THE OXIDATIVE STRESS AND ANTIOXIDANT DEFENSE IN THE BLOOD OF THE JUMPING HORSES DURING EXERCISE

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ABSTRACT

The aim of the present study was to investigate the effect of intensive exercise on levels of oxidative stress biomarkers (alkyl hydroperoxides, middle molecules, 2-thiobarbituric acid reactive substances, the carbonyl derivatives of oxidatively modified proteins) and antioxidant defenses (superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase activity, ceruloplasmin level, total antioxidant capacity) in well-trained Ukrainian warmblood horses involved in equestrian show jumping. Our results suggest that training session results in different consequences on oxidative stress biomarkers in the plasma and erythrocytes of horses. The decrease in plasma TBARS level in jumping horses after exercise could be attributed as adaptive mechanism to regular exercise with activation of the antioxidant defense. Significant decrease of ketonic derivatives in the erythrocytes of horses after training session is result of exercise-induced adaptation. This is an important adaptive response because the induction of a repair mechanism – proteasome complex – is regarded as a repair enzyme. The significant increase of catalase and glutathione reductase activity in horses after exercise is a manifestation of the compensatory and adaptive reaction of the organism to training session. High level of total antioxidant capacity is determined by superoxide dismutase, catalase, glutathione reductase activity and
causes to decrease of ketonic derivatives of oxidatively modified proteins with increase of catalase and glutathione reductase activity in the blood of horses before exercise. High level of total antioxidant capacity in the erythrocytes of jumping horses is determined by glutathione reductase activity. Catalase activity is in synergic relation with glutathione reductase activity and causes to decrease of superoxide dismutase activity in the blood of horses after exercise.

Key words: jumping horses, exercise, alkyl hydroperoxide, middle molecules, lipid peroxidation, protein damage, antioxidant defense, blood

INTRODUCTION

Horses are superb athletes, but still require regular exercise to improve their athletic abilities (Hinchcliff and Geor 2008). Depending on the increased metabolic activity during exercise, the use of oxygen and electron leakage from the mitochondrial electron transport chain is increased, resulting in an increase in many reactive oxygen species including superoxide, hydrogen peroxide and hydroxyl radicals (Akkuş 2011, Ji and Leichtweis 1997, Deaton and Marlin 2003). Several studies have reported that intense physical activity may shift the balance between reactive oxygen species (ROS) production and ROS inactivation in favor of oxidative stress (Williams et al. 2004, Gondim et al. 2009, Kinnunen et al. 2005, Williams and Burk 2012). The athletic ability of a horse can be compromised by high levels of oxidants and their by-products (Deaton and Marlin 2003, Kinnunen et al. 2005; Kirschvink et al. 2008).

Professional training in equestrian sport involves repeated bouts of exercise, and high volume of physically demanding in practice sessions and competitive games may lead to decline of performance, induce of oxidative stress, inflammation etc. (Kirschvink et al. 2008). Numerous studies have shown that exercise-induced oxidant/antioxidant changes in trained horses are vary with exercise type (Williams et al. 2004, Gondim et al. 2009, Kinnunen et al. 2005, Kirschvink et al. 2008), training status (De Moffarts et al. 2005, Muñoz-Escassi 2006, Soares et al. 2011, Williams and Burk 2012), ambient conditions and presence of disease (Art and Lekeux 2005, Lamprecht and Williams 2012), vitamins, trails element and others naturals antioxidant might have beneficial effect on the oxidant/antioxidant equilibrium (Williams et al. 2004, De Moffarts 2005, Williams and Burk 2010).

