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# Exercise and Oxidative Stress

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## 1. Introduction

Reactive oxygen species (ROS), as well as reactive nitrogen species (RNS), are products of normal cellular metabolism. ROS and RNS are well recognized for playing a dual role as both deleterious and beneficial species, since they can be either harmful or beneficial to living systems (Valko et al., 2006). Beneficial effects of ROS occur at low/moderate concentrations and involve physiological roles in cellular responses to noxia, as for example in defense against infectious agents and in the function of a number of cellular signaling systems. One further beneficial example of ROS at low/moderate concentrations is the induction of a mitogenic response.

On the other hand, high levels of free radicals may cause biological damage, which is termed oxidative stress and nitrosative stress (Ridnour et al., 2005). This occurs in biological systems when there is an overproduction of ROS/RNS or/and some kind of deficiency in antioxidant defense system (ADS). Generally, oxidative stress results from the metabolic reactions that use oxygen and represents a disturbance in the equilibrium status of prooxidant/antioxidant reactions in living organisms. The excess ROS can damage cellular lipids, proteins, or DNA and inhibit their normal function. Because of this, oxidative stress has been implicated in a number of human diseases as well as in the aging process. The delicate balance between physiological and pathophysiological effects of ROS is achieved by mechanisms called “redox regulation”. The process of “redox regulation” protects living organisms from various oxidative stresses and maintains “redox homeostasis” by controlling the redox status in vivo (Droge, 2002).

Free radicals, as one form of ROS, can be defined as molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbitals (Halliwell & Gutteridge, 1999). This unpaired electron(s) usually gives a considerable degree of reactivity to the free radical. Radicals derived from oxygen represent the most important class of radical species generated in living systems. Molecular oxygen (dioxygen) has a unique electronic configuration and itself is a radical. Superoxide anion ($O_2^{\bullet -}$), , arising either

through metabolic processes or following oxygen "activation" by physical irradiation, is considered the "primary" ROS, and can further interact with other molecules to generate "secondary" ROS, either directly or indirectly via enzymatic or nonenzymatic reactions (Valko et al., 2005). The production of superoxide occurs mostly within the mitochondria of a cell. Superoxide is produced from both Complexes I and III of the electron transport chain, and once in its anionic form it readily crosses the inner mitochondrial membrane. Recently, it has been demonstrated that Complex I-produced superoxide is exclusively released into the matrix and that no detectable levels escape from intact mitochondria (Muller et al., 2004). In addition, Complex III is responsible for extramitochondrial release of superoxide, but less then 50 % of total production. Other half is directly released to the mitochondrial matrix.

The hydroxyl radical ($^{\bullet}OH$) is the neutral form of the hydroxide ion and has a high reactivity, making it a very dangerous radical with a very short *in vivo* half-life (Pastor et al., 2000). The redox state of the cell is largely linked to an iron (and copper) redox couple and is maintained within strict physiological limits. The released $Fe^{2+}$ can participate in the Fenton reaction, generating highly reactive hydroxyl radical ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$). Also, the superoxide radical participates in the Haber–Weiss reaction ($O_2^{\bullet -} + H_2O_2 \rightarrow O_2 + {}^{\bullet}OH + OH^-$) which combines a Fenton reaction and the reduction of $Fe^{3+}$ by superoxide, yielding $Fe^{2+}$ and oxygen ($Fe^{3+} + O_2^{\bullet -} \rightarrow Fe^{2+} + O_2$) (Liochev & Fridovich, 2002). The most realistic *in vivo* production of hydroxyl radical according to the Fenton reaction occurs when $Mn^+$ is iron, copper, chromium, or cobalt. Although Fenton chemistry is known to occur *in vitro*, its significance under physiological conditions is not clear, noting particularly the negligible availability of "free catalytic iron" due to its effective sequestration by the various metal-binding proteins (Kakhlon & Cabantchik, 2002). However, organisms overloaded by iron (as in the conditions of hemochromatosis, b-thalassemia, hemodialysis) contain higher amounts of "free available iron" and this can have deleterious effects. "Free-iron" is transported into an intermediate, labile iron pool (LIP), which represents a steady state exchangeable and readily chelatable iron compartment (Kakhlon & Cabantchik, 2002).

Additional reactive radicals derived from oxygen that can be formed in living systems are peroxyl radicals ($ROO^{\bullet}$). The simplest peroxyl radical is $HOO^{\bullet}$, which is the protonated form (conjugate acid; p*K*a ~4.8) of superoxide ($O_2^{\bullet -}$) and is usually termed either hydroperoxyl radical or perhydroxyl radical. Hydroperoxyl radical is the most important in initiation of lipid peroxidation by two parallel pathways: fatty acid hydroperoxide (LOOH)-independent and LOOH-dependent. The LOOH-dependent pathway of $HO_2^{\bullet -}$ initiated fatty acid peroxidation may be relevant to mechanisms of lipid peroxidation initiation *in vivo*. This process is in physiological conditions mostly scavenged by action of antidoxidant enzymes, basically by superoxide dismutase (SOD), catalase (CAT), glutathione perooxidase (GPx), glutathione reductase (GR), as well as xanthine oxidoreductase (XOR) (Vorbach et al., 2003). XOR is the key enzyme in purine catabolism, by catalyzing oxidative hydroxylation of hypoxanthine to xanthine and subsequently of xanthine to uric acid. Uric acid acts as a potent antioxidant and free radical scavenger. XOR therefore has important functions as a cellular defense enzyme against oxidative stress. Thus, the synthesis of both an antioxidant (uric acid) and numerous prooxidants (ROS and RNS) makes XOR an important regulator of the cellular redox potential.

Peroxisomes are known to produce $H_2O_2$, but not $O_2^{\bullet -}$, under physiological conditions. Oxygen consumption in the peroxisome leads to $H_2O_2$ production, which is then used to

oxidize a variety of molecules. $H_2O_2$ is physiologically produced by action of SOD, who is specifically scavenging $O_2^{\bullet -}$ and released $H_2O_2$ is one end product. Cytosolic CAT and GPx can prevent overproduction of $H_2O_2$ in physiological conditions. Peroxisomes also contain catalase, which decomposes hydrogen peroxide and presumably prevents accumulation of this toxic compound. Thus, the peroxisomes maintain a delicate balance with respect to the relative concentrations or activities of these enzymes to ensure no net production of ROS. How the organelle maintains this equilibrium is unclear. When peroxisomes are damaged and their $H_2O_2$ consuming enzymes downregulated, $H_2O_2$ releases into the cytosol which is significantly contributing to oxidative stress.

Phagocytic cells, exposed to a stimulus, have the ability to recognize the foreign particle and undergo a series of reactions called the respiratory burst (De Coursey & Ligeti, 2005). Nicotine adenine dinucleotide phosphate (NAD(P)H) oxidase, one of enzymatic components in this process, is best characterized in neutrophils, where its production of $O_2^{\bullet -}$ generates the respiratory burst necessary for bacterial destruction. The nonphagocytic NAD(P)H oxidases produce superoxide at a fraction (1–10%) of the levels produced in neutrophils and are thought to function in intracellular signaling pathways.

### 1.1 Reactive Nitrogen Species (RNS)

Nitric oxide ($^{\bullet}NO$) is a small molecule that contains one unpaired electron on the antibonding orbital and therefore is a radical. $^{\bullet}NO$ is generated in biological tissues by specific 3 isomers of nitric oxide synthases (NOSs):

1. NOSI (neuronal NOS - nNOS) and
2. NOSIII (endothelial NOS – eNOS)

These are both constitutive and responsible for production of physiological amount of $^{\bullet}NO$, while

3. NOSII (inducible NOS – iNOS)

is inducible and responsible for cytotoxyc $^{\bullet}NO$ production and acts as ROS in both physiological (i.e. immune response) or different pathophysiologocial conditions.

All NOS isoforms metabolize L-arginine to L-citrulline with the formation of $^{\bullet}NO$ *via* five electron oxidative reaction (Ghafourifar & Cadenas, 2005). $^{\bullet}NO$ is an abundant reactive radical that acts as an important oxidative biological signaling molecule in a large variety of diverse physiological processes, including neurotransmission, blood pressure regulation, defense mechanisms, smooth muscle relaxation and immune regulation. $^{\bullet}NO$ has a half-life of only a few seconds in an aqueous environment. $^{\bullet}NO$ has greater stability in an environment with a lower oxygen concentration (half-life >15 s). However, since it is soluble in both aqueous and lipid media, it readily diffuses through the cytoplasm and plasma membranes (Chiueh, 1999). $^{\bullet}NO$ has effects on neuronal transmission as well as on synaptic plasticity in the central nervous system. In the extracellular milieu, $^{\bullet}NO$ reacts with oxygen and water to form nitrate and nitrite anions.

Overproduction of reactive nitrogen species is called nitrosative stress (Ridnour et al., 2004). This may occur when the generation of reactive nitrogen species in a system exceeds the system's ability to neutralise and eliminate them. Nitrosative stress may lead to nitrosylation reactions that can alter the structure of proteins and so inhibit their normal

function. Cells of the immune system produce both the superoxide anion and nitric oxide during the oxidative burst triggered during inflammatory processes. Under these conditions, nitric oxide and the superoxide anion may react together to produce significant amounts of a much more oxidatively active molecule, peroxynitrite anion (ONOO−), which is a potent oxidizing agent that can cause DNA fragmentation and lipid oxidation:

$$^{\bullet}NO + O_2^{\bullet -} \rightarrow ONOO^- \quad (1)$$

Nitric oxide readily binds certain transition metal ions; in fact many physiological effects of $^{\bullet}NO$ are exerted as a result of its initial binding to $Fe^{2+}$-Haem groups in the enzyme soluble guanylyl cyclase (sGC). The product is represented here as *{*$Fe^{3+}$*–*$^{\bullet}$*NO}*, however, *{*$Fe^{3+}$*–*NO*−}* is also commonly seen. The convention *{*FeNO*}*$^{7}$, where the superscript is the sum of the metal d electron count (here 6 or 5) and the occupancy of the relevant NO-* orbital (here 1 or 2), is 50 *M* often employed to avoid specific assignment of oxidation states.

### 1.2 Oxidative damage

Reactive species produced by activated blood cells can be released extracellularly and oxidize several plasma proteins. In addition, the same reactive species can attack the leukocytes generating them. The concentration of most amino acids is higher in neutrophils than in the surrounding plasma. The major proteins of neutrophils include those of the structural matrix, proteins required for its locomotion, chemotactic properties and adhesiveness, as well as the many granule proteins with bactericidal, hydrolytic and inflammatory functions (Smith et al., 2005). As it is the case with other cells, plasma membrane and membranes of the intracellular organelles of leukocytes are rich in lipids. Five percent of the neutrophils wet weight is lipid, which is distributed among various classes. Neutrophils and lymphocytes contain approximately 32 % and 28% PUFA of the total fatty acids by weight, respectively (Kew et al., 2004). A number of studies reported increases of malondialdehyde (an index of lipid peroxidation) after exercise in neutrophils (Ferrer et al., 2009) and lymphocytes (Sureda et al., 2008), whereas others reported no change (Ferrer et al., 2009; Tauler et al., 2008). Interestingly, it has been reported that exercise increased the percentage contribution of PUFA in neutrophils after exercise, supporting the idea that exercise may modulate neutrophil function through alterations in its fatty acid composition (Lagranha et al., 2008). On the other hand, it has been also suggested that changes in neutrophil fatty acid composition does not always lead to changes in neutrophil redox function, such as $O_2^{\bullet -}$ generation. The reactive species biology of platelets is not well studied and the physiological importance of reactive species produced by these blood cells (primarily $O_2^{\bullet -}$ and $^{\bullet}NO$) is uncertain (Halliwell & Gutteridge, 2007).

