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The influence of exogenous antioxidants and physical exercise on some parameters associated with production and removal of free radicals

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Metabolic processes generate chemically active forms of oxygen, among which a prominent role is played by the superoxide ion. Cells are equipped with defence systems against the effects of superoxide radicals, superoxide dismutase is the most impertant one. The organism depends on the delivery of exogenous antioxidants, like selenium, vitamins E and C. Physical exercise triggers the production of superoxide radicals, which can at least partly be responsible for muscular damage. This work has studied the effect of Protecton Zellaktiv® (Smith Kline Beecham, Fink Naturarznei GmbH), a preparation containing selenium, vitamins C, E, B_2, niacin and β-carotene on the activities of superoxide dismutase and catalase, levels of glutathione, malondialdehyde, selenium, iron, zinc, triglicerides, total cholesterol, HDL- and LDL-cholesterol, before and after physical exercise. Muscle status was monitored by the activities of lactic dehydrogenase and creatine kinase. Protecton Zellaktiv® was administered orally for one month, the measurements were repeated and the results before and after treatment were compared. It was found that treatment diminished the levels of malondialdehyde and zinc in serum, as well as cholesterol and triglicerides. Physical exercise before treatment decreased the levels of reduced glutatione, zinc and triglycerides. As expected, the levels of selenium were increased by the preparation. Protecton Zellaktiv® suppressed the production of malondialdehyde during physical exercise. The preparation had a beneficial effect on lipid levels and it is inferred that lipid peroxidation was suppressed.

1. Introduction

Research interest in physical exercise has traditionally focused on issues concerned with the uptake, distribution and consumption of oxygen in the organism. It has been thought since long that oxygen is as good to the organism as it is indispensable, until biochemical studies on physical exercise demonstrated that oxygen is harmful as well [1]. Adverse effects of oxygen arise from its ability to form reactive molecules, such as singlet oxygen ($^1O_2$) and the free radical (O_2^-) also called superoxide ion. McCord and Fridovich [2] were the first to report that production of superoxide ions is an intrinsic feature of normal metabolism. Later, McCord [3] demonstrated that superoxides are an important participant in inflammatory reactions. Apart from immunology, much research has been done on the metabolism and mechanisms of action of superoxides generated in mechanical processes [4] and following drug administration [5]. Recently, more attention is being devoted to the involvement of superoxide ions in aging [6].

Superoxides are able to react with almost any constituent of the cell but their principal target are polyunsaturated fatty acids, a very important structural element of cellular membranes [7]. Loss of hydrogen atoms creates organic radicals, which react with neighbouring polyunsaturated fatty acids. This peroxidation reaction continues until antioxidative agents, such as vitamin E, interfere. Peroxidation of lipids leads to reduced membrane fluidity, changes in its permeability and in the activities of membrane-bound enzymes [8]. Furthermore, lipid peroxidation generates other compounds able to leave the cell and damage external structures. One of them is malondialdehyde, a potentially carcinogenic and mutagenic agent [9, 10].

Superoxide ions arise during aerobic metabolism, which presents itself as addition of oxygen, removal of hydrogen or electron transfer and is overwhelmingly located in the mitochondrium. The mitochondrial respiratory chain is an electron transfer system at the end of which oxygen is combined with hydrogen. However, due to electron escape from the respiratory chain approximately 4–5% of oxygen is converted to the superoxide form. Production of superoxides rises whenever electrons accumulate in the respiratory chain, as it happens during respiration with limited supply of ADP [11].

Due to their detrimental properties, superoxides must be efficiently removed. Some react spontaneously forming hydrogen peroxide but most are processed enzymatically [12]. The key enzyme for their removal is superoxide dismutase, which produces hydrogen peroxide and molecular oxygen. Enzymes of this class differ depending on the metal in the catalytic center. Superoxide dismutase in the cytoplasm and extra-cellular fluid contains two atoms of copper and two of zinc. The enzyme of the mitochondrial matrix contains one atom of manganese [13, 14].

Hydrogen peroxide is transformed either by glutathione peroxidase or catalase into water and molecular oxygen. The former enzyme seems to offer the main defence against hydrogen peroxide [15]. Most tissues contain a selenium-dependent and selenium-independent glutathione peroxidase activity, the latter probably attributable to glutathione reductase [16]. Glutathione peroxidase reduces hydrogen peroxide by oxidizing glutathione to its disulphide. Oxidized glutathione is subsequently reduced by glutathione reductase [17]. Besides hydrogen peroxide, glutathione peroxidase is capable of reducing a variety of hydroperoxides [18]. The activity of the enzyme increases in parallel to the selenium content in blood, to a level above which further intake of selenium is without effect [19]. In man this level is equal to 10 μg/ml [20]. Selenium is also found in selenocysteine and selenomethionine amino acids with antioxidative properties [21].