High performance equestrian sport, like show jumping demanding regular intensive training which may induce to accelerated generation of ROS. ROS result of lipid peroxidation (LPO) and protein oxidation which induces adverse effects on the health status and performance of horses (Muñoz-Escassi 2006, Krumrych 2010; Soares et al. 2011). After intense exercise in horses, oxidative stress can occur and lead to cellular and muscular damage (Art and Lekeux 2005, Kirschvink et al. 2008, Fazio et al. 2014). Despite the increased ROS production during exercises, growing evidence derived from epidemiological and prospective studies strongly indicates that moderate physical activity reduces the exercise-induced oxidative stress (Goto and Radák 2007, Radák et al. 2008, Silva et al. 2009, Pinho et al. 2012, Andriichuk et al. 2012, 2013a, b, c, 2014, 2016). Recent studies of equine exercise physiology have focused mainly on determining the usefulness of biochemical parameters for
evaluating physiological capacity and adaptation to increasing loads (Krumrych 2010, Janicki et al. 2013, Fazio et al. 2014). Therefore, biochemical determinations of blood give a more complete picture of the horse’s response to physical loads and its adaptation to training program. In particular, many studies have been carried out with the purpose of defining the pattern of some physiological, haematological and biochemical alterations in jumping horses during training and physical exercise (Fazio et al. 2014, Soares et al. 2011) showing significant variations of some of these parameters after training or event. Since show jumping represents an intense muscular exertion which requires some anaerobic metabolism, physiological and metabolic responses in athlete horses participating in show jumping competitions have been presented (Krumrych 2006, Piccione et al. 2007, Fazio et al. 2014). However, some controversy exists in terms of poorly reproducible and even contradictory results regarding to consequences of regular intense physical activity on transitory effects of oxidative stress and enhanced ability of adaptation to increasing loads in jumping horses. Therefore, the aim of the present study was to investigate the effect of intensive exercise on levels of oxidative stress biomarkers (alkyl hydroperoxides, middle molecules, 2-thiobarbituric acid reactive substances, the carbonyl derivatives of oxidatively modification of proteins) and antioxidant defenses (superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase activity, ceruloplasmin level, total antioxidant capacity) in well-trained Ukrainian warmblood horses involved in equestrian show jumping.

MATERIALS AND METHODS

**Horses.** A total 9 equine athletes were used in this study. All horses have performed regularly in national jumping competitions and had been in regular training for several years. Nine well-trained Ukrainian Warmblood horses (3 mares, 4 geldings, 2 stallions) aged 8.3 ± 1.6 years; body weight 484.10 ± 27.96 kg; mean withers height, 166.44 ± 4.27 cm; mean chest volume, 188.55 ± 3.57 cm; mean body length, 161.88 ± 2.60 cm; mean body condition score, 3.5). All horses were involved in training for show jumping. The animals were selected on the basis of clinical examination performed by qualified veterinarian and hematological analysis to exclude pathologic conditions. The females were non-pregnant. All the horses were dewormed, vaccinated at similar time. All the animals underwent fitness training for several years, 6 days per week with a rest day on Sundays. Training started at 9 AM every day and lasted 1-1.5 h. All horses were participated in the same daily training program. They had three times per week a higher-level exercise bout consisted of the warm-up (10 min of the walk, 30 min of the trot, and 10 min of the gallop) and series of jumping over obstacles with an average height of 110 ± 10 cm. Each horse was trained according to its individual abilities, as assessed by the trainer, to achieve each horse’s optimal performance. The riders and trainers provided information that each horse was trained according to its individual’s abilities, as assessed by the trainer, to achieve each horse optimal fitness. A subjective appreciation of the level of performance of each horse was made by the trainer. During the observation period none of the horses participated to official competitions.

**Diet.** All horses were housed in the same environment in individual boxes at natural indoor temperature (18-20ºC). All horses had the same diet. This diet was composed
of grass meadow hay (6 kg), oats (6 kg), carrot (1 kg) and wheat bran (2 kg), distributed in three times per day (digestible energy 11.4 MJ; 6.71% digestible protein; 3.16% crude fat; 14.7% crude fiber) and provided in average intake of β-carotene, 157 mg; 320.46 mg vitamin E; 0.0225 mg vitamin D; 63.5 g lysine; 57.1 g calcium; 172.4 g phosphorus; 86.1 mg Cu; 426.4 mg Zn; 15.2 mg Na; 51.3 mg Cl (based on analysis of hay, oats, carrot and wheat bran). Salt and water were available ad libitum.

**Exercise test.** The study design consisted of one exercise test according to training program. This test consisted of a series of physical exercise of high intensity including series of jumping over obstacles. The exercise test consisted of the walk (5 min), the trot (10 min), the walk (5 min), the gallop (15-20 min) with a series of jumps over obstacles height 80-120 cm, and the walk (15 min). The exercise test was done indoor riding hall with sandy cove. Exercise test and general animal care were carried out by professional staff not associated with the research team. The exercise test was made by professional riders. They are able to teach both horse and have themselves competed in high level equine sport. During the different exercise gait, the riders hand controlled race speed by using reference points placed along the standard arena is 20 m by 60 m within a certain time window.