## 2. Oxidative stress and strenuous exercise

### 2.1 Aerobic exercise

Aerobic exercise is accompanied by increased oxygen consumption ($VO_2$), wand consequently increased ROS production. Aerobic exercise increases $VO_2$, which, in turn, may increase ROS production. Therefore, many studies suggested that such physical activity enhanced ROS production both in animals and in humans (Mastaloudis et al., 2001).

However, this phenomenon cannot occur with low exercise intensity (< 50 % of maximum oxygen consumption [$VO_{2max}$]). In such a case, antioxidant capacity is not overwhelmed and ROS-induced damage does not appear. Moreover, the more intense the exercise is, the more important the ROS production and the oxidative stress are. This is confirmed by some studies that show a correlation between $VO_2$ and oxidative stress. However, other studies show that oxidative stress does not increase after intense aerobic exercise (Chevion et al., 2003). Such contradictory results can be explained by antioxidant nutritional status. Effectively, these studies are done with trained subjects and because of this complexity, no single effects such as ROS production can be connected so simple. However, trained subjects can exhibit oxidative stress as well as sedentary subjects (Pincemail et al., 2000; Palazzetti et al 2003). Moreover, some differences can be explained by the methods used for the measurement of oxidative stress.

### 2.2 Anaerobic exercise

Anaerobic exercise is a type of exercise that includes a large variety of sport activities (e.g. sprints, jumps or resistance exercise). Information on the production of ROS as a result of acute anaerobic exercise is lacking compared with aerobic exercise (Groussard, 2003). However, these studies generally show an increase of the oxidative stress after supramaximal exercise such as intermittent running, sprints, jumps or sets of jumps, resistance or Wingate tests on an ergocycle (Groussard, 2003; Chen et al., 2001; Kayatekin et al., 2002; Goldfarb et al., 2005, Ramel et al., 2004).

The increase of FR production specific for the anaerobic exercise may be mediated through various pathways in addition to electron leakage which is thought to be the main source of ROS due to aerobic exercise (Groussard, 2003). In this phenomenon the dominant role belongs to xanthine oxidase and NADPH oxidase. It seems possible that ischemic reperfusion of the active muscle is greatly involved in oxidative stress during and after anaerobic exercise. Precisely, this type of exercise significantly enhances the catabolism of purins and provokes a fast deoxygenation (phenomenon of ischaemia reperfusion). These two phenomena are known to increase the activity of xanthine oxidase, which accelerates FR production. Xanthine oxidase has been demonstrated to generate FR during ischemia reperfusion, but direct evidence for xanthine oxidase as a radical generator in muscle during exercise is lacking. In ischemic tissues, it has been proposed that the xanthine dehydrogenase undergoes proteolytic conpreserve to the oxidase form, which uses $O_2$ as electron acceptor. It is known that xanthine oxidase in the presence of the substrates hypoxanthine or xanthine reduces molecular oxygen to $O_2^{\bullet -}$ and $H_2O_2$. Recently, it has been demonstrated that the enzyme can further reduce $H_2O_2$ to $OH^{\bullet -}$. Thus, it has been hypothesized that xanthine oxidase and its requisite substrates would be present in high concentrations in reperfused tissue and consequently would result in oxygen FR generation upon reperfusion.

Moreover, the increase of lactic acid, acidosis, catecholamine and post-exercise inflammation also represent important factors in increased ROS production. Inflammation and cellular damage often happen after traumatizing exercise such as impact sports or eccentric exercises (Childs et al., 2001).

## 3. Methods for exercise testing and redox status determination during exercise

Exercise testing allows measurement of basic and specific skills that are known or assumed to influence the creation of the final results in a particular sport. The testing of physical activity provides insight into the simultaneous cellular response and cardiovascular system under conditions of precisely controlled metabolic stress. The activity requires the coordinated function of the level of the heart, lungs, peripheral and pulmonary circulation with the common goal of satisfying the increased cellular respiration.

For proper testing it is necessary to provide specific conditions. Optimally equipped laboratory obtain a large number of parameters from the testing process (that must be controlled and reproducible) with continuous monitoring of blood pressure and pulse, and continuous monitoring of gas exchange, electrocardiogram and blood sampling during the test and recovery. Laboratory testing must possess certain characteristics of the microclimate with exactly defined values of temperature of 18-22 degrees Celsius and humidity of 60 %. Testing should be carried out in the morning. An athlete must have breakfast from 1 to 1.5 hour before the test, and be dressed comfortably. If blood is taken, the necessary equipment for the extraction of blood must be placed at appropriate place, in order to avoid confusion and unnecessary movement during the test. The number of people involved in testing must be limited to those who perform testing and those that are responsible for the safety of athletes.

### 3.1 Protocol for estimation of fitness status

The experiments performed by our investigation team usually start between 8 and 9 AM. Before breakfast the first blood sample is taken from venous blood by needle which is placed to all subjects. At the same time, the capillary blood and urine samples are taken too. After that, subjects have light and lean breakfast. The conventional medical examination is performed to make sure that all subjects are healthy without known acute and chronic diseases. During two hours of resting the participants are explained the aim and protocol of testing process. Then the written inform consent is obtained from all subjects.

Pulmonary $VO_2$, $CO_2$ production ($VCO_2$), and expired minute ventilation ($V_E$) are measured continuously using an automated metabolic cart (Quark b2, Cosmed Srl., Rome, Italy). Before each test ambient conditions are measured, and then the gas analyzer and the flow meter are calibrated with high precision gases. During submaximal and maximal exercise the $VO_2$ values are recorded as averages of 15 s. The participants state their subjective feeling of exhaustion by using Borg's CR10 exhaustion scale (Borg, 1982). Heart rate is monitored continuously and it is recorded as average of 15 s using a Polar Sport Tester (HRM, Finland). We hypothesize that maximal oxygen consumption $VO_{2max}$ is reached when oxygen consumption get its plateau (the time when increasing of workload can not affect increase of oxygen consumption) (Howley et al., 1995).

Anaerobic threshold (ANT) is defined as the level of $VO_2$ during exercise above which aerobic energy production is supplemented by anaerobic mechanisms and is reflected by an increase in lactate and lactate/pyruvate ratio in muscle and arterial blood. During maximal exercise test, which aims to achieve maximal oxygen uptake, there are three phases and two

types of anaerobic threshold. The first aerobic threshold is determined by V-slope method. The assessment of this threshold is obtained by cutting the two regression lines (S1, S2), that are the transition from aerobic metabolism, where $VCO_2$ increases linearly with $VO_2$ (curve S1), to anaerobic plus aerobic metabolism where the curve S2 receives a value greater than 1. This point represents the beginning of isocapnic buffering where values of ventilation (VE) grow proportionately to the concentration of $CO_2$ ($VCO_2$), and therefore ventilatory equivalent for $CO_2$ does not change at the level of aerobic threshold. The second anaerobic threshold is determined by ventilatory equivalent method by visual inspection of the breakpoints in the inflection of VE versus $VCO_2$, non linear increase of $VE/VCO_2$ versus work load and deflection point of the end tidal $CO_2$ pressure ($P_{ET}CO_2$) data curves (Wasserman et al., 1973; Caiozzo et al., 1982). Mean $VO_2$ and mean HR values are then expressed as a percentage of $VO_{2max}$ and percentage of $HR_{max}$ at which the anaerobic threshold occured (% $ANT_{VO2}$; % $ANT_{HR}$).

- Phase I represents a time between rest period and the first aerobic threshold. The intensity of this phase is characterized as very light physical activity. Achieved percentage of oxygen consumption in this stage is in the range of 45-55 % of $VO_{2max}$ or 60-70 % of maximum heart rate. The concentration of lactate in this stage of the test does not exceed 2 mmol/l (BL: <2 mmol/l). The value of this level of RPE Borg replies to 6-9. In the contemporary theory of sports training this zone is referred to as E1 (Endurance Zone 1)
- Phase II represents a time between two determined thresholds. Furthermore, this phase is divided into:

a. The intensity of this interval corresponds to light physical activity. Achieved percentage of oxygen consumption is in the range of 55-75 % of $VO_{2max}$ or 70-80 % of maximum heart rate. The concentration of lactate is between 2.0-3.0 mmol/l. The value of this level of RPE is 10-12. Applied to the training process, this zone is referred to as E2 (Endurance 2 zones).
b. The intensity of this interval is defined as a medium heavy action. Achieved percentage of oxygen consumption is in the range of 70-80 % of $VO_{2max}$ or 80-90 % of maximum heart rate. The concentration of lactate is between 3.0-4.0 mmol/l. The value of this level of RPE Borg replies to 13-14. Applied to the training process, this zone is referred to as E3 (Endurance 3 zones).

- Phase III represents a time interval between the second anaerobic threshold and the beginning of recovery phase. The intensity of this phase is characterized as heavy physical activity. Achieved percentage of oxygen consumption of this stage is between 80 % of $VO_{2max}$, or 90 % of maximum heart rate. The concentration of lactate at this stage of the test exceeds 4 mmol/l (BL: > 4 mmol/l). The value of this level of RPE Borg replies to > 14. In the contemporary theory of sports training this zone is referred to as E4 (Endurance 4 zones).

The exercise protocol for rowers that were the subjects of one of our studies mentioned later in this chapter was performed on a rowing ergometer (Indoor rower Concept 2, Canada) and consisted of 15 minutes of warm-up period on the individual level intensity, following which the workload was increased during the next four stages until exhaustion. Duration of each interval was 3+3+2+1 minute. Workloads were individually graded according to 500m time lap, which was the workload for the second interval of the test. The value of the first

and the third interval was 3 seconds above and below the time in the second interval. Hypothetically, the second interval time or intensity is enough for entering anaerobic metabolism.

The exercise protocol for taekwondo subjects that will also be mentioned later in this chapter was performed on treadmill (HP Cosmos, Germany) and consisted of a 15 minute of warm-up period. Intensity was measured by treadmill velocity on which participant reach the value of heart rate 150 beats/min during warm-up period. It was the starting velocity for the maximal test. Workload was increased by 2km/h every three minutes with constant elevation of 3 %, according to individual starting velocity.

The exercise protocol for cycling was performed on a cycle-ergometer (Ergo win 8008) and consisted of a 15 min warm-up period at 100W and cadence held at constant 90 rpm, following which the workload was increased by 40W every 2 min until exhaustion. The cycling position was standardized with a $160^{o}$ knee angle with pedal in lowest position.

The exercise protocol for handball players that were subjects of a few investigations of our team was also performed on a cycle-ergometer using modified Astrand method for determining workload while measuring pulmonary parameters using an automated metabolic cart mentioned earlier in the text.

The exercise protocol for football players who will be mentioned later was performed on a treadmill using modified Ellestad protocol while measuring pulmonary parameters using an automated metabolic cart.

Morphological characteristics (body composition parameters) of subjects in our investigations are obtained using apparatus for bioelectrical impedance analysis In Body 720 (Biospace, Korea) whose validity was previously confirmed (Lim et al., 2009).

### 3.2 Biochemical methods for determination of redox status

Determination of oxidative stress parameters from blood samples begins with separation of plasma and erythrocytes from whole blood in procedure known as 'washing' of erythrocytes. In the first step, blood is centrifuged (10 min on 3000 rpm) for extraction of plasma (usual volume 1-2 ml). The rest of the plasma is aspirated in order to keep only erythrocytes. In step two, saline is added to erythrocytes (ratio 2:1) and this is mixed on the vortex machine. Then, it is centrifuged three times (10 min on 3000 rpm). After every centrifugation, supernatant is aspirated. When last centrifugation is finished, 1 ml of erythrocytes is taken and put in new test tube. Then 3 ml of cold distilled water are added (ratio 3:1). In the final step solution must be put into cold water jacket for 30 min.

