometry.

Direct measurements of maximal oxygen uptake

It is well known that the peak VO_2 values reflect true VO_{2max} as the limits of oxygen delivery.[20] Peak VO_2 was determined from expired gas measurements during the test. Expired gase (O_2 and CO_2) levels were analyzed breath by breath on a SensorMedics 2900 C Metabolic Measurement Cart (Sensormedics Corporation, Anaheim, CA, USA). Samples were analyzed for O_2 and CO_2 content by zirconia oxide and infrared analyzers, respectively. The system was calibrated before each test with standard gases of known O_2 and CO_2 concentrations.

Biochemical measurements

In the present study, blood samples were collected from the 25 members of both the control and footballer groups. Heparinized blood samples were intravenously collected from the subjects at rest under the same time and space conditions. The subjects were told to fast for at least 8 hours before the measurement. The collection of blood samples of football players was performed after the end of the first 5-month-period of the competitive season, and on the first day of the resting interval. The blood samples were stored at -80℃ until undergoing biochemical investigations.

Analysis of SOD activity

From the collected blood samples, erythrocytes were washed with physiological saline 3 times and suspended with lysate. SOD activity was determined by the spectrophotometric method of Sun, et al.[21] Briefly, SOD activity involves inhibition of nitro blue tetrazolium (NBT) reduction, with xanthine-xanthine oxidase used as a superoxide generated at pH 10.2. Under these conditions, one international unit of SOD is defined as the amount of protein that inhibits the rate of NBT reduction by 50% at 560 nm, spectrophotometrically. The erythrocyte SOD activity results were expressed as U/gr Hb.

Analysis of MDA levels

Thiobarbituric acid reactive substances (TBARs) were measured spectrophotometrically by the modified method of Buege and Aust.[8,22] In this modified method, butylated hydroxy toluene (BHT) was added to the reaction mixture to prevent lipid peroxidation during heating. Absorbance values at 572 nm were subtracted from those at 532 nm to correct background absorption. The results were expressed in μmol/L.

Analysis of minerals

Five milliliter of whole blood was collected in a heparinized tube and centrifuged at 3000 × g for 15 min. Plasma was removed and stored in polypropylene tubes at -80°. Plasma zinc and copper were measured by flame atomic absorption spectrometry (FAAS) (Shimadzu 680 AA, Tokyo, Japan).[23,24]

Measurement of Hb, Hct and RBC count

Hct values, Hb levels and RBC count were

measured using an autoanalyzer (Coulter Hmx Hematology Flow Cytometer, Beckman Inc., CA, USA) from heparinized blood samples.

Statistical analysis

All results are from both groups (50 subjects). Correlations were analyzed using Spearman's test. Results were expressed as Mean ± standard error of mean (SEM). Comparisons between the two groups were performed by using the Student's t-test and $p<0.05$ was considered significant.

RESULTS

The subjects in both groups were age, height and weight matched (Table 1). VO_{2max}, which is a measure of functional capacity, of the footballer group was significantly higher than that of the control group ($p<0.001$) (Table 1). Measured haematological parameters including RBC count and Hb levels, were significantly lower than those of the control group ($p<0.001$, for both). There was no statistically significant difference of Hct values between the groups ($p>0.05$) (Table 2). Both groups had RBC, Hb and Hct values within physiological ranges.[23,25]

Plasma copper concentration of the footballer group was significantly lower than that of the control group ($p<0.001$). But, no significant difference of plasma zinc concentrations between the groups was observed. The Zn/Cu ratio of the footballer group was also significantly higher than that of the control group ($p<0.001$) (Table 3). Both groups had Zn and Cu levels within physiological ranges (burtis).[22]

The plasma MDA levels and erythrocyte SOD activity values in both groups are shown in Table 4, along with their statistical comparison. As seen in Table 4, the MDA level is lower ($p<0.001$) while the SOD activity is higher ($p<0.001$) in the footballer group than in the control group.