**Blood Samples.** Blood was drawn from jugular veins of the animals in the morning, 90 minutes after feeding, while the horses were in the stables (between 8:30 and 10 AM), and immediately after exercise session (between 11:00 AM and 4 PM). Blood was stored into tubes with potassium ethylenediaminetetraacetate and held on ice until centrifugation at 3,000 g for 15 minutes. The plasma was removed; the erythrocytes suspension (one volume) was washed with five volumes of saline solution three times and centrifuged at 3,000g for 15 minutes. Plasma aliquots were frozen and stored at -25°C until analyzed. Plasma was used for the determination of 2-thiobarbituric acid reactive substances, acyl hydroperoxide, middle molecules, carbonyl derivatives of protein oxidative modification, catalase activity and ceruloplasmin (CP) level; erythrocyte suspensions were used for 2-thiobarbituric acid reactive substances, carbonyl derivatives of protein oxidative modification, total antioxidant capacity (TAC), superoxide dismutase, glutathione reductase, and glutathione peroxidase activity.

**Biomarker analysis.** 2-Thiobarbituric acid (TBA), oxidized and reduced glutathione (GSSG and GSH), NADPH, and 5,5-dithiobis-2-nitrobenzoic acid (DTNB), ethylenediaminetetraacetic acid (EDTA), thricloroacetic acid (TCA), quercetin, tetramethylenediamine (TEMED), hydrogen peroxide, ammonium molybdate, sodium aside, t-butylhydroperoxide, Tween 80, urea acid, 2,4-dinitrophenyl hydrazine (DNFH) were obtained from Fluka (Buchs, Switzerland). All other chemicals were of analytical grade. All enzymatic assays were carried out at 22 ± 0.5°C using a Specol 11 spectrophotometer (Carl Zeiss Jena, Germany). The enzymatic reactions were started by adding the blood, erythrocyte suspension or plasma samples. The specific assay conditions are presented subsequently. Each sample was analyzed in duplicate.

**Lipid hydroperoxides level assay.** The method was described by Kamysnykov (2004). To 0.2 ml of plasma was added 4 ml “heptan-isopropanol” mixture and vortexed vigorously. Then 1 ml of HCl (pH 2.0), and 2 ml of heptane reagent were added, vortexed, and centrifuged at 3,000 rpm for 5 min. The lipid hydroperoxides level
was read spectrophotometrically at 233 nm and expressed as $A_{233}$ per ml. In blank, mixture with distilled water was used.

**Middle molecules level assay.** The concentration of middle molecules in the serum was determined spectrophotometrically and was expressed in mg/l. The method is based on the precipitation of high blood plasma proteins using perchloric acid and ethanol, followed by photometry at a wavelength of 210 nm (Kamyshnikov 2004).

**Thiobarbituric acid reactive substances (TBARS) assay.** The level of lipid peroxidation was determined by quantifying the concentration of TBARS with the Kamyshnikov (2004) method for determining the malondialdehyde (MDA) concentration. This method is based on the reaction of the degradation of lipid peroxidation product, MDA, with TBA under high temperature and acidity to generate a colored adduct that is measured spectrophotometrically. Briefly, 0.1 ml of sample (plasma, and erythrocytes’ suspension) was added to 2 ml of distilled water, 1 ml of 20% TCA and 1 ml of 0.8% TBA. The mixture was heated in a boiling water bath for 10 minutes. After cooling, the mixture was centrifuged at 3,000 g for 10 minutes. The µmol of MDA per l l was calculated by using $1.56 \cdot 10^5$ mM$^{-1}$·cm$^{-1}$ as extinction coefficient.

**The carbonyl derivatives content of protein oxidatively modification (OMP) assay.** The rate of protein oxidative destruction was estimated from the reaction of the resultant carbonyl derivatives of amino acid reaction with DNPH as described by Levine et al. (1990) and as modified by Dubinina et al. (1995). DNPH was used for determining carbonyl content in soluble and insoluble proteins. Briefly, 1 ml of 0.1 M DNPH (dissolved in 2 M HCl) was added to 0.1 ml of the sample (plasma and erythrocytes’ suspension) after denaturation of proteins by 20% TCA. After addition of the DNPH solution (or 2 M HCl to the blanks), the tubes were incubated for a period of 1 h at 37°C. The tubes were spun in a centrifuge for 20 min at 3,000 g. After centrifugation, the supernatant was decanted and 1 ml of ethanol-ethylacetate solution was added to each tube. Following mechanical disruption of the pellet the tubes were allowed to stand for 10 min and then spun again (20 min at 3,000 g). The supernatant was decanted and the pellet washed thrice with ethanol-ethylacetate. After the final wash, the protein was solubilized in 2.5 ml of 8 M urea solution. To speed up the solubilization process, the samples were incubated in at 90°C water bath for 10-15 min. The final solution was centrifuged to remove any insoluble material. The carbonyl content was calculated from the absorbance measurement at 370 nm and 430 nm, and an absorption coefficient 22,000 M$^{-1}$·cm$^{-1}$. Carbonyl groups were determined spectrophotometrically from the difference in absorbance at 370 nm (aldehyde derivatives, OMP$^{370}$) and 430 nm (ketonic derivatives, OMP$^{430}$).