#### 3.2.1 Superoxide anion radical ($O_2^{\bullet -}$) determination

Determination of superoxide anion radical ($O_2^{\bullet -}$) plasma concentration is based on reaction of $O_2^{\bullet -}$ with Nitro Blue Tetrazolium (NBT) and forms nitro blue formazan (Auclair & Voisin, 1985). Maximum absorption for measuring is $\lambda_{max}$=550 nm. Assay mixture consists of 50 mM TRIS-HCl buffer (pH=8.6), 0.1 mM EDTA, 0.1 mg/ml of gelatin and 0.1 mM of NBT. Before using, solution needs to be gassed with nitrogen (under pressure) for 60 minutes. In the test

tubes (12 x 100) 50 µl of plasma and 950 µl of assay mixture are pipetted, and the reaction starts. For blank probe (instead of plasma) adequate volume of distilled water is used. At the beginning of the reaction, extinction of mixture is measured and noted as $E_1$. Mixing is performed (with plastic stick) every 60 sec and extinction is noted after mixing until it is stable, which considers two consecutive, approximately the same extinctions. Last extinction is noted as $E_2$. The same procedure is applied for measuring the blank probe. The concentration of released $O_2^{\bullet -}$ is calculated using the following equation:

$$\Delta E_s = E_{2s} - E_{1s} \text{ (for sample)}$$

$$\Delta E_{bp} = E_{2bp} - E_{1bp} \text{ (for blank probe)}$$

$$\Delta E = \Delta E_s - \Delta E_{bp}$$

$$\text{nmol } O_2^{\bullet -}/\text{ ml plasma} = \Delta E/0.015 \times 1/0.05$$

### 3.2.2 Hydrogen peroxide ($H_2O_2$) determination

Determination of hydrogen peroxide ($H_2O_2$) plasma concentration is based on oxidation of phenol red using hydrogen peroxide, in reaction catalised by enzyme peroxidase from horse radish (HRPO) (Pick & Keisari, 1980). This reaction results in forming of compound with maximum absorption of $\lambda_{max}$=610 nm. Linear dependence of $H_2O_2$ concentration absorbance on 610 nm is stabile for 1-60 mM of ratio concentration (1-60 nmol/ml). This method allows us opportunity to determinate forming and releasing of $H_2O_2$ in time interval from 5-60 min. Two hundred ml of plasma and 800 ml of fresh made phenol red solution (PRS) consisting of 140 mM NaCl, 10 mM potassium phosphate buffer (pH=7), 5.5 mM D(+) glucose and 0,28 mM phenol red are pippeted in the test tubes (12 x 100). Then 10 ml (1:20) HRPO, made *ex tempore*, is added. Samples are left on room temperature for 10 min, and pH is adjusted to >12 using 1M NaOH. For blank probe (instead of plasma) adequate volume of distilled water is used. Concentration of released $H_2O_2$ in venous blood is calculated using calibration diagram (standard curve) for each assay. For constructing standard curve standard (stock) $H_2O_2$ solution is used, after checking concentration (for 10 mM $H_2O_2$, A230=0.810). Five, ten and twenty ml of 1 mM $H_2O_2$ solution are pippeted in three test tubes (instead of plasma), together with 200 µl distilled water, 800 µl phenol red solution and 10 ml (1:20) HRPO. After 10 min on room temperature, pH>12 is adjusted using 1M NaOH (10 ml). Concentration and volume of released $H_2O_2$ in coronary venous effluent are calculated using factor of absorbance (F)/nmol $H_2O_2$:

$$F = \frac{\Delta A}{\text{nmol } H_2O_2/\text{cuv}}$$

On the basis of the sample absorbance ($A_s$) at $\lambda_{max}$=610 nm and comparing it with blank probe, final absorbance is calculated ($\Delta A$) ($A = A_s - A_{bp}$). For calculating plasma concentration and volume of $H_2O_2$ the following formula is used:

$$\text{nmol } H_2O_2/\text{ml plasma} = \Delta A/F$$

### 3.2.3 Index of lipid peroxidation (TBARS) determination

Level of lipid peroxidation is determined indirectly via products of lipid peroxidation reaction with thiobarbituric acid (TBA) - (Thiobarbituric Acid Reactive Substances - TBARS). This method is based on determination of level of one of lipid peroxides (malonildialdehide - MDA) with thiobarbituric acid (Ohkawa et al., 1979). Extract is obtained by combining 400 µl of 28 % TCA (Trichloroacetic acid) and 800 µl plasma. Then it is incubated in cold water jacket (-4°C) for 10 min. After incubation the samples are centrifuged on 15000 rpm for 4 min to form supernatant. In test tubes (12 x 100) 800 µl plasma extract and 200 µl 1% TBA in 0,05 M NaOH are pippeted, and this is incubated in water jacket 100°C for 15 min. As a blank probe (instead of plasma) adequate volume of distilled water is used. Measuring is performing at λ=530 nm. The concentration of released TBARS is calculated using the following equation:

$$\text{nmol TBARS/ml plasma} = \Delta A\ (A_s - A_{bp})/1.56 \times 1.25$$

$A_s$ = absorbance of sample
$A_{bp}$ = absorbance of blank probe
1.56, 1.25 – correction factors for this assay

### 3.2.4 Nitric Oxide ($^{\bullet}$NO) determination

Nitric oxide (•NO) decomposes rapidly to form stable metabolite nitrite/nitrate products. Considering that •NO in reaction with molecular oxygen forms equimolar amount of nitrites ($\bullet NO + \frac{1}{2}O_2 \rightarrow NO_2^-$), we can assert with great certainty that amount of released nitrites represent amount of released •NO. The method for detection of the plasma nitrate and nitrite levels is based on the Griess reaction. Nitrite ($NO_2^-$) is determined as an index of nitric oxide production with Griess reagent (forms purple diazo-complex) (Green et al., 1982). Griess reagent is prepared *ex tempore* just before the experiment by mixing equal amounts of stocks: 1 % (w/v) sulfanil-amide dissolved in 5 % HCL and 0.1% (w/v) aqueous solution of N-1-naphtyl-ethylene-diamine-dihydrochloride (N-NEDA). Extraction is obtained by combining 100 µl 3 M PCA (Perchloride acid), 400 µl 20 mM EDTA (ethylenediamonoetetraacetic acid) and 200 µl plasma, put on ice for 15 min, and then centrifuged for 15 min at 6000 rpm. After pouring off the supernatant, 220 µl $K_2CO_3$ is added to set pH at 7.4.

Two hundred µl plasma extract (previously described), 250 µl Griess reagent, and amonium buffer (pH=9) (containing amonium chloride - $NH_4Cl$ and sodium tetraborat - $Na_2B_4O_7$) is then pippeted in test tubes (12 x 100) in order to stabilize diazo-complex. As a blank probe adequate amount of distilled water (instead of plasma) is used. The concentration of released nitrites is calculated using the calibration curve. Calibration curve is constructed from samples extinction (which consists of specific concentration of nitrites, after their reaction with Griess reagent and in the presence of amonium buffer). For that purpose, different amount of 1 mM $NaNO_2$ (3, 6, 12, 24 µl) in 1 ml of distilled water is pippeted. After color stabilization on room temperature (5-10 min), spectrophotometrical measuring at λ=550 nm starts. The concentration and amount of released nitrites is calculated via determination of standard factor (F):

$$\frac{\text{Extinction of blank probe}}{\text{Concentration of } NaNO_2 \text{ in standard}}$$

For each standard (F1-F4), mean is calculated:

$$\text{nmol } NO_2/\text{ml extract} = \Delta E\ (E_s - E_{bp})/F$$

### 3.2.5 Superoxide Dismutase (SOD) activity determination

Determination of superoxide dismutase (SOD) activity is based on epinephrine method. This method belongs to 'negative' type group of methods, since it monitors decrease of autooxidation speed in alkaline medium, which is dependent of $O_2^{\bullet -}$ (Misra & Fridovich, 1972). SOD removes $O_2^{\bullet -}$ and thus inhibits autooxidation of epinephrine. Speed of epinephrine autooxidation is detectable spectrophotometrically via changing the absorbance on 480 nm. The accumulation of epinephrine induces increase of absorbance on 480 nm. The percentage of inhibition is used as unit for measuring catalitical activity of this enzyme. The speed of epinephrine autooxidation in absence of SOD, represents reference (control), while the speed of epinephrine autooxidation in presence of SOD (i.e. protein in cytosol) represents part of referent values. In 3.2 ml of reaction assay, containing 3 ml carbonate buffer (pH = 12) and 0.1 ml of epinephrine solution, add 0.01 ml of supernatant is added. Autooxidation of epinephrine is monitored for 4 min at λ=480 nm. Reaction is stable at temperature ratio 26-30°C. Control reaction is simultaneously performed. For calculating SOD activity, percentage of epinephrine autooxidation inhibition in presence of SOD from sample versus control reaction is used. The amount of SOD is expressed in units of SOD activity per gram of Hb (unit/gHb). This unit is defined as volume of proteins which induces 50% of epinephrine autooxidation speed inhibition in linear part of absorption increase. The concentration of SOD activity is calculated using following equation:

$$\text{SOD-1} = \frac{2(\Delta K - \Delta A)xR}{V \text{ x } Hb \text{ x } \Delta K}$$

ΔK – change of control reaction absorption per minute
ΔA - change of sample reaction absorption per minute
V – sample volume (ml)
Hb – amount of haemoglobin (g/100ml lysate)
R – dilution

### 3.2.6 Catalase (CAT) activity determination

Determination of catalase (CAT) activity in sonificate is based on method by Beutler (Beutler, 1982). This method considers spectophotometrical monitoring of hydrogen peroxide degradation speed in presence of catalase (CAT) at 230 nm. Concentration of hydrogen peroxide is calculated as follows: with regard to absorption of diluted buffer solution (1:10) (as zero), absorption of solution containing 0.9 ml diluted buffer and 0.1 ml diluted solution 30 % $H_2O_2$ (1:100), is read. Concentration of hydrogen peroxide is calculated via coefficient of extinction (for $H_2O_2$ – 0.071, at 230 nm), using formula:

$$C = \frac{\Delta A}{0.071}$$

This concentration is diluted until 10 mM.

Reaction assay: In quartz cuvette that contains 50 µl of buffer, 5 to 50 µl of sample (depending from CAT activity) is added. Reaction starts by adding 1 ml 10 mM hydrogen peroxide solution. The decrease of absorbance is monitored spectrophotometrically at 230 nm, for 3 min. The CAT activity is expressed in unit/mg protein, and this unit is defined as amount of reduced $H_2O_2$ (µM per minute). For calculation the following equation is used:

$$CAT = \frac{\Delta A \cdot R}{0.71 \cdot Low \cdot V}$$

ΔA – change of absorbance per minute
R – dilution
V – sample of volume (ml)
Low – amount of protein (mg/ml sonificate)

### 3.2.7 Reduced glutathione activity (GSH) determination

The level of reduced glutathione (GSH) in red blood cells (RBC) is determined spectrophotomatrically according to Beutler (Beutler, 1982) which is based on GSH oxidation via 5,5 dithio-bis-6,2-nitrobenzioc acid (DTNB). GSH extract is obtained by combining 0.1 ml 0.1 % EDTA, 400 µl and 750 µl precipitation solution (containing 1.67 g meta-phosphoric acid, 0.2 g EDTA, 30 g NaCl and filled with distilled water until 100 ml; solution is stable for 3 weeks on +4°C). After vortexing and extraction on cold ice (15 min), centrifugation on 4000 rpm takes place (10 min). For measuring, quartz cuvette (1ml) is used. Two hundred µl extract, 750 µl $Na_2HPO_4$ and 100 µl DTNB (1mg DTNB/ml 1 % sodium citrate) is pippeted in test tubes. As a blank probe distilled water is used. Concentration and amount of reduced glutathione in plasma is determined on the basis of calibration diagram (standard curve) for each assay. For standard curve construction standard stock-solution of GSH (concentration 1,5 mmol/l) is used. In order to determine concentration of glutathione in standard samples (nmol/GSH/ml), in 4 test tubes (instead of plasma) 10, 20, 30 and 40 µl 1 mM GSH solutions and 300 µl distilled water are pippeted. Measuring of absorbance (A) is performed at $\lambda_{max}$=420 nm. For obtaining final absorbance (ΔA), value of blank probe absorbance (B) is subtracted from absorbance (A). To calculate GSH concentration in plasma following formula is used:

$$\text{nmolGSH/ml RBC} = \Delta A / F$$

$$F = \frac{\Delta A}{nmol\,GSH\,/\,cuv}$$

## 4. Adaptive responses to free radical formation in exercise

### 4.1 The acute effects of exercise on redox status of athletes

The relationship between exercise and oxidative stress has been a topic of intensive scientific research for more than 3 decades. Since the early work of Dillard and colleagues (Dillard et al., 1978), who were the first to connect physical activity with free radical production,

hundreds of original papers have been published, but the topic of exercise-induced oxidative stress still receives considerable scientific attention. Data on the acute effects of exercise on redox homeostasis in humans are equivocal because of the many types of exercise and experimental conditions used in previous studies. Although there is some inconsistency present in the literature, it is now clear that both acute aerobic and anaerobic exercises have the potential to result in increased free radical production, which may or may not result in acute oxidative stress (Fisher-Wellman & Bloomer, 2009). It is thought that the extent of oxidative stress induced by an acute bout of exercise depends on many factors, such as exercise mode, intensity and duration, the participant's state of training, gender, age, nutrition habits, etc (Bloomer & Fisher-Wellman, 2008). Having in mind the above-mentioned factors, we designed a study that had two objectives: 1) to compare the effects of acute exercise on redox state of trained and untrained subjects and 2) to compare the effects of two exercise sessions that differ by exercise mode, intensity and duration among group of athletes.