Apart from the enzyme systems described above, there are low molecular weight substances that act as "scavengers" of radicals in the cell. This class of compounds includes vitamins A, C, E and β-carotene.

Vitamin E (tocopherol) is an important antioxidant taking part in cellular defence against free radicals. The link between the metabolism of vitamin E and selenium has been established since long and oxidative damage to many tissues is potentiated by shortage of both substances [22, 23]. Vitamin E prevents the peroxidation of lipids in lipo-

proteins and biological membranes but its antioxidative properties strictly depend on the presence of another antioxidant – vitamin C [24]. Vitamin C is the basic antioxidant in plasma and there it exerts most if its effects [25]. β-Carotene is another antioxidant, acting synergistically with tocopherol to protect lipids of lipoproteins and of biological membranes against peroxidation. Antioxidative properties are shared by coenzyme Q_{10}, present in relatively small amounts in lipoproteins, capable of regenerating reduced tocopherol and acting synergistically with it.

In spite of the complex defence against free radicals, cells sometimes are subjected to oxidative stress, which represents a disequilibrium between the generation of free radicals and their removal. This condition can be observed when, for example, superoxide dismutase is inhibited by hydrogen peroxide [26]. Catalase and glutathione peroxidase are inhibited by superoxides [27, 28]. Furthermore, in the presence of some transition metals like iron or copper, the generation of very reactive free hydroxyl radicals is observed. These radicals deserve special attention because no effective system for their removal has so far been revealed [29].

It has been documented that physical exercise triggers the production of free radicals [30–32]. Intense muscular activity often results in muscle damage and loss of normal function, a process that can be explained by oxidative stress in the muscle due to the action of free radicals [30]. During physical exercise of various intensity and character an increase in the peroxidation of lipids has been observed [33]. Adaptation to physical exercise is manifested i.a. by reduction in the level of free radicals. Markedly increased generation of free radicals upon engagement in intense exercise suggests that free radicals play an important role in normal muscular function [34, 35]. Preliminary data indicate at the same time that regeneration after physical exercise is much faster and more thorough in subjects treated with ascorbic acid [36].

Taking into account the possible link between free radicals, lipid peroxidation and muscular function it was decided to examine the effect of Protecton Zellaktiv®, a preparation containing selenium, vitamins B_2, C, E, niacin and β-carotene on some biochemical changes during intense physical exercise in healthy untrained individuals. Activities of superoxide dismutase and catalase, levels of glutathione, malondialdehyde, selenium, iron, zinc, triglycerides, total cholesterol, HDL- and LDL-cholesterol were measured before and after exercise. Muscle status was monitored by the activities of lactic dehydrogenase and creatine kinase. Protecton Zellaktiv® was administered orally for one month, the measurements were repeated and the results before and after treatment were compared.

2. Investigations, results and discussion

2.1. Influence of exercise on biochemical changes in erythrocytes and in serum

As shown in the Table, exercise led to a significant reduction in the level of reduced glutatione, zinc and triglycerides and to an increase in the level of selenium.

No significant changes in the activities of superoxide dismutase, catalase, lactic dehydrogenase and creatine kinase, as well as in the remaining parameters were observed.

2.2. Influence of Protecton Zellaktiv on biochemical changes in erythrocytes and serum

Treatment with the preparation resulted in a lowering of serum levels of malondialdehyde, zinc, total cholesterol and triglycerides. As expected, the level of selenium in-