**Superoxide dismutase activity assay.** Superoxide dismutase (SOD, E.C. 1.15.1.1) activity was assessed by its ability to dismutate superoxide produced during quercetin auto-oxidation in an alkaline medium (pH 10.0) by Kostiuk et al. (1990) method. Briefly, 1.0 ml of C reagent was mixed with 0.1 ml of blood sample (dilution in water 1 : 1000). C reagent was made ex tempore (mixture of equal volumes of 0.1 M K, Na-phosphate buffer, pH 7.8 and 0.08 M EDTA solution); pH of C reagent was adjusted to 10.0 by adding TEMED. Distilled water (0.1 ml) was added to blank vials instead of blood sample. The total volume of all samples was brought up to 2.4 ml
using distilled water. The reaction was initiated by adding 0.1 ml of quercetin (1.4 \( \mu \text{M} \) dissolved in dimethyl sulphoxide). Absorbance at 406 nm was measured immediately and after 20 min addition of quercetin solution. Activity is expressed in units of SOD per ml of blood.

**Catalase activity assay.** Catalase (CAT, E.C. 1.11.1.6) activity was determined by measuring the decrease of \( \text{H}_2\text{O}_2 \) in the reaction mixture using a spectrophotometer at the wavelength of 410 nm by the method of Koroliuk et al. (1988). The reaction was initialized by adding 0.1 ml of plasma into the incubation medium (2 ml of 0.03% \( \text{H}_2\text{O}_2 \) solution) and to 1.0 ml of 4% ammonium molybdate dissolved in 12.5 mM \( \text{H}_2\text{SO}_4 \) solution (blank sample). The duration of reaction was 10 min at room temperature. The reaction was terminated by rapid adding 1.0 ml of 4% ammonium molybdate dissolved in 12.5 mM \( \text{H}_2\text{SO}_4 \) solution to incubation medium and 1 ml of 125 mM \( \text{H}_2\text{SO}_4 \) to all samples. All samples were centrifuged at 3,000 g for 5 min. The absorbance of the obtained solution was measured at 410 nm and compared with that of the blank. One unit of catalase activity is defined as the amount of enzyme required for decomposition of 1 \( \mu \text{mol} \) \( \text{H}_2\text{O}_2 \) per min per l of blood.

**Glutathione reductase activity assay.** Glutathione reductase (GR, EC 1.6.4.2) activity in the blood was measured according to the method described by Glatzle et al. (1974). The enzymatic activity was assayed spectrophotometrically by measuring NADPH consumption. In the presence of GSSG and NADPH, GR reduces GSSG and oxidizes NADPH, resulting in a decrease in the absorbance at 340 nm. The enzyme assay mixture contained 2.4 ml of 67 mM sodium phosphate buffer (pH 6.6), 0.2 ml of 7.5 mM oxidized glutathione, and 0.1 ml of hemolyzed erythrocytes (1 : 20). The rate of NADPH oxidation was followed spectrophotometrically at 340 nm. Quantification was performed based on a molar extinction coefficient of 6.22 mM\(^{-1} \cdot \text{cm}^{-1}\). The activity was expressed as nmol NADPH per min per ml of blood.

**Glutathione peroxidase activity assay.** Glutathione peroxidase (GPx, EC 1.11.1.9) activity was determined by detecting the nonenzymatic utilization of GSH (the reacting substrate) at an absorbance of 412 nm after incubation with 5,5-dithiobis-2-nitrobenzoic acid (DTNB) according by the method of Moin (1986). The assay mixture contained 0.8 ml of 0.1 M Tris-HCl buffer with 6 mM EDTA and 12 mM sodium azide (pH 8.9), 0.1 ml of 4.8 mM GSH, 0.2 ml of hemolysed erythrocytes (1 : 20), 1 ml of 20 mM \( \text{t-butylhydroperoxide} \), and 0.1 ml of 0.01 M 5,5-dithiobis-2-nitrobenzoic acid. The rate of GSH reduction was followed spectrophotometrically at 412 nm. GPx activity is expressed as \( \mu \text{mol} \) GSH per min per ml of blood.

**Ceruloplasmin level assay.** The ceruloplasmin (CP, EC 1.16.3.1) level in the plasma was measured spectrophotometrically at 540 nm, as described by Ravin (1961). The assay mixture contained 0.1 ml of plasma, 0.4 M sodium acetate buffer (pH 5.5), and 0.5% \( \text{p-phenylenediamine} \). The mixture was incubated at 37°C for 60 min. Before cooling at 4°C for 30 min, the mixture was added to 3% sodium fluoride for inhibition. Ceruloplasmin was expressed in mg per l of plasma.