### 4.1.1 Comparison of blood pro/antioxidant levels before and after acute exercise in athletes and nonathletes

Many studies have compared the antioxidant status of trained and untrained subjects at rest (Ørtenblad et al., 1997; Balakrishnan & Anuradh 1998; Brites et al., 1999; Evelson et al., 2002; Cazzola et al., 2003; Gougoura et al., 2007; Yamaner, 2010), but, to our knowledge, except the study of Ørtenblad and coworkers (1997) that analyzed blood antioxidant status in untrained and jump-trained humans following six bouts of 30-s continuous jumping, no study has compared the athletes' and non-athletes' pro/antioxidant responses to acute exercise of maximal intensity. The hypothesis of the first part of that study (Djordjevic, 2011) was that athletes would have not only higher activity of antioxidants in rest, but that the extent of redox disturbance induced by a maximal progressive exercise test would be lower in athletes compared with non-athletes. A group of 58 young handball players (age 16 - 19 years) and 37 age-matched adolescents who did not perform regular physical activity took part in this research. They were subjected to a maximal progressive exercise test on a cycle ergometer and taken blood sample immediately before and after exercise test.

At rest (before the exercise test), athletes had significantly higher superoxide dismutase (SOD) and catalase (CAT) activity, higher levels of glutathione (GSH) and nitric oxide ($^{\bullet}NO$) and lower levels of lipid peroxidation (TBARS) compared with non-athletes. The results regarding redox status of athletes showed that athletes really do gain desirable changes of antioxidant defense system, which alleviates the risk of oxidative stress in rest. A maximal exercise test induced statistically significant rise of superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide ($H_2O_2$) and $^{\bullet}NO$ levels in non-athletes, while TBARS levels decreased. Athletes experienced the fall in $^{\bullet}NO$ levels and the fall in CAT activity. After exercise, athletes had significantly lower levels of $O_2^{\bullet-}$ compared with non-athletes. Two way repeated measures ANOVA showed that exercise-induced changes of $O_2^{\bullet-}$, $^{\bullet}NO$ and TBARS were dependent on combination of factors - sports engagement and exercise test.

The exercise test induced the statistically significant increase of $O_2^{\bullet-}$ levels only in non-athletes who, as already mentioned, had lower levels of SOD compared with athletes. CAT and GPx are both engaged in $H_2O_2$ elimination, but their affinity for $H_2O_2$ is different and dose dependent. Affinity of GPx for $H_2O_2$ is higher at low $H_2O_2$ levels, while CAT's affinity rises with the increase of $H_2O_2$ levels. Non-athletes in our study experienced a significant

rise of $H_2O_2$ with exercise, but neither GSH levels nor CAT activity changed significantly. On the other hand, athletes did not experience the rise in $H_2O_2$ production, but CAT activity was decreased after exercise test. It may be that increased $H_2O_2$ production in non-athletes is a consequence of their less efficient ADS, while athletes' significantly higher basal GSH levels and CAT activity provided efficient elimination of excess exercise-produced $H_2O_2$. Subjects with a favorable blood glutathione redox status at rest maintain a more favorable redox status in response to exercise-induced oxidative stress (Laaksonen et al., 1999).

Resting TBARS levels of athletes in this study were significantly lower than resting TBARS levels of non-athletes. Since blood GSH was shown to be a determinant of plasma TBARS at rest (Laaksonen et al., 1999), we hypothesize that lower resting TBARS levels in athletes compared with non-athletes are a consequence of significantly higher GSH levels in athletes' blood. Maximal exercise test induced the fall of TBARS levels in group of non-athletes, which was quite unexpected if taken into consideration the behavior of other three prooxidative parameters. Namely, levels of $O_2^{\bullet -}$, $H_2O_2$ and $^{\bullet}NO$ increased after exercise test in group of non-athletes, but although the reactions between $O_2^{\bullet -}$ and other two prooxidants may lead towards formation of hydroxyl radical, a powerful inducer of lipid peroxidation, TBARS as index of lipid peroxidation was decreased.

Athletes in our study had significantly higher basal levels of $^{\bullet}NO$ compared with non-athletes. It is in accordance with numerous previous studies that showed that regular physical activity increases the bioavailability of $^{\bullet}NO$ (Kingwell et al., 1997; Jungersten et al., 1997; Maeda et al., 2001; Maiorana et al., 2003) and that physically active people have greater basal $^{\bullet}NO$ production compared with a sedentary population (Green et al., 2004; Poveda et al., 1997; Banfi et al., 2006). Studies that investigated the effects of acute exercise on $^{\bullet}NO$ production yielded various results. Some studies reported $^{\bullet}NO$ increase with exercise, some reported no change in $^{\bullet}NO$ production and some reported a decrease in $^{\bullet}NO$ production with exercise (Jungersten et al., 1997; Rassaf et al., 2007; Allen et al., 2006; Allen et al., 2009; Djordjevic et al., 2010a; Poveda et al., 1997; Djordjevic et al., 2010b; Jakovljevic et al., 2011; Cubrilo et al., 2011). Those differences are probably due to different protocols, i.e. different characteristics of subjects (age, physical activity, and health), different training and tests (type, intensity, duration of exercise tests or training), various methods of measuring RONS production, etc. The exercise test in this research induced the fall in $^{\bullet}NO$ production in athletes but the rise of $^{\bullet}NO$ levels in non-athletes. Two way repeated measures ANOVA showed that exercise-induced changes of $^{\bullet}NO$ were dependent on sports engagement of subjects. The rise of $^{\bullet}NO$ levels in non-athletes may be explained by effects of shear stress, while the response of athletes may be explained by endothelium preconditioning achieved by chronic exposure to shear stress during exercise trainings and i.e. structural and functional adaptations of endothelium (Tinken et al., 2008; Kingwell, 2000). It seems that the effects of chronic exercise on the basal NO production is more important than effects of $^{\bullet}NO$ production on tolerance of physical activity.

It should be noticed that there was statistically significant difference in total exercise test time and load between athletes and non-athletes, which may be the reason for observed biochemical responses, but the aim of our study was to assess the effects of a maximal exercise test, and the test was maximal for every participant. The differences in redox status post exercise are probably a function of the exercise-induced mechanical damage to muscle fibers and the subsequent inflammatory cascade in unaccustomed subjects.

### 4.1.2 Changes in athlete's redox state following sport specific and sport nonspecific bout of exercise

As previously mentioned, it is thought that the extent of redox state disturbance following acute bout of exercise depends on, among other factors, exercise mode, intensity and duration. Thus, the aim of our investigation was to compare the effects of sport non-specific exercise i.e. maximal progressive exercise test on a cycle ergometer and sport specific exercise session i.e. specific handball training (Djordjevic, 2011). Subjects (58 young handball players) were taken a blood sample immediately before and after both exercise bouts. Laboratory exercise test that lasted 10.57 ± 0.24 minutes and in which levels of athlete's heart rate corresponded to a submaximal and maximal intensity zone for 5.58 ± 1.27 minutes induced significant decrease of catalase activity, while one and a half hour handball training during which players spent 44.71 ± 10.52 minutes in submaximal and maximal zone of intensity induced significant decrease of superoxide dismutase activity and glutathione levels. Although handball training did not induce changes of prooxidative parameters, the fact that both exercise sessions induced depletion of antioxidants suggests that athletes experienced exercise-induced oxidative stress regardless of mode, intensity and duration of exercise.

### 4.2 Oxidative stress and nitric oxide evaluation during progressive maximal exercise test

As previously mentioned, last three decades brought hundreds of papers on relationship between acute exercise and oxidative stress, but it should be noticed that vast number of those studies were performed *in vitro*, i.e. in conditions that are not adequate to the ones in an activated muscle, so they have to be accepted with limitations. On the other hand, *in vivo* studies mostly measured free radical production after a bout of exercise, so the real extent of their production during the exercise remained unknown (Ji, 1999). The reaction between superoxide anion radical and nitric oxide represents one of the fastest reactions in the human body. This reaction not only decreases bioavailability of nitric oxide, but generates one of the most toxic reactive nitrogen species – peroxynitrite. The only limiting factor for this reaction to happen is the probability that these two species crash into one another in space. The reaction between superoxide anion radical and nitric oxide is 3 times faster than SOD-catalysed dismutation of superoxide anion radical, and the extent of their reaction increases as the levels of NO increase into the elevated nanomolar range and approach the local concentrations of SOD (Pryor et al., 1995).

Given the importance of these two species and their interaction in cardiovascular physiology, the aim of one of our previously published investigations was to assess the time-course of plasma nitric oxide ($^{\bullet}NO$) and superoxide anion radical ($O_2^{\bullet -}$) production during progressive exercise test, as well as to analyze the cause and result of changes in their production in basal conditions, during exercise and recovery. 19 elite football players were subjected to maximal progressive exercise test on treadmill. A small cathether was inserted into their antecubital vein for venous blood sampling and blood samples were taken immediately before the exercise test, in last 10 seconds of each level of the exercise testing (5 three minutes stages) without test interrupting, as well as in the 90th and 180th second of the recovery.

### 4.2.1 $^{\bullet}NO$ and $O_2^{\bullet-}$ - concentrations during the exercise test

Statistically significant difference in $^{\bullet}NO$ production (estimated through nitrites $NO_2$-) was found between resting state and grade I (increase in $^{\bullet}NO$ production), and between stage I and stage II (decrease in $^{\bullet}NO$ production). $^{\bullet}NO$ stayed decreased (lower than basal) until the end of testing and recovery period. Regarding time course of $O_2^{\bullet-}$ production, statistically significant increase in $O_2^{\bullet-}$ production was also found between the resting state and grade I (increase in $O_2^{\bullet-}$ production), then between the grade IV and V which was the time when athletes' anaerobic threshold was observed (increase in $O_2^{\bullet-}$ production), and between the grade IV and the first phase of the recovery during which levels of $O_2^{\bullet-}$ continued to rise, while in the second phase of recovery it's production started falling down.