Investigating the footballers' data with Spearman's correlation analyses, the correlation coefficients (r) between Zn/Cu and SOD (r=0.44; $p<0.05$)

Table 1. General Subject Characteristics

	Footballer group n=25	Control group n=25
Age (Years)	19.0 ± 0.3	18.6 ± 0.2 (NS)
Height (cm)	176.9 ± 1.1	178.5 ± 1.2 (NS)
Body mass (kg)	68.5 ± 0.8	70.2 ± 1.5 (NS)
VO_{2max} ($ml.min^{-1}.kg^{-1}$)	54.69 ± 0.44*	46.56 ± 0.39

Values are expressed as mean ± SE. Comparisons were performed using Student's t-test.
*$p<0.001$, in comparison with groups.
NS=not significant.

Table 2. Values of Haematologic Parameters in the Groups

	Footballer group n=25	Control group n=25	Physiologic values in male blood
RBC ($10^6/mm^3$)	4.96 ± 0.08	5.19 ± 0.05*	5.2 ± 0.3
Haemoglobin (g/dl)	14.77 ± 0.11	15.29 ± 0.16*	15 ± 2
Haematocrit (%)	44.67 ± 0.49	44.73 ± 0.15 (NS)	45 ± 4

Values are expressed as mean ± SE. Comparisons were performed using Student's t-test.
*$p<0.01$, in comparison with groups.
NS=not significant.

Table 3. Mineral Indices in the Groups

	Footballer group n=25	Control group n=25	Physiologic values in male plasma (μg/dl)
Copper (μg/dl)	74.89 ± 1.5	137.69 ± 4.1*	70 - 140
Zinc (μg/dl)	111.8 ± 1.9	115.82 ± 3.1 (NS)	70 - 120
Zn/Cu Ratio	1.50 ± 0.03	0.86 ± 0.03*	

Values are expressed as mean ± SE. Comparisons were performed using Student's t-test.
*$p<0.001$, in comparison with groups.
NS=not significant

Table 4. SOD and MDA Values in the Groups

Parameters	Footballer group n=25	Control group n=25
SOD (U/gr Hb)	2477.57 ± 419.17*	2196 ± 158.00
MDA (μmol/L)	1.655 ± 0.316*	2.178 ± 0.356

Values are expressed as mean ± SE. Comparisons were performed using Student's t-test.
*$p<0.001$, in comparison with groups.

and between VO_{2max} and SOD (r=0.42; $p<0.05$) were both positive.

DISCUSSION

Our study was carried out on subjects having regular football training. Footballers perform regular aerobic and anaerobic training programmes to improve functional capacity, encompassing power, sprint and endurance training during a competitive season. The oxidant stress which can develop during these types of exercise has been shown to raise the organism's antioxidant capacity for subsequent physical efforts at higher levels.[26] Brite, et al. also investigated the plasma antioxidant status in a group of soccer players engaged in a regular training programme. An increase in plasma SOD activity with a general increase in antioxidant status were observed in relation to exercise.[27]

In the present study, probably due to the elevation of oxidative stress caused by regular strenuous training in the footballer group, the organism maintains its own antioxidant enzyme system more active, and the SOD activity increases. Human tissues contain two forms of SOD: Cu/Zn SOD is located primarily in the cytoplasm, whereas MnSOD, a structurally distinct protein encoded by a different nuclear gene,[28] is located primarily in the mitochondria. Previous studies reported that both immunoreactive Mn SOD and Cu/Zn SOD contents were upregulated by endurance training[29] and that the increased activities in both SOD isoenzymes seem to be due, mainly, to the increases of the enzyme content.[30] Higuchi, et al. and Ji, et al. demonstrated that Mn SOD was primarily responsible for the increased SOD activity with training.[31,32] Ohishi et al. also studied the relationship among SOD isoenzyme activity in the rat soleus muscle after endurance training, and found that resting Cu/Zn SOD activity was significantly increased with training whereas enzyme protein and mRNA levels were not altered.[33] These data suggest that training induction of both SOD isoenzymes is caused by post-transcriptional mechanisms and that post translational modulation may play a role in Cu/Zn SOD.[31-33]

It is known that mineral nutritional status has an important role in facilitating the development of peak physical performance.[34] In our study, although both groups had Zn and Cu levels within physiological ranges, the plasma copper

concentration of the footballer group was lower than in the control group. However, these lower levels did not negatively affect the physical performances and plasma SOD activities of the footballer group.