**Total antioxidant capacity assay.** The TAC level in the plasma was estimated by measuring the TBARS level following Tween 80 oxidation. This level was determined spectrophotometrically at 532 nm (Galaktionova et al. 1998). Plasma inhibits
the Fe^{2+}/ascorbate-induced oxidation of Tween 80, resulting in a decrease in the TBARS level. Briefly, 0.1 ml of plasma sample were added to 2 ml of 1% Tween 80 reagent, 0.2 ml of 1 mM FeSO_{4}, and 0.2 mM of 10 mM ascorbic acid. In the blank assay, 0.1 ml of distilled water were used instead of the sample. The mixture was heated in a boiling water bath for 48 h at 37°C. After cooling, 1 ml of 20% TCA was added. The mixture was centrifuged at 3,000 \cdot g for 10 min. After centrifugation, 2 ml of supernatant and 2 ml of 0.25% of TBA reagent were mixed. The mixture was heated in a boiling water bath at 95°C for 15 min. The absorbance of the obtained solution was measured at 532 nm. The absorbance of the blank was defined as 100%. The level of TAC in the sample (%) was calculated with respect to the absorbance of the blank.

**Statistical analysis.** Results are expressed as mean ± S.E.M. All variables were tested for normal distribution using the Kolmogorov-Smirnov test (p > 0.05). In order to find significant differences (significance level, p < 0.05) between states at the rest and after exercise training, Wilcoxon signed-rank test was applied to the data (Zar 1999). All statistical analyses were performed using Statistica 10.0 software (StatSoft, Poland). In addition, the relationships between oxidative stress biomarkers and enzymes activities of all individuals were evaluated using Spearman's correlation analysis (Zar 1999).

**RESULTS AND DISCUSSION**

Conjugated dienes (CD), or alkyl hydroperoxide, which absorb ultraviolet light at 230-235 nm, are one of the first products of the peroxidation of unsaturated fatty acids and are considered accurate and repeatable measurements of lipid peroxidation (Clarkson and Thompson 2000). Lipid hydroperoxides level (LOOH) an index of membrane lipid peroxidation and many studies have used LOOH as a marker of oxidative stress during exercises (Urso and Clarkson 2003, Kinnunen et al. 2005, George and Osharechiren 2009). LOOH are formed earlier in the pathway leading to malondialdehyde (MDA) and chemiluminescence (HPLC) or enzymatic methods are used to detect LOOH in blood and tissue (Han et al. 1997). The exercised-induced alterations of lipid hydroperoxides level are shown in Fig. 1.

![Fig. 1. Alkyl hydroperoxide content (A_{233} \cdot ml^{-1}) in plasma of jumping horses (n = 9) recorded before and after exercise session. Values are means ± S.E.M Source: own research](image-url)
The conjugated dienes indicating early events of lipid peroxidation did not change significantly during the exercises. There were non-significance decreased levels of dienes conjugated by 17.5% (p = 0.514) after exercise. Conjugated dienes have been used to assess low-density lipoprotein oxidation in vitro (Urso and Clarkson 2003). However, researchers consider that use of conjugated dienes as a marker of oxidative stress in humans and animals must therefore be interpreted with caution (Clarkson and Thompson 2000). Measurement of conjugated dienes has produced less consistent results (Vollaard et al. 2005). A number of studies have demonstrated an increase in levels of CD after exercise (Marzatico et al. 1997, Ramel et al. 2004) whereas others did not observe such an effect (Liu et al. 1999). It has been shown that CD formation were reduced after exercise suggesting increased oxidative stress (Liu et al. 1999), but remained non-changed (Vasankari et al. 1997), or even increased in other studies (Ginsburf et al. 1996, Case et al. 2004). In our previous study, no significant differences of values of conjugated dienes in Holsteiner horses during the exercise test were noted (Andriichuk et al. 2014a).

Middle molecules are a large heterogeneous group of compounds which includes hormones, neuropeptides, and neurotransmitters of immune response and many other products of protein metabolism, which generally define high biological activity. The fraction of middle molecules or low-molecular-weight metabolites includes all those organic compounds of biological origin with a molecular weight below 900 Dalton (Hadacek and Bachmann 2015). Low-molecular-weight metabolites may to serve as organic ligands in coordination complexes with various inorganic metals as central atoms. Especially the transition metals Fe, Cu, and Mn can catalyze one electron reduction of molecular oxygen, which results in formation of free radical species and reactive follow-up reaction products (Hadacek and Bachmann 2015). Depending on the chemical environment, the same low-molecular-weight metabolites or middle molecules can act as pro-oxidants by reducing molecular oxygen (Hadacek and Bachmann 2015). Thereby, we have used the definition of middle molecules as markers of oxidative stress. The level of middle molecules before and after exercise test are shown in Fig. 2.