Increased $^{\bullet}NO$ production at the beginning of the exercise test can be explained by increased blood flow and shear stress (Wollin, 2000) while the subsequent drop in $^{\bullet}NO$ production may be explained by effects of ROS ($O_2^{\bullet-}$) on $^{\bullet}NO$ bioavailability (Jackson et al., 2007). The increase of $O_2^{\bullet-}$ levels with the beginning of the test and latter $O_2^{\bullet-}$ decrease could be explained by mitochondrial properties to produce free radicals – mitochondrial free radical production increases during state 4 respiration (low $VO_2$; high membrane potential; low ATP production), while during state 3 respiration (high $VO_2$; lower membrane potential; high ATP production) it decreases (Rassaf et al., 2007). That is in accordance with results of Herrero and Barja (1997), who confirmed the decrease of radical production as $VO_2$ and ATP synthesis increase, and observed the lowest $O_2^{\bullet-}$ concentration at the point of anaerobic threshold (our subjects achieved their anaerobic threshold somewhere near the end of the stage IV). After the point of respiratory compensation (anaerobic threshold), $O_2^{\bullet-}$ concentration started to increase again, which partly can be explained by U-shaped radical formation related to the mitochondrial $pO_2$ and/or an alternative mechanism – mechanism of xanthine oxidase $O_2^{\bullet-}$ production during ischaemia and reperfusion (Ji, 1999). Also, high intensity exercise is often accompanied by increased secretion of the catecholamines which can undergo autooxidation, with and without oxygen, and produce $O_2^{\bullet-}$ (Allen et al., 2005).

### 4.2.2 $^{\bullet}NO$ and $O_2^{\bullet-}$ - interaction during the exercise test

By following the ratio of $^{\bullet}NO$ and $O_2^{\bullet-}$ production during the exercise test, we detected the point of their most intensive reaction, which turns to be near the grade IV, at the level of anaerobic threshold. It suggests that lactate threshold could be of a crucial importance not only in anaerobic and aerobic metabolism but in mechanisms of signal transductions as well.

### 4.2.3 Timecourse of $^{\bullet}NO$ and $O_2^{\bullet-}$ - production during the exercise test – Dependence on their basal levels

As basal $^{\bullet}NO$ values of athletes varied to the great extent, we divided them into three groups (depending on their basal $^{\bullet}NO$ value - B1: $^{\bullet}NO$ > 20 nmol/ml, B2: $^{\bullet}NO$ 10-20 nmol/ml, B3: $^{\bullet}NO$ < 10 nmol/ml), in order to detect differences in time-course of $^{\bullet}NO$ production between groups, i. e. to see whether basal value of $^{\bullet}NO$ affects time-course of $^{\bullet}NO$ production during the test. The results showed that groups produced higher or lower concentrations of $^{\bullet}NO$, but the pattern of $^{\bullet}NO$ production during the test was similar. According to basal $O_2^{\bullet-}$ production subjects were also divided into three groups (B1: $O_2^{\bullet-}$ > 10 nmol/ml, B2: $O_2^{\bullet-}$ = 5-10 nmol/ml and B3: $O_2^{\bullet-}$ < 5 nmol/ml). Results showed that $O_2^{\bullet-}$ production was not related either to levels of effort or to belonging to one of these three groups.

### 4.2.4 Timecourse of $^{\bullet}NO$ and $O_2^-$ production during the exercise test – Dependence on aerobic capacity of athletes

In order to determine whether dynamics of $^{\bullet}NO$ and $O_2^{\bullet-}$-production during the exercise test depends on the level of aerobic capacity of athletes, we divided athletes into three groups, based on the $VO_{2max}$ achieved on the test – G1: $VO_{2max}$ > 65 ml/kg, G2: $VO_{2max}$ = 60-65 ml/kg, and G3: $VO_{2max}$ < 60 ml/kg. We didn't find significant variations in time-course of $^{\bullet}NO$ production between groups, i.e. there wasn't significant correlation between $VO_{2max}$ and time-course of $^{\bullet}NO$ production during exercise, but that there was significant correlation between $VO_{2max}$ and $^{\bullet}NO$ basal production, since athletes who had basal $^{\bullet}NO$ production > 20 nmol/ml achieved higher $VO_{2max}$ on the test (mean value $VO_{2max}$ = 65.84 ml/kg) compared to athletes who had $^{\bullet}NO$ basal production < 20 nmol/ml (mean value $VO_{2max}$ = 60.82 ml/kg).

Correlation between $^{\bullet}NO$ bioavailability and exercise capacity was also shown in many other studies. Rassaf and colleagues showed that the capacity of the vasculature to produce $^{\bullet}NO$, and thus nitrite, predicts maximal power and duration of exercise in 55 healthy subjects (Rassaf et al, 2007). Hambrecht and colleagues suggested that improvement in $^{\bullet}NO$ -mediated vascular function improves cardiac function and $VO_{2max}$ in patients with chronic heart failure (Hambrecht et al, 1998). Allen and coworkers also showed significant relation between $VO_{2max}$ and baseline levels of nitrite and nitrate ($NO_x$), but he also found correlation between $VO_{2max}$ and the increase in $NO_2^-$ or NOx from baseline to recovery (Allen et al., 2006; Allen et al., 2005; Allen et al., 2009), which is opposite to our results (both nitrite and nitrate increased during exercise in his studies, which is opposite to our results, too). The decrease in $^{\bullet}NO$ production during the exercise test found in our study is also not in compliance with several other studies that found increased $^{\bullet}NO$ production during exercise (Rassaf et al., 2007; Allen et al., 2005, 2006, 2009), or found that $^{\bullet}NO$ didn't change during or after exercise bout (Poveda et al., 1997), but the differences are probably due to different protocols, i.e. different characteristics of subjects (age, physical activity, health), training and tests (type, intensity, duration of exercise tests or training), various methods of measuring RONS production.

Analysis of $O_2^{\bullet-}$ production during exercise showed that there were differences in time-course of $O_2^{\bullet-}$ production between group with the lowest $VO_{2max}$ and other two groups – groups with higher aerobic capacity showed decrease in $O_2^{\bullet-}$ production after the initial increase in the beginning of the test, while in group with lower $VO_{2max}$ concentrations of $O_2^{\bullet-}$ continued to rise until the stage III. Trend of decrease in $O_2^{\bullet-}$ production in groups with high $VO_{2max}$ may be explained by metabolic, cardiovascular, respiratory and endocrine adaptations that developed simultaneously with aerobic capacity development (Jones & Carter, 2000). It was shown that antioxidants appear in blood within less than 5 minutes after occurrence of free radicals (Ji & Fu, 1993), i.e. during the stage II of the exercise test, so decrease in $O_2^{\bullet-}$ production may also be explained by activity of antioxidants.

### 4.3 Oxidative stress and nitrite dynamics under maximal load in elite athletes: Relation to sport type

Maximal workload in elite athletes may result in increased generation of reactive oxygen/nitrogen species (RONS) and oxidative stress. The primary objective of our

investigation was to evaluate the effects of regular engagement in different sports on basal oxidative stress and •NO level as well as to compare their dynamics during maximal exercise testing (sport specific). The basal $NO_2^-$ (as a marker of •NO) concentrations were significantly different between examined groups of athletes. It seems that individual training led to adaptation and establishing of a new basal levels, different for different types of sport (and type of exercise). Basal values of •NO in rowers and cyclists were similar to values observed in young handball players (Djordjevic et al., 2011), while the values of taekwondo athletes corresponded to those of senior-level soccer players (Djordjevic et al., 2010). If we assume that mean nutritional intake per kg of body weight was similar in all three groups of athletes, the observed lower basal levels of •NO in taekwondo athletes compared to levels of cyclists and rowers may be due to smaller length of training or extended training cessations (more than 6 weeks) (Djordjevic et al., 2010). Furthermore, taekwondo is an anaerobic performance, and oxygen supply comes after workload without additional systemic demands for •NO-mediated vasodilatation. In aerobic workload, demands for oxygen delivery are high and depend on blood flow and endothelium-dependent vasodilatation through an increased •NO production. In our study, rowers had the highest •NO and lowest TBARS basal levels among examined sportsmen suggesting more circulating •NO and lower oxidative pressure in these athletes. The elevation in basal •NO in rowers could be related to the main mechanism that leads to the increase in •NO bioavailability and that is the decrease in •NO inactivation by ROS (Jones & Carter, 2004). Since basal levels of superoxide are similar in examined groups it seems that circulating hydrogen peroxide and lipid peroxides are effective ROS mediators of oxidative stress in these athletes. In any group of athletes, •NO levels did not change significantly due to maximal workload. Even during recovery period, measured levels were similar to values in rest.

Baseline TBARS values in the group of rowers correspond to those obtained in our previous research on young handball players (Djordjevic et al., 2011). High-basal level of TBARS in cyclists suggests that ROS production overwhelmed antioxidative defense despite possible adaptation (Miyazaki et al., 2001). These results indicate that the values of maximal oxygen uptake can not be an important predictive factor in terms of the TBARS level of athletes engaged in different sports. Only in the case of ranking athletes in one sport, $VO_2max$ value may indicate the specific dynamics of TBARS level (Djordjevic et al., 2011). Baseline values of taekwondo athletes compared with a group of cyclists once again call into question the significance of aerobic capacity level on the level of the TBARS, due to significantly lower values in the group of taekwondo athletes. Comparing the results of other researchers who have studied the impact of aerobic activity on markers of oxidative stress after a $VO_{2max}$ test, we conclude that in all of them there was an increase in TBARS concentration after a maximal exercise test on a treadmill or on bicycle (Miyazaki et al., 2001; Groussard et al,. 2003). However, comparing the results of research that examines the influence anaerobic activity on markers of oxidative stress, we recognize that anaerobic activity did not lead to an increase in TBARS concentration and/or led to its decline (Groussard et al., 2003). In our study, lower $VO_{2max}$ value in taekwondo athletes could be connected with low •NO levels supporting again notation that anaerobic and/or lower oxygen demanding performance do not request additional systemic demands for •NO-mediated vasodilatation.

Concentrations of superoxide in blood plasma were similar in rest, at the maximal intensity of exercise, and during the recovery period, in all the examined sportsman groups. Values obtained in our study were significantly higher compared to the results obtained in our previous research when analyzing the oxidative status of football and handball players (Djordjevic et al., 2011; Jakovljevic et al., 2011). It is possible that prolonged vigorous exercises cause an immediate inflammatory response and probably result in an infiltration of mononuclear cells and neutrophils in tissues. During and after exercise, this process can generate a substantial amount of ROS and, consequently, these may attack lipids and proteins (Vollaard et al., 2005). It could be that recovery period in our study was insufficient for measurable changes since examined parameters are end products of several multistep balanced processes including respiration, free radical-mediated oxidation of cellular molecules, •NO dynamics, the activity of antioxidant enzymes, and levels of circulating antioxidants and inflammation.

In summary, the data found in this study allow us to conclude that regular, long term, different training strategies (aerobic, anaerobic or aerobic-anaerobic) are able to provoke training induced upregulation of nitrite level, as well as the products of lipid peroxidation. Furthermore, maximal, progressive exercise of sport specific intensities does not influence nitrite and oxidative stress parameters level in maximal load and the first 10 minutes of recovery no matter which sport individuals perform.

### 4.4 The influence of pre-exercise superoxide dismutase activity on pro/antioxidant response to acute exercise

Since superoxide dismutase (SOD), the first line defense enzyme in red blood cells (RBCs), was the most commonly found to be the one that changes under the influence of both acute and chronic exercise (Miyazaki et al., 2001; Groussard et al., 2003; Ookawara et al., 2003) and the one that differentiates between well trained subjects and controls (Evelson et al., 2002; Metin et al., 2003; Brites et al, 1999), we hypothesized that the level of its pre-exercise (basal) activity may determine the extent of oxidative stress induced by acute exercise. So, one of our recent papers dealt with the differences in response to acute exercise in subjects with different basal level of SOD activity (Djordjevic et al., 2010a). 24 young handball players were subjected to maximal graded exercise test and taken blood samples immediately before and after exercise. Maximal progressive exercise test induced significant changes in five out of six investigated parameters of redox state ($O_2^{\bullet-}$ was not changed significantly while $H_2O_2$, •NO, TBARS, SOD, CAT were) which suggests that either this kind of exercise is a potent oxidative stress inducer, or antioxidative defense system of our subjects was not efficient enough to resist the generated prooxidants. Interestingly, when analyzing the changes of these biochemical parameters in groups of athletes with different basal SOD activity (8 athletes with the lowest, 8 athletes with average and 8 athletes with the highest levels of basal SOD activity), $H_2O_2$, •NO, SOD and CAT were changed after exercise only in group of athletes with the lowest basal SOD activity. In other two investigated groups of athletes only TBARS changed significantly. So, statistical significance of the changes seen in the whole group of investigated athletes had its roots in the group of athletes with the lowest basal SOD activity which points out to the role of pre-exercise SOD activity in maintaining desirable redox state, both in exercise-related and exercise-nonrelated conditions.