The evacuation of copper consumed in the diet is by means of feces. In addition, a very small amount is evacuated by means of sweat and urine.[35] The low level of plasma Cu in the footballer group may be due to increased sweat amounts and urine concentrations because of their physical activity. Studies of athletes during training, as compared to nontraining control subjects, indicate the potential for increased loss of minerals in sweat and urine.[18] In addition, a recent study reported that the mean urinary concentrations of Zn and Cu increased significantly after sessions with physical exercise.[36]

Besides, the copper ions in Cu/Zn SODs appear to function in the dismutation reaction by undergoing alternate oxidation and reduction.[37] In this context, the low Cu levels of footballers are well-matched with their raised SOD activities. This situation may be another reason to explain the low level of Cu. Lukaski, et al. also reported that increases in erythrocyte SOD activity without an increase in dietary copper were a functional adaptation of copper metabolism to aerobic training.[38]

On the other hand, the Zn/Cu ratio of the footballer group was significantly higher than that of the control group. In addition, the correlation coefficient between Zn/Cu and SOD was positive in the footballers. The importance of the relationship between Zn/Cu ratios and SOD activities has been investigated in different studies. In a recent study which was characterized with oxidative stress, it was shown that the low levels of blood Zn/Cu ratios and SOD activities were associated with cancer or all causes mortality.[39] In addition, copper causes an acceleration to lipid peroxidation in human erythrocytes.[40,41] Moreover copper can also generate highly reactive hydroxyl radicals ($^{\cdot}OH$).[42] In our study, when we compared sedentary volunteers with footballers, we found that the blood Cu levels were high while Zn levels remained constant and while plasma SOD activities of the footballer group were high. These results may indicate the protective effects of exercise training in oxidative stress with high levels of plasma SOD activity in the footballers. Besides SOD activity, exercise may also increase the activities of CAT, GPX, and glutathione reductase in rat heart and skeletal muscle, although it seemed to decrease the content of vitamin E and glutathione redox state (GSH/GSSG) in muscle mitochondria.[2,4] It can be concluded that antioxidant supplementation (vitamin E, vitamin C) might be beneficial to improve antioxidant defence machinery and increase the GSH/GSSG ratio.

Miyazaki, et al. previously reported that high-intensity endurance training can elevate antioxidant enzyme activities in erythrocytes and decrease neutrophil superoxide anion (O_2^-) production in response to exhausting exercise. In addition, at rest, SOD and GPX activities were also increased after training programme.[43] Furthermore, this up-regulation in antioxidant defences was accompanied by a reduction in exercise-induced lipid peroxidation in the erythrocyte membrane.[43] Our results may show that regular training has beneficial effects by inducing SOD activity and by detoxificating oxygen radicals to prevent the production of $^{\cdot}OH$.

As was expected for aerobic training exercises, VO_{2max} values were increased in the footballer group, and the correlation between VO_{2max} and SOD was also positive. Jenkins, et al. reported the relation of oxygen uptake to antioxidant defense enzyme activity (CAT and SOD activities) in humans and rats.[44] Similar data also demonstrated that the ability to quench free radicals in serum increased in relation to the maximum ability to consume oxygen.[45]

There have been reports of "sports anaemia" associated with intensive physical exercise.[46-49] Gastrointestinal and urinary tract bleeding, iron deficiency, insufficient erythropoiesis and intravascular hemolysis are the main mechanisms which have been proposed to explain this situation.[50-52] Intravascular hemolysis is the most emphasized mechanism. Mechanical trauma (footstrike or compression of erythrocytes in capillaries within the contracting muscles), elevated body temperature, dehydration, hemoconcentration and oxidative stress may cause intravascular hemolysis during regular or sporadic bouts of exercise

and/or recovery period.[47,49] In our study, the lower haematological values found in the footballer group when compared with the control group were probably due to their intensive and chronic physical activity. However, these values were within physiologic ranges and can not be accepted as 'sports anemia'.

In conclusion, during endurance and strenuous physical loads, the primary function of the organism is to provide homeostasis, i.e. to supply the body with oxygen, detoxify cytotoxic ROS and excrete metabolites. An integral assessment of organism adaptation may be achieved by evaluating the reserve capacities to maintain this level of homeostasis. The results of the present study revealed that regular exercise may improve antioxidant enzyme activity and can be beneficial to inhibit lipid peroxidation.

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