![Fig. 2. Middle molecules content (mg · l⁻¹) in the plasma of jumping horses (n = 9) recorded before and after exercise session. Values are means ± S.E.M Source: own research](image-url)
Non-significant increase of middle molecules level by 34% \( (p = 0.08) \) after exercise test was observed. It has been shown the ability of some low-molecular-weight metabolites to reduce of molecular oxygen by one-electron transfers which regarded as the major mechanism contributing to their cytotoxicity (Kappus and Sies 1981). Some of middle molecules, as low-molecular-weight metabolites, may play a role microenvironmental factors in bloodstream that can influence the functional state of erythrocyte membranes, inducing the oxidative stress (Hadacek and Bachmann 2015).

Studies have shown that intense exercise causes oxidative stress in animals and humans (Chiaradia et al. 1998, Marin et al. 2013, Williams et al. 2004, Gondim et al. 2009, Kinnunen et al. 2005, Williams and Burk 2012). This may be related to chronic fatigue syndrome (Berryman 2010, Nunes-Silva and Freitas-Lima 2015), delayed onset muscle soreness and tissue lesions (Chiaradia et al. 1998, Kirshvink et al. 2008, Cheung et al. 2003). Exercise induces an increase in oxygen consumption and energy demand by inducing increased production of ROS (Ji and Leichtweis 1997, Nunes-Silva and Freitas-Lima 2015). The increased production of ROS associated with the inefficiency of neutralization by the antioxidant defense system and can lead to oxidative stress. Under these circumstances, ROS react with cellular structures, inducing high levels of oxidation by altering the function and impairing intracellular homeostasis (Nunes-Silva 2015 and Freitas-Lima). Generation of ROS during physical activity lead to lipid peroxidation, which induced adverse effects on the health status and performance of horses (Chiaradia et al. 1998, Williams et al. 2004, Kirschvink et al. 2008).

Byproducts of lipid peroxidation are the most frequently studied markers of oxidative tissue damage during exercise (Banerjee et al. 2003, Soares et al. 2011). The most common method used to assess changes in MDA during exercise training is the 2-thiobarbituric acid reactive substances (TBARS) assay. MDA content was found to be increased during exercise in a variety of tissues, and the extent of lipid peroxidation also appears to depend on exercise intensity (Chiaradia et al. 1998, Muñoz-Escassi et al. 2006, Kirshvink et al. 2008, Gondim et al. 2009, Soares et al. 2011). The lipid peroxidation before and after exercise test was measured through analysis of the TBARS level and shown in Fig. 3.

![Fig. 3. Effect of the exercise session on the TBARS level in the erythrocytes and plasma of jumping horses (n = 9). Values are means ± S.E.M](image)

* The level of significance is set at \( p < 0.05 \), paired samples by Wilcoxon test

Source: own research
After exercise test, TBARS level in plasma of jump horses was decreased by 24% (p = 0.030). However, there were no significant alterations in erythrocytes' TBARS level after exercise test. It has been shown that increased TBARS level in plasma after exercise is a consequence of leakage of peroxides from tissues, especially from muscle into plasma (Ilhan et al. 2004). Oxidative modification of plasma constituents was an expression of oxidative damage that occurred in tissues (Witt et al. 1992). However, accumulating evidence suggest an association between oxidative stress and regular physical exercise as well as the beneficial effects of chronic exercise training in physical condition (Radak et al. 2008, Gomes et al. 2012, Nunes-Silva and Freitas-Lima 2015). This beneficial effect is due to the fact that exercise-induced ROS production is necessary for oxidative stress-related adaptations. It has been suggested that acute and irregular exercises have negative effects, whereas regular physical activity creates an advanced antioxidant system and decreases oxidative damage (Radak et al. 2008, Gomes et al. 2012, Radak et al. 2013, Nunes-Silva and Freitas-Lima 2015). Falone and co-authors (2010) have reported that exhaustive exercise lead to reduce TBARS levels in trained subjects, while resulting in increased levels of TBARS in untrained subjects. This phenomenon is not a paradox; it is a result of exercise-induced adaptation. The adaptation process involves activation of the antioxidant system, interferes with the oxidative damage repair/eliminating systems, and influences redox-sensitive transcription, hence the gene expression and protein assembly (Radak et al. 2008, Radak et al. 2013). Recent studies indicated that ROS generated during exercise are initiation of two important redox-sensitive signaling pathways including nuclear factor κB (NF-κB) and mitogen activated protein kinase (MAPK) (Fisher-Wellman and Bloomer 2009, Radak et al. 2013). Activation of these pathways lead to induction of antioxidant enzymes including mitochondrial superoxide dismutase (SOD) and glutathione peroxidase (Fisher-Wellman and Bloomer 2009). The exercise-induced ROS generation caused to increase activity of enzymatic antioxidants, which then results in increased resistance to oxidative damage (Gomes et al. 2012, Nunes-Silva and Freitas-Lima 2015). Based in our results it is possible to affirm that the reduced TBARS level in trained horses involved is show jumping could be attributed to exercise-induced adaptation for minimize the transitory oxidative stress occasioned by an exercise.