## 5. The chronic effects of exercise on redox status of athletes

### 5.1 Assessment of the redox state of athletes and non-athletes

During sports training athletes are continously exposed to various kind of stress. Adaptations to stress occur on numerous levels: from adaptations on subcellular, cellular and tissue level, to adaptations of organs and the whole organism of an athlete. Adaptations to stressors, i.e., structural and functional changes, enable improvement to occur in an athlete's sports performance. Since energy demands and oxygen consumption increase several-fold during exercise, it is thought that production of reactive oxygen and nitrogen species (RONS) also increases. Although much of early research viewed exercise-induced RONS production as a potential detriment to physiological function, more recent work investigates an alternative role for RONS production in regards to favorable exercise-induced adaptations (Fisher-Wellman & Bloomer, 2009). It is thought that the basic principle of exercise, stress-adaptation, also takes place in events related to exercise-induced oxidative stress. The exercise-induced increase in free radical production can be seen as no different from other responses to exercise: a certain load disturbs homeostasis, resulting in adaptations in the body to be able to cope with a similar load in the future (Vollaard et al., 2005). The oxidative challenge-related adaptive process of exercise is systemic and includes increased antioxidant/damage repair enzyme activity, lower oxidative damage, and increased resistance to oxidative stress (Radak et al., 2008a). The upregulation of antioxidant system provides adaptive protection from RONS during subsequent training sessions, as well as during non-exercise related conditions.

Previously published papers on exercise-induced changes in redox state of athletes mainly explored redox state of athletes engaged in dominantly aerobic or anaerobic training protocols, while mixed (aerobic-anaerobic) sports were not so explored. According to some studies, anaerobically trained subjects have a better antioxidant enzyme activity in blood, in tissues and especially in working muscle (Evelson et al., 2002; Marzatiko et al., 1997; (Radak et al., 2008b). It was also shown that a controlled protocol of endurance training is followed by an increase in antioxidant enzyme activity in plasma and other tissues (Tanskanen et al., 2010; Selamoglu et al., 2000; Lekhi et al., 2007). Regarding mixed (aerobic-anaerobic) training, there are only a few studies suggesting that trained football and rugby players show lower oxidative stress at rest than sedentary subjects, and that there is a correlation between redox status and fitness level (Chang et al., 2002; Metin et al., 2003; Cazzola et al., 2003). Currently, there is little information available regarding exercise-induced adaptations of the antioxidant defense system in adolescent and child athletes. The absence of studies investigating the effect of exercise on young population is surprising considering the numerous metabolic and physiologic differences between children, adolescents and adults (Cooper et al., 2004; Armstrong et al., 2008; Boisseau et al., 2000). The lack of information on young individuals' biochemical responses to exercise is mainly attributable to ethical concerns and methodological constraints that limit invasive research in children/adolescents. Thus, our team performed a set of investigations on exercise induced changes in redox state of young, adolescent handball players. Effects of acute exercise on their redox state were described earlier in this chapter. Here we'll focus on relationship between duration of training experience, aerobic capacities and biochemical parameters that represent factors of redox homeostasis. The first objective of our investigation was to explore the relationship between sports engagment and redox state so we compared redox

state of 33 young handball players (age 16 - 19 years) with redox state of 14 age-matched adolescents who do not have regular physical activity (Djordjevic et al., 2011). The results showed that athletes and non-athletes did not differ significantly in levels of investigated prooxidants ($O_2^{\bullet-}$, $H_2O_2$, $^{\bullet}NO$, TBARS), but athletes had significantly higher levels of SOD and lower level of CAT activity. Higher SOD activity in athletes was also found in other studies, including studies involving karate athletes (Naghizadeh et al, 2009) soccer players (Briites et al., 1999; Cazzola et al., 2003), rugby players (Evelson et al., 2002;), jump-trained subjects (Ortenblad et al., 1997) and students of Physical Education and Sports Sciences (Balakrishnan & Anuradh, 1998). Regarding CAT activity, there is a number of previous studies that also found that it's acitivity is decreased in athletes compared with controls, but there are also a number of studies that reported no change in CAT activity as a consequence of exercise training (Miyazaki et al., 2001; Lekhi et al., 2007; Metin et al., 2003; Ortenblad et al., 1997; Balakrishnan & Anuradh, 1998). According to numerous results from previous papers, it can be concluded that SOD behaviour as a consequence of chronic exercise training is clear (its' activity increases), while CAT has variant tendencies. The second objective of that work was to compare redox state of athletes with different duration of training experience. The results showed that there was no significant correlation between duration of sports engagement and redox state of athletes. The final objective of this work related to the correlation between morphofunctional characteristics and redox state of subjects. Athletes were divided into 3 groups based on ther aerobic capacity expressed through maximal oxygen consumption ((1) athletes with poor cardiorespiratory fitness ($VO_{2max}$ < 38.3 ml/kg/min), (2) athletes with average cardiorespiratory fitness ($VO_{2max}$ = 38.4– 45.1 ml/kg/min), and (3) athletes with good cardiorespiratory fitness ($VO_2$max > 45.2 ml/kg/min)). Interestingly, athletes with low aerobic capacity had higher levels of $H_2O_2$ than athletes with average or high aerobic capacity, but lower levels of TBARS. Positive correlation was found between muscle percentage and TBARS. We hypothesize that the highest levels of TBARS in athletes with the highest aerobic power may be a consequence of the higher working capacity and consequently increased oxidative stress in working musculature of these athletes.

### 5.2 Systemic adaptation to oxidative stress induced by regular long term exercise

#### 5.2.1 Morphofunctional effects of redox disturbance homeostasis induced by long term exercise

The overall positive impact of exercise on growth and development of children and youth is one of the generally accepted facts. From the aspect of physiology, adaptation to muscle activity is presented through a systematic response in order to provide the best possible performance with the lowest energy deficit. Adaptation is an universal, common characteristic of all living beings, responsible for the survival of the organism under different conditions.

Football, as a team game of simple rules without significant financial investment in equipment and space to play, is one of the most popular sports today, played by all nations, both sexes and various ages, regardless of skill level (Stolen, 2005). According to the intensity of football loads it can be classified as a highly intensive intermittent team sport (Bangsbo, 1994). For these reasons, it is evident that players are expected to have a high level of aerobic capacity and aerobic endurance. There is an evidently progressive trend of

aerobic capacity in the last 20 years (Casajus, 2001), compared with results from eighties of the twentieth century (Ekblom, 1986; Faina et al., 1988). One of our studies was focused on analysis of the aerobic capacity of young soccer players of different age (14-15 versus 16-17 years old) and length of the sports experience (Cubrilo, 2009). Surveys conducted to date show that, when compared to the seniors, young players show lower values of maximum oxygen consumption, lower than 60 ml/kg/min (Stolen, 2005), which is consistent with the results of our research. In fact, comparative analysis of our research between the categories of young athletes in relation to age and length of the sports experience showed that aerobic capacity is significantly changed in terms of age, while the length of training does not affect the value of this parameter. The values of oxygen consumption of 51.05 ± 2.39 ml/kg/min in a group of older athletes with 10-11 years of sport experience approach the values achieved at the level of elite senior players rank of Serbia (Ostojić, 2000; Ponorac, 2005). These results confirm the fact that during the development of top athletes aerobic capacity reaches its maximum between 17-22 years, then linearly decreases with age (Shephard, 1999), which would practically mean that the beginning of a process of adaptation to level of stroke volume, or end-diastolic volume as a key factor of aerobic capacity (Levine, 2008), takes place only after 16 years of age. The level of aerobic capacity in the older group of athletes positively correlated with the $H_2O_2$ concentrations, as opposed to a group of young athletes where the level of aerobic capacity negatively correlated with the level of SOD activity. Due to the higher values of SOD in the group of young athletes, a negative correlation could be interpreted as an adaptive response to significantly lower values of aerobic capacity.

The percentages of fat mass (% FM) and fat free mass (% FFM) were not statistically different in the observed groups of athletes, while body mass index (BMI) was higher in older compared to younger group of athletes. However, correlations of measured parameters were age specific. In group of older athletes, fat mass percentage positively correlated with TBARS and $H_2O_2$ levels in older group while BMI positively correlated with SOD levels and negatively with CAT levels and GPx levels. Percentage of fat free mass positively correlated with $H_2O_2$ in younger athletes. Since there was no statistically significant differences in $H_2O_2$ concentrations between groups of younger and older players, observed positive correlation between the concentration of $H_2O_2$ in plasma and valuable asset of maximal oxygen uptake in a group of older athletes is especially interesting. Given that in the older group of athletes only TBARS plasma concentrations correlated with ECG parameters at rest, one might assume that the concentration of hydrogen peroxide in plasma indirectly mediates the induction of morphological changes of the left ventricle, with resultant effects on the regulation of myocardial inotropic properties.

Maintenance of optimal amounts of reactive species formed from molecular oxygen in the homeostatic balance is of great importance for preserving health. High levels of ROS are viewed as a toxic mediator of cell and tissue injury. Erythrocytes are the cells most exposed to possible damage caused by reactive oxygen species (Tappel, 1953). The results of correlation analysis in our work indicated that CAT and GPx significantly positively correlated in the group of young players. SOD significantly negatively correlated with CAT and GPx in older players tested. In erythrocytes, a major role in maintaining homeostatic balance of ROS belongs to enzymatic antioxidant system (AOS). Studies in healthy people have shown the expression of antioxidant enzymes is in a positive correlation with SOD activity, CAT and GR. A study of 220 healthy subjects from Danish population showed that

erythrocyte SOD positively correlated with CAT, but there is no correlation between GSH-Px and other antioxidant enzymes (Andersen, 1997). The appearance of a negative correlation between SOD, CAT and GPx in the group of older players in our study resulted from the decrease in SOD activity in the older group, compared to the younger players. Reduction of SOD activity is most likely a result of inhibition of CuZn SOD by hydrogen peroxide. It is known that evidently increased antioxidant activity observed under the influence of the training process is the result of altered gene expression and influence on both the mRNA and at the level of protein synthesis. The results of these studies indicate that changes in markers of oxidative stress and antioxidant defense may occur within the normal variation in training and food intake rich in antioxidants. This means that variations in diet can affect markers of oxidative stress and antioxidant defense (for example if food is richer or poorer in antioxidants). All this indicates that under conditions of intense exercise there are numerous sources of hydrogen peroxide and nitric oxide present in the circulation and the potentiated heart and that some of these reactive species can have a detrimental effect on circulating blood elements by propagating systemic damage. Increased amounts of prooxidants in erythrocytes in the circulation may cause vascular spasm which further emphasizes the production of free radicals.