Table: Biochemical results in erythocytes and serum before and after treatment

Parameter (units)	Before treatment		After treatment		Statistical significance
	Resting (1)	Exercise (2)	Resting (3)	Exercise (4)	
SOD (U/l)	1114.4 ± 143.5	1102.4 ± 169.1	1190.1 ± 191.1	1096.7 ± 202.2	n.s.
CAT (U/l)	318.9 ± 65.1	248.7 ± 103.4	318.9 ± 77.1	204.1 ± 91.2	(1): vs (4)*, (3): vs (4)*
GSH (mmol/l)	10.78 ± 2.33	6.49 ± 0.94	9.30 ± 1.93	6.76 ± 1.69	(1): vs (2)*, (1): vs (4)* (2): vs (3)*, (3): vs (4)*
MDA (mmol/l)	1.67 ± 0.76	2.33 ± 1.55	0.94 ± 0.49	1.82 ± 0.85	(1): vs (3)*, (2): vs (3)* (3): vs (4)*
Se (μg/ml)	55.55 ± 21.62	79.71 ± 12.52	92.60 ± 11.61	92.25 ± 16.09	(1): vs (2)*, (1): vs (3)*, (1): vs (4)*
Fe (μg/ml)	133.2 ± 62.0	121.6 ± 30.9	130.5 ± 49.6	119.4 ± 40.3	n.s.
Zn (μg/ml)	96.3 ± 13.8	83.0 ± 12.0	74.4 ± 11.3	61.1 ± 12.1	(1): vs (2)*, (3): vs (4)*, (1): vs (3)*, (1): vs (4)*, (2): vs (4)*
TCh (mg/dl)	198.6 ± 50.2	204.4 ± 51.6	153.0 ± 38.5	160.7 ± 43.6	(1): vs (3)*, (2): vs (4)*, (2): vs (3)*
HDL-Ch (mg/dl)	50.5 ± 10.3	53.6 ± 12.7	45.2 ± 10.6	47.0 ± 11.4	n.s.
LDL-Ch (mg/dl)	124.1 ± 45.1	127.3 ± 45.5	94.8 ± 34.9	97.3 ± 40.7	n.s.
TG (mg/dl)	181.8 ± 61.1	119.7 ± 67.7	85.2 ± 46.4	107.1 ± 52.0	(1): vs (2)*, (1): vs (3)*, (1): vs (4)*
LDH (U/l)	159.4 ± 47.5	172.8 ± 42.9	197.2 ± 54.5	213.2 ± 82.2	(1): vs (4)*
CK (U/l)	78.6 ± 38.7	78.7 ± 46.8	64.6 ± 34.6	83.0 ± 50.7	n.s.

for abbreviations see Experimental
* $p < 0.05$
n.s. – not significantly

creased. When exercise was undertaken a significant loss of catalase activity was seen, the level of selenium remained constant but otherwise the pattern of changes resembled that before treatment. Again, no significant changes in the activities of superoxide dismutase, lactic dehydrogenase and creatine kinase were noted.

3. Discussion

Intense physical exercise is accompanied by a spate of biochemical changes, some of which have been observed in the present study. However, the activity of superoxide dismutase (SOD) in erythrocytes remained unchanged by physical exercise and administration of exogenous antioxidants (Protecton Zellaktiv®). This is in contrast to the increased activity of this enzyme in the liver and skeletal muscles following intense exercise [37, 38]. This finding can be explained by the absence of aerobic metabolism in erythrocytes, the principal source of free radicals. Regardless of the volume of oxygen consumed by other tissues the level of free radicals in erythrocytes would remain steady. In the liver and skeletal muscles higher oxygen consumption is accompanied by a more intense production of free radicals which could be met by a rise in the activity of SOD. Treatment was without effect on the activity of SOD probably because erythrocytes contain the cytosolic isoenzyme which does not require selenium.

The activity of catalase in erythrocytes was reduced by exercise, although the change was significant only after treatment. Controversies concerning the activity of this enzyme in muscles and liver have been reported [37, 39]. Storz et al. [40] have observed a rise in the activity of catalase in Procaryota, caused by activation of the oxyR gene during oxidative stress. Nevertheless, our result is unexpected considering the observation of Rokitzki et al. [41] that catalase activity was unchanged after exercise in runners receiving α-tocopherol and ascorbic acid.

Reduced levels of glutathione in erythrocytes after exercise are a clear indication of increased production of free radicals. Lower levels of reduced glutathione (GSH) after exercise are in agreement with the study of Dufaux et al. [42]. Treatment did not change the level of GSH before exercise, unlike in the study of Tessier et al. [43]. The same authors found increased activities of glutathione peroxidase after 10 weeks of submaximal exercise. Somani and Hussain [44] noted that the affinity of glutathione peroxidase for glutathione is higher following exercise. These changes might be part of an adaptive mechanism to meet greater production of free radicals during exercise.

Resting levels of malondialdehyde in serum were significantly reduced after one month of treatment. In view of reduced levels of cholesterol and triglycerides in serum this result can be attributed both to inhibition of lipid peroxidation due to the antioxidant properties of the drug, as well as to an improvement in lipid metabolism. Following exercise, MDA levels in serum rose sharply, in agreement with the study of Jenkins et al. [45] showing that the electrically stimulated muscle is a source of this compound.