Reactive free radicals can modify amino acid residues of proteins and lead to cross-linking alterations in conformation and loss of function (Radak et al. 2000). Protein carbonyl content, an indicator of irreversible oxidative damage leading to protein oxidation may have lasting detrimental effects on cells and tissues (Dalle-Donne et al. 2003). The effect of exercise test on carbonyl contents in the erythrocyte and plasma samples of horses are shown in Fig. 4.

After exercise test, ketonic derivatives of OMP in erythrocytes of UWB horses were decreased by 60% (p = 0.038) compared to period before exercise. No significant alterations of protein carbonyl oxidation in the plasma of trained horses before and after exercise test were observed. It has been shown that regular physical exercise leads to increase the activity of the proteasome complex involved in the removal of oxidatively modified proteins in the muscle of old well-trained rats (Radak et al. 2008, 2013). This is an important adaptive response because the induction of a repair mechanism – proteasome complex – is regarded as a repair enzyme. This could be due with decrease in the accumulation of potentially
Fig. 4. Carbonyl content of oxidatively modified proteins (OMP, nmol · ml⁻¹), measured by quantity of aldehyde and ketonic derivatives, in erythrocyte suspension and plasma of jumping horses (n = 9) recorded before and after exercise. Values are means ± S.E.M
* The level of significance is set at p < 0.05, paired samples by Wilcoxon test
Source: own research

harmful post-translationally modified proteins. Exercise can induce the activity of the proteasome complex involved significantly in the degradation of oxidatively modified proteins (Radak et al. 2008, Gomes et al. 2012, Radak et al. 2013, Nunes-Silva and Freitas-Lima 2015). Increased activity of proteasome could be an important factor that affects the rate of protein turnover and the remodeling of skeletal muscle after injury (Ji et al. 2008). An increased rate of protein turnover with exercise training decreases the accumulation of oxidative damage, hence beneficially affecting the physiological function of proteins. The proteasome complex plays a critical role in this process (Radak et al. 2008, Ji et al. 2008, Radak et al. 2013). Thus, in our study, significant decrease of carbonyl derivatives in erythrocytes’ suspension of UWB horses, seems is the result of enhanced activities of antioxidant system and activity of the proteasome complex.

The total antioxidant capacity assay offers many advantages and is considered a useful tool for detecting oxidative stress phenomena in bodily fluids and tissues (Palmieri and Sblendorio 2007).

Fig. 5. Effect of the exercise session on total antioxidant capacity (TAC) level of erythrocyte and plasma of jumping horses (n = 9). Values are means ± S.E.M
Source: own research
No significant alterations of TAC level in erythrocytes and plasma of horses before and after exercise test was observed (Fig.5). Several studies have used TAC activity assay to detect increases in oxidative stress after exercise (Santos-Silva et al. 2001, Urso and Clarkson 2003). However, Alessio and co-workers (1997) found that plasma total antioxidant capacity not increased in response to a 30 min exercise, despite an increase in MDA.

Antioxidant enzymes may be activated during strenuous exercise depending on the oxidative stress imposed on the specific tissues as well as the intrinsic antioxidant defense capacity. The main enzymatic antioxidants include superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT). Each of these enzymes is responsible for the reduction of a different ROS, and they are located in different cellular compartments (Nunes-Silva and Freitas-Lima 2015). Antioxidant defense in the blood of well-trained Ukrainian warmblood horses before and after exercise test are shown in Table 1.