### 5.2.2 The cardiac conduction system and redox status

Young athletes in constant competition and training process represent a specific subgroup of healthy people with specific lifestyles, who gained the ability to overcome supramaximal physical efforts (Maron, 2003, Maron & Zipes 2005, Maron et al., 1996). "Athlete's heart" was first described by Henchen in the European literature of the 1899th. Applying percutaneus method, he found an increase in the heart of racers–runners (Henchen, 1899). The growing interest in studying the effects of intensive physical loads on the cardiovascular system, and enormous progress in the field of noninvasive cardiac diagnostics, especially echocardiography in the last 30 years, have led to significant process of observing and noticing of heart remodeling. This fact is the focus of scientists in monitoring and understanding of specific changes in the heart of an athlete, known as "athlete's heart" (Morganroth et al., 1975; Martin et al., 1986; Pelliccia et al., 1991; Pelliccia et al., 1996; Douglas et al., 1997; Sharma et al., 2002; Pelliccia et al., 2002; Fagard, 2003; Pelliccia et al., 2005; Maron & Pelliccia, 2006; Pelliccia et al., 2007). Electrophysiological changes associated with athlete's heart syndrome, reflected by changes in heart rhythm, change in heart conductivity, repolarization, and precordial voltage changes, are consequently manifested by changes on ECG. Previous studies related to the search for the causes of these changes were mainly oriented towards the study of the autonomous regulation of heart rate due to changes in terms of increased vagal tone on the one hand and the suppression of sympathetic nerve regulation on the other. It is evident that intensive training influences the autonomic control and intrinsic cardiac pacemaker function (Huston et al., 1985). For these reasons, there is growing interest in more complete understanding of systemic change in the heart of an athlete, with emphasis on differentiation of changes in terms of age, or the influence of sports training and competition. Another, no less important reason is related to the strict classification of ECG changes in terms of successful differentiation of athlete's heart from cardiovascular disease and the possible development of sudden cardiac death of athletes.

Understanding the specific adaptations of the sport and its specific mechanisms and regularity, especially in a group of young athletes, requires an interdisciplinary approach, which should answer many questions related to the optimization of training, the athletes' health and morbidity in terms of preventing injury and sudden cardiac death in the field. Regular, intense physical activity is associated with increased dimensions of the heart muscle, where these changes are interpreted as benign (Maron, 1998, Maron & Pelliccia, 2006). In addition, it was shown that cardiac dimensions and functional abilities tend to change over training cycle as an adaptation to the specific requirements of a particular sports season (Crouse et al., 1992). The degree of cardiac adaptations can be determined by various factors including age, sex, type and intensity of exercise (Pelliccia et al., 2000), as well as ethnicity and race (Crouse et al., 2009).

Analysis of electrocardiograms of athletes in our study (Cubrilo, 2009), who were included in regular, intense physical activity, showed that some ECG changes may occur in athletes under the influence of training on the one hand or the regular process of growth and development during the normal training cycle on the other. It is shown that athletes' age significantly affects the dynamics of change in P wave voltage to the lead D3, the amount of ST elevation in V1 lead, and the duration of PQ, QRS and QT intervals. On the other hand, the length of the sports experience showed a statistically significant effect on the mean QRS vector, P wave voltage to the lead D2, as well as the value of the sum of Σ (RD1 + SD3). Also, in older athletes it has been reported higher incidence in percentage of sinus arrhythmia and an incomplete right bundle branch block, regardless of the length of the sports experience. The results of our research in terms of classification of ECG findings, based on generally accepted clinical criteria (Pelliccia et al., 2000), show that athletes 57 % of both age groups can be classified as with normal ECG findings while 41 % is classified as partially normal and 2 % significantly abnormal findings with potential structural changes and clinical significance. The largest number of research on electrocardiographic changes include athletes between 18 and 35 years, while very few studies focused on the monitoring of young athletes with the aim of determining the difference in the adaptation of the heart to the training process in relation to the age. In our study ECG voltage criteria of LVH (RD1 + SD3> 25 mm) (Gubner & Ungerleider, 1943) showed statistically significant difference compared to the length of the sports experience. Consideration of the dynamics of average values of this parameter in the observed groups of athletes showed a decreasing trend with increasing length of the sports experience in both age groups. Average values of P waves in leads D2 and D3 showed statistically significant differences observed among groups of athletes, where the dynamics of P waves in the lead D2 showed a statistically significant difference compared to the length of the sports experience, as opposed to the dynamics of the P wave to the lead D3, where it was observed statistically significant differences by athletes age. By analyzing the dynamics of average values of P wave in D2 lead, voltage reduction is observed with increasing length of the sports experience. The mean QRS vector in our study shows a statistically significant upward trend in this parameter with increasing length of the sport experience in both groups.

Increased oxidative stress is involved in the pathophysiology of diverse diseases such as atherosclerosis, neurodegeneration, renal disease and cancer. Over the past 20 years, significant evidence has suggested a role for increased oxidative stress in the pathophysiology of congestive heart failure (CHF). In previous scientific work focus is mainly put on the analysis of adaptive response to redox disturbance during physical

activity, where the subject matter referred to the response of skeletal muscle, liver and brain (Radak, 2008). The potential connection of ROS as second-messenger in terms of transmission of biological information through the modulation of signaling molecules, enzymes and proteins at the level of the heart muscle and heart conduction system of young athletes, represented through ECG changes, in current literature has not yet been processed. The concept of ROS as normal and necessary components of the cellular milieu is emerging as an important homeostatic mechanism that participates in the control of multiple cellular processes (Finkel, 1998; Lander, 1997). Skeletal muscles produce ROS at a rate that is activity dependent. This, plus evidence of ROS induced cellular damage, lead to idea that ROS may participate in the development of fatigue and/or activity induced injury as well as processes of cardiac contraction and relaxation (intracellular calcium cycle). More recently, various steps of the contractile process have proven to be susceptible to redox modulation. First, the opening probability of isolated sarcoplasmic reticulum (SR) $Ca^{2+}$ release channels of the ryanodine receptors increases upon oxidation of accessible protein thiols. Moreover, SR $Ca^{2+}$ reuptake is inhibited by high concentration of $H_2O_2$. Finally, oxidants alter myofibrillar $Ca^{2+}$ sensitivity in a time and concentration dependent fashion. This so-called redox signalling function is especially true for the ROS, $H_2O_2$, which is more stable and diffusible than radical species such as $O_2^{\bullet -}$, but also applies to nitric oxide. An important consideration in the experimental use of oxidants such as $H_2O_2$ is whether the observed changes are physiological or are the results of overt oxidative stress. This issue has become topical in our research on young football players (children of 14-15 years old) where $H_2O_2$ positively correlated with the percentage of muscle, while in the older group of athletes demonstrated (16-17 years old) a positive correlation between $H_2O_2$ and the percentage of fat was found. A positive correlation between $H_2O_2$ and the sum of Σ(SV1 + RV5) was also observed.

The observed positive correlation between TBARS concentration and QT interval duration, and a negative correlation between the concentration of $^{\bullet}NO$ and PQ interval duration can be observed in the light of altered $Ca^{2+}$ homeostasis as an indicator of oxidative stress. Andrade and coworkers showed that continuous exposure to relatively high concentrations of $H_2O_2$ for extended periods of time caused resting $Ca^{2+}$ to increase and slowed the return of $Ca^{2+}$ to resting levels after stimulation ended (Andrade et al., 1998). Prolonged QT syndrome is a functional abnormality probably coupled with neurological severe impacts that can cause lethal arrhythmias (Schwartz et al., 1975). The acquired forms of prolonged QT interval may be due to idiosyncrasies of medicines (antiarrhythmics and psychotropic drugs), electrolyte abnormalities, hypothermia, toxic substances and injuries of the central nervous system (Bhadari & Scheinman, 1985). Correlations obtained in our study may include oxidative stress as the cause of the prolonged QT interval in young athletes. TBARS as index of lipid peroxidation could play a significant role in the modulation of cardiac systolic phase in which the mechanism of action can be linked to $Ca^{2+}$ homeostasis and the $Ca^{2+}$ influx through slow calcium channels. TBARS could act on the $Ca^{2+}$ dependent slow action potential, which is its negative inotropic effect: reduced $Ca^{2+}$ current with prolongation of the action potential slow phase.

Heart conductivity disorders are very common finding in active athletes. First-degree atrioventricular block is represented with 6-33 % (Puffer, 2001) or 35-40 % (Crisafulli et al., 2002) in athletes, which is significantly higher than in the general population (0.65 %). The observed negative correlation between the concentration of $^{\bullet}NO$ and PQ interval duration in young athletes called into question a possible link with the myocardial (atrial) contractile

•NO effect. Conductivity at the AV (atrioventricular) node is mediated by increased parasympathetic tone and/or reduced sympathetic tone in rest. So far some of the most important controversies surrounding the myocardial contractile effect of •NO were if •NO exert a myocardial contractile effect under baseline conditions or only following adrenergic or cholinergic stimulation on the one hand, or can contractile effects of •NO be labeled as positively or negatively inotropic on the other. One might assume that •NO might play an important role in the contraction of the atria (presystolic phase) and the filling of the ventriculi in the physiological resting conditions of the athlete's heart.

Changes in QRS complexes voltage are very common finding in the sports population. However there is considerable variation in the percentage of literature data which goes up to 8-76 %, thus the problem of standardization of criteria for their registration exists. Intensive, long-practicing activity is associated with morphological changes in heart muscle, including increased volume, as well as increased thickness of the walls of the heart (Huston et al., 1985; Fagard, 2003). The main difference between pathologycal and physiological hypertrophy is the nature of the stimulus for the growth of cells, as well as the duration of a given stimulus. For the development of physiological hypertrophy, the most common stimulus is training process, with effect from time to time, episodic, and largely through the sympathetic neurotransmitter.

Several studies showed high incidence (80 %) sportsmen who met electrocardiographic criteria for left ventricular hypertrophy using the Sokolow and Lyon criteria (S wave in V1 + R wave in V5> 35 mm) (Sharma et al., 1999; Pelliccia et al., 2000). The percentage of the right ventricle was 18-69 %, where the sum is taken as a criterion RV1 and SV5 deflection greater than 10.5 mm. ECG voltage criteria of LVH (Gubner & Ungerleider, 1943), ( RD1 + SD3> 25 mm), in our study showed a statistically significant difference compared to the length of the sports experience. Consideration of the dynamics of average values of this parameter in the observed groups of athletes observed a decreasing trend with increasing length of the sports experience in both age groups.

Electrical heart axis in sport depends on age, chest structure and position of heart in chest cavity. It is manifested by the development of left ventricular hypertrophy. In about 60-70 % of all athletes electrical cardiac axis is between 30-70 degrees, and turn 90 degrees to the right and more encounters with 10-20 % of athletes. Turning electrical axis of 30 degrees to the left and more are found in 15-25 % of athletes. Thus, it was determined that it often turns to the right of athletes involved in endurance sports type, while in athletes involved in power sports-type it often turns to the left (Sharma et al., 2002). The literature has shown that the shaft QRS complex becomes more vertical with increasing levels of well trained, often associated with right bundle branch block incomplete which can be explained by increased myocardial mass at the top of the right ventricle. The mean QRS vector in our study shows a statistically significant upward trend (verticalisation) with increasing length of sport experience of the athlete in both groups. In the group of younger athletes, negative corelation between $O_2^{\bullet-}$ concentration in plasma and P wave voltage in inferior bipolar leads was observed and/or negative correlation with the mean QRS vector. On the other hand, in the older group of athletes a positive correlation between TBARS in plasma with values voltage criteria for LVH (RD1 + SD3) was observed, and negative correlation with the mean QRS vector. The presence of these correlations confirms the assumptions about the role of ROS as signaling molecules ideal, where the level of their production during and after physical activity could be a crucial link between exercise and consequently disturbed

homeostasis caused by the influence of adaptation at the level of gene transcription (Vollaard et al., 2005).

The following research could provide a clearer picture of the possible diagnostic and therapeutic importance (individual antioxidant supplementation) of these correlations in a group of young athletes of both ages. Mechanical stretch and neurohumoral factors induce changes in intracellular signaling pathways resulting in increase protein synthesis and the activation of specific genes promoting growth and potentially leading to ventricular remodeling. Our results demonstrated that oxidative stress and body (systemic) redox state influence heart functionality, but the effects are age specific.