Intense exercise resulted in higher serum levels of selenium, unchanged levels of iron and lower levels of zinc. After one month of treatment resting levels of selenium increased, while those of zinc decreased. Iron levels remained unaltered. An increased serum concentration of Se is a consequence of the fact that Protecton Zellaktiv® contains selenium. Supplementation of this element seems to stabilize the selenium-dependent systems so that exercise is not accompanied by an increased demand for Se. One can only speculate that exercise leads to increased synthesis of enzymes containing zinc and thus reduces the level of this element in serum.

Exercise was accompanied by a sharp decrease in the level of triglycerides (TG). This finding was abolished by treatment possibly because Protecton Zellaktiv® reduced the TG levels in serum by more than half. The preparation also reduced the level of total cholesterol (TCh). No change in HDL- and LDL-cholesterol was noted after treatment or after exercise. Similarly, the activities of lactic dehydrogenase (LDH) and creative kinase (CK) remained essentially unaltered. It appears that exercise was not accompanied by significant muscular injury, precluding the possibility of observing a beneficial effect of the preparation on muscular function.

In summary, Protecton Zellaktiv® has had a beneficial effect on serum lipids. Lower lipid levels could have been accompanied by an inhibition of lipid peroxidation, reflected by decreased levels of malon dialdehyde (MDA) in serum. The drug increased the level of selenium, possibly contributing to a more efficient removal of free radicals and thus to a further decrease in lipid peroxidation.

4. Experimental

The study was performed in 20 healthy male volunteers who gave their written informed consent to participate in the experiment, aged 19–49 years (mean 29.15 ± 10.8), body weight 60–99 kg (mean 78.2 ± 10.9), without history of disease, non-smokers, not on any drugs and not consuming alcohol since at least two weeks before the study. The study protocol was approved by the Ethics Committee of the Pomeranian Medical University in Szczecin. Normal health status was confirmed before the experiment by history taking, physical examination, resting ECG, CXR and laboratory tests: hematology, ESR, PCV, serum total protein, albumins, glucose, urea, creatinine, bilirubin, AspAT and AlAT activities, systolic and diastolic blood pressures.

Activities of superoxide dismutase (SOD), calatase (CAT) and concentration of reduced glutathione (GSH) in erythrocytes, as well as serum concentrations of malondialdehyde (MDA), selenium (Se), iron (Fe) and zinc (Zn) ions, triglycerides (TG), total cholesterol (TCh) HDL-cholesterol (HDL-Ch), LDL-cholesterol (LDL-Ch) and activities of creatine kinase (CK) and lactic dehydrogenase (LDH) were measured before and after one month of the administration of the drug, each time before and 3 min after physical exercise. For this aim blood was drawn from the cubital vein. Coffee and food were withheld 12 hours before exercise.

The activity of superoxide dismutase was assessed using the method of McCord and Fridovich and Misra [2, 46]. Content of GSH was determined according to the method of Beutler [47] and the concentration of MDA according to the method of Wasowicz [48]. Activities of CK and LDH in serum were obtained using the method of Sznajd and Idzior-Walus [49]. The concentration of Se was obtained using the method of Danach [50]. The concentrations of Fe and Zn ions were measured according to the method of Tomaszewski [51]. Concentrations of TG, TCh, HDL- and LDL-ch were determined using the method of Tustanowski et al. [52].

Standardized physical exercise was performed until fatigue on a threadmill from Tracmaster. Exercise intensity was increased every 3 min by increasing the speed and slope of the threadmill as follows: (1) V = 3 km/h, angle < 5% (50 W); (2) V = 3 km/h, angle < 10% (75 W); (3) V = 4.5 km/h, angle < 12% (100 W); (4) V = 6 km/h, angle < 14% (150 W); (5) V = = 7 km/h, angle < 16% (250W); (6) V = 8 km/h, angle < 18% (300 W); (7) V = 9 km/h, angle < 20% (350 W); (8) V = 10 km/h, angle < 22% (400 W). Before the test each subject rested for 20 min at a temperature of 20 °C and humidity of approx. 50%. The test was performed twice: before and after one month of treatment.

The drug administered was Protecton Zellaktiv® (SmithKline Beecham, Fink Naturarznei GmbH). Each capsule (0.45 g) contained: 100 mg vitamin C, 25 mg selenium, 22 mg niacin, 18 mg vitamin E, 7.5 mg β-carotene, 2.25 mg vitamin B_2. The drug was administered orally, one capsule twice daily after meals.

Statistical analysis was done using ANOVA and Tukey's test. The level of significance was taken as 0.05.

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