## 6. Effect of different supplementation strategies on nitric oxide and oxidative stress parameters dynamics during maximal exercise testing

### 6.1 The influence of L-Arginin supplementation on nitric oxide and oxidative stress parameters dynamics during maximal exercise testing

Arginine is one of the 20 amino acids (AA) necessary for protein synthesis and coded by DNA. Plasma arginine concentrations are therefore maintained mostly by protein catabolism (≈ 85 %) (Morris et al., 2006) or by synthesis from other AA. Of the total synthesis, de novo synthesis accounts for < 15 %, while 60 % of arginine is derived from citrulline (Wu et al., 1998). Arginine metabolism may give rise to several other AA and molecules indispensable for life. Exogenous arginine is largely destroyed (≈ 40 %) during absorption by the gut itself. Much attention was given to arginine availability due to the discovery in the 1980's that endothelium-dependent relaxing factor' is a gas, nitric oxide (•NO), produced from arginine by endothelial •NO synthase (eNOS) (Ignarro et al., 1987) in a reaction that gives also rise to citrulline. In endothelial cells, eNOS and the two sequential enzymes argininosuccinate lyase (ASL) and argininosuccinate synthase (ASsynth), necessary for recycling citrulline to arginine, are co-localized in the caveolae (Li et al., 2005), a fraction of cell membranes that provides an efficient environment for maintaining arginine available to eNOS. The cytoplasm of endothelial cells has a concentration of arginine largely saturating eNOS, and yet the acute introduction of exogenous arginine elicits an increase in •NO production, a puzzling finding known as "the arginine paradox" (Kurz et al., 1997). Another puzzling finding is the peculiar regulation of arginine transport and its effects on •NO. The transport of arginine into cells is mediated by the cationic AA transporter 1 (CAT1). Over-expression of CAT1 enhances arginine uptake sixfold, and •NO production twofold. The presence of AA competing with arginine decreases arginine uptake, but not •NO production (Li et al., 2005). Moreover, citrulline succeeds in stimulating NO production even in a medium containing saturating levels of arginine, and extracellular citrulline does not influence intracellular arginine levels. Therefore, •NO production depends mostly on the efficient recycling of arginine-derived citrulline back to arginine, and not so much on exogenous arginine supply. Thus, the presence of a "micro-environment", where arginine metabolism and recycling are independently regulated and only partially in balance with plasma arginine concentrations, is the most consistent explanation for the endothelial arginine paradox (Flam et al., 2007).

#### 6.1.1 Endothelial vasomotion and the exogenous arginine paradox

To understand the clinical paradox of arginine, i.e. the fact that the much needed arginine is ineffective or actually detrimental when supplemented chronically, we have to focus on

another pathway of the complex arginine metabolism, which is controlled by the ubiquitous enzymes arginases (types, 1 and 2), which compete with NOS for arginine as a substrate. The effect of both arginases is to catalyze the cleavage of urea from arginine, thus forming ornithine. Urea controls osmolarity and water content of plasma and cells. The other product of the reaction, ornithine, may be recycled to citrulline and also synthesized de novo by the liver from glutamine, and then transformed by the gut into citrulline. The kidneys provide further recycling of citrulline to arginine, which is finally released into the plasma (Van de Poll et al., 2007). Arginase 1 (ARG 1) is a cytosolic enzyme, mostly expressed in the liver and red blood cells. Deletion of the ARG 1 gene, as occasionally found in humans, is incompatible with prolonged life, and the accompanying hyperargininemia is associated with a several-fold increase in the activity of the mitochondrial arginase (ARG 2), which accounts for the persistent ureagenesis in those patients (Grody et al., 1989). ARG 2 is widely expressed, mostly in the kidneys, gut and brain. ARG 2 overexpression plays a critical role in the pathophysiology of cholesterol-mediated endothelial dysfunction (Schulman et al., 2006). Arginases and NOS compete for arginine, and – under any conditions – arginase activity exceeds NOS activity at all NOS/arginase molar ratios (Santhanam et al., 2008). Moreover, although the $K_M$ of arginases is 100-fold higher than that of NOS, the enzymes compete for arginine because the maximal catalytic rate of arginases is more than 1000 times higher than that of NOS (Wu et al., 1998; Topal et al., 2006). Therefore, increased expression and/or activity of ARG have a deep impact on NOS efficiency. As an example, arginase activity is increased in type 2 diabetic subjects with impaired NOS activity, and such impairment correlates with the degree of hyperglycemia and is reduced by insulin (Kashyap et al., 2008). The problem is more interesting because results of experimental and clinical studies are quite controversial and can not definitely support relevant conclusions. There are only few experimental studies dealing with this problem.

The study of Maxwell and coworkers (Maxwell et al., 2001) was performed to determine whether supplementation with L-arginine would prevent the decline in aerobic capacity observed in hypercholesterolemic mice. In one previous study the same authors determined the exercise capacity of wild-type (E1) and apolipoprotein E-deficient mice (E2) at 8 week of age when the cholesterol levels of both strains are low. The observed decline in $VO_{2max}$ is associated with endothelial vasodilator dysfunction and reduced urinary nitrate excretion. This study was designed with the intention of averting the impairment in aerobic capacity associated with hypercholesterolemia through chronic supplementation of L-arginine. Those results clearly showed that administration of L-arginine restores exercise-induced EDNO synthesis and normalizes aerobic capacity in hypercholesterolemic mice. In normal mice, L-arginine enhances exercise-induced EDNO synthesis and aerobic capacity. As we noted above, investigation on humans do not clearly support exact role of L-arginine/NO system in exercise-induced vasodilation in healthy subjects, athletes or patients in some pathophysiological statement. Increased vasodilation and large artery distensibility occur after dynamic aerobic exercise (Kingwell et al., 1997; Naka et al., 2003) probably attributed in part to augment $^{\bullet}$NO release (Green et al., 2002; Gilligan et al., 1994). Although the contribution of $^{\bullet}$NO to large artery stiffness, wave reflection (AIx), and pressure amplification during exercise has never been tested, it is an important consideration relevant to cardiac structure and cardiovascular risk (Sharman et al., 2007). If reduced $^{\bullet}$NO bioavailability underlies abnormal exercise hemodynamics in a fashion similar to that found in men with hypercholesterolemia, we may expect that induction of endothelial dysfunction

in healthy men (by blockade of •NO synthase) should lead to increased AIx and reduced PP amplification. Sharman and coworkers (Sharman et al., 2008) compared this hypothesis in young men who performed submaximal cycle exercise after intravenous infusion of L-NMMA with an •NO-independent control vasoconstrictor (noradrenaline [NE]) and placebo (saline). This study supports modulatory role of •NO in large artery stiffness, pressure amplification, and myocardial loading under resting conditions. However, this is the first study to report that •NO does not affect large artery hemodynamics and wave reflection such that ventricular-vascular interaction is substantially altered during low-intensity aerobic exercise. Systemic infusion of L-NAME (an inhibitor of NOS) caused significant increase in MAP, but not in $VO_{2max}$ in healthy volunteers at submaximal exercise. Furthermore, that reduction was significant in maximal exercise (Jones et al., 2004). These finding suggest that muscle blood flow was well preserved during submaximal exercise following NOS inhibition, indicating multifactorial influence on blood flow regulation during exercise.

On the other hand, one other study on healthy subjects showed that L-NAME decreases lactate production during exercise, which is quite logical, taken into consideration well known fact that •NO stimulates glycolysis. These data indicated that skeletal muscle •NO production represents an important limitation to the acceleration of oxidative metabolism following the onset of supramaximal exercise in humans (Wilkerson et al., 2004).

In conclusion, the study by Taddei and coworkers (Taddei et al., 2000) demonstrated that regular physical training protects the vascular endothelium from aging-related alterations. The beneficial effect of exercise is related to preservation of •NO availability by a mechanism probably linked to the prevention of oxidative stress and the consequent •NO breakdown. This beneficial effect could be important in accounting for the positive impact of regular exercise on cardiovascular risk in the elderly population. Arginine and glutamine are two non-essential amino acids than can become "conditionally essential" because of elevated needs during pathological conditions, and metabolism may not be able to maintain their concentrations at sufficient levels to match metabolic requirements. Chronic exogenous arginine supplementation has not proven to exert positive clinical effects in different trials, and sequential articulation of the knowledge of introduction of arginine-driven transcriptional, translational, and epigenetic adaptations may give us a key for interpreting those controversial results. Study by Lerman and coworkers demonstrated that oral L-arginine supplementation for 6 months improves coronary endothelial function in association with improvement in symptoms in patients with non-significant coronary artery stenosis. This study proposes a therapeutic role for L-arginine in patients with chest pain and coronary endothelial dysfunction (Lerman et al., 1998).

The role of L-arginine supplementation in cardiac patients remains controversial. Furthermore, it is also unclear if arginine supplementation in the sedentary population can have the same results. Further research will be needed to assess the interaction of these factors and to determine the effects of prolonged administration of arginine and antioxidants on exercise performance. Supporting that, a recent study in trained athletes, who were without any cardiovascular problems, showed beneficial effect of L-arginine in elderly physically active subjects (Chen et al., 2010). An arginine and antioxidant-containing supplement increased the anaerobic threshold and the work at anaerobic threshold at both week one and week three in elderly cyclists. No effect on $VO_{2max}$ was observed. This study

indicates a potential role of L-arginine and antioxidant supplementation in improving exercise performance in elderly.

Our data about different supplementation in different sports are in accordance with those results on elderly cyclist (Cubrilo, 2006). So, both vitamins and combination of vitamins+amino acids (L-arginine+L-citruline, as potential improvement of L-arginine/NO system) increased basal level of •NO in different sports: cycling, rowing and taekwondo. Furthermore, two months supplementation by vitamins+amino acids increased release of •NO and showed antioxidant properties in acute exercise, what can be start point for further strategies in systemic supplementation in elite athletes (Cubrilo, 2006).

## 7. Conslusions and perspectives

Regarding to all notes listed above, general conclusion about role of oxidative stress in exercise suggest that reactive oxygen species play in part role in oxidative damage during exercise. Taken into consideration controversial results of many studies, as well as our own results, we can not clearly conclude which ROS is responsible for such events in strenuous exercise. Furthermore, therapeutic interventions with different antioxidants and other supplements are, also, controversial. Based on our results, SOD might be important marker for acute adaptation on oxidative damage, but this note need further support with serious studies with large number of well randomized participants. Also, based on recent results only measurement of isoprostans, 8-Br-Deoxyguanosine and allantoin in body fluids can support conclusion about oxidative damage. Anyway, we are in a big open field and usage of redox status as potential new therapeutic target needs further big clinical trials.

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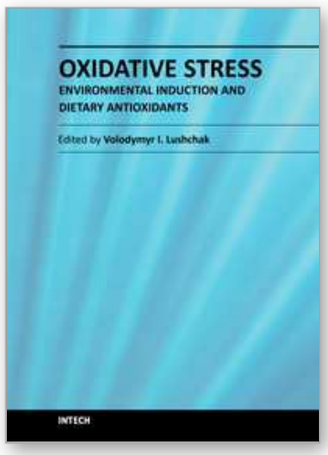


**Oxidative Stress - Environmental Induction and Dietary Antioxidants**

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This book focuses on the numerous applications of oxidative stress theory in effects of environmental factors on biological systems. The topics reviewed cover induction of oxidative stress by physical, chemical, and biological factors in humans, animals, plants and fungi. The physical factors include temperature, light and exercise. Chemical induction is related to metal ions and pesticides, whereas the biological one highlights host-pathogen interaction and stress effects on secretory systems. Antioxidants, represented by a large range of individual compounds and their mixtures of natural origin and those chemically synthesized to prevent or fix negative effects of reactive species are also described in the book. This volume will be a useful source of information on induction and effects of oxidative stress on living organisms for graduate and postgraduate students, researchers, physicians, and environmentalists.



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