Table 1
Antioxidant enzymes activities in the blood of jumping horses before and after exercise session (n = 9)

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Before exercise</th>
<th>After exercise</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD, U·ml⁻¹</td>
<td>12.70 ± 1.17</td>
<td>17.19 ± 1.65</td>
</tr>
<tr>
<td>CAT, µmol·min⁻¹·l⁻¹</td>
<td>3.87 ± 1.59</td>
<td>5.12 ± 1.59*</td>
</tr>
<tr>
<td>GR, nmol·min⁻¹·ml⁻¹</td>
<td>2.09 ± 1.14</td>
<td>3.91 ± 1.90*</td>
</tr>
<tr>
<td>GPx, µmol·min⁻¹·ml⁻¹</td>
<td>0.84 ± 0.22</td>
<td>0.88 ± 0.14</td>
</tr>
<tr>
<td>CP, mg·l⁻¹</td>
<td>25.08 ± 6.59</td>
<td>49.19 ± 11.79</td>
</tr>
</tbody>
</table>

Values expressed as mean ± S.E.M
* the significant change was shown as p < 0.05 when compared between values before and after exercise (Wilcoxon signed-rank test)

Source: own research

There were no statistically significant changes in the activities of antioxidant defenses instead CAT and GR activity in the blood of horses involved in show jumping (Tab. 1). The CAT activity was higher by 32% (p = 0.028) after exercise test. The GR activity after exercise test was also increased by 87% (p = 0.007) compared to the resting period. SOD acts on superoxide radicals to form oxygen and the lesser reactive non-radical species, hydrogen peroxide, while GR can regenerate oxidized glutathione (GSSG, glutathione disulfide) to reduced glutathione (GSH). The CAT is located mainly in peroxisomes and mitochondria and also removes H₂O₂. CAT requires iron as a cofactor, and similar to GPx and SOD, its activity is highest in highly oxidative muscle fibers (Gomes et al. 2012). In our study, increase in erythrocyte glutathione reductase activity caused by specific response to the greater anion superoxide production induced by exercise test. The increased catalase activity could contribute to the rise in glutathione reductase activity when the superoxide anion production overwhelmed of elimination of free radicals. Erythrocyte CAT activity showed an increase following training, suggesting the capacity to overcome hypoxia in the trained rats (Devi et al. 2005). Increase of erythrocytes’ glutathione reductase
activity indicates that regular exercises may be effective to induce GR activity and hence effective in scavenging of free radicals. Our results are consistent with the previous findings where endurance training of horses resulted in increased of GR activity (Kinnunen et al. 2005, Gondim et al. 2009). CAT is widely distributed in all animal tissues and erythrocytes have high activity. This preventive enzymatic antioxidant plays a profound role in protecting erythrocytes against oxidative stress (Kolanjiappan et al. 2002). High activity of CAT in red blood cell has been reported to play a crucial role in protecting red blood cells against oxidative damage. Administration of catalase has been shown to have an important role in protection against H₂O₂-mediated lipid peroxidation (Kolanjiappan et al. 2002). Marin and co-workers found increased CAT activity in erythrocytes in handball athletes during six weeks training loads. Our results are in agreement with results of other researchers. It also has been shown that exhaustive exercises induced significant increases in CAT and GR activities in cyclists (Aguiló et al. 2005). Frasier and co-authors showed adaptive response to exercises through redox-dependent modifications in GR which were involved in exercise cardioprotection (Frasier et al. 2013).

Correlative dependences between oxidative stress biomarkers and antioxidant defenses in the blood of jumping horses before exercise are presented in Figs 6 and 7. High level of TAC is determined by SOD activity (r = 0.689, p = 0.040) (Fig. 6A), CAT activity (r = 0.756, p = 0.0185) (Fig. 6D), GR activity (r = 0.826, p = 0.006) (Fig. 6D) and cause to decrease of ketonic derivatives of oxidatively modified proteins (r = -0.913, p = 0.0006) (Fig. 6A) with increase of CAT activity (r = -0.844, p = 0.0042) (Fig. 6B) and GR activity (r = -0.872, p = 0.0022) (Fig. 6C).

![Graph A](image_url)
OMP_{430} plasma: TAC erythrocytes: $y = 145.09 - 4.436^*x$; $r = -0.913; p = 0.0006; r^2 = 0.8339$

OMP_{430} plasma: Catalase: $y = 68.498 - 3.499^*x$; $r = -0.844; p = 0.0042; r^2 = 0.7129$

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GR: OMP_{370} plasma: $y = 12.697 - 0.323^*x$; $r = -0.890; p = 0.0013; r^2 = 0.792$

GR: OMP_{430} plasma: $y = 19.089 - 0.295^*x$; $r = -0.872; p = 0.0022; r^2 = 0.7600$
Fig. 6. Correlative dependences between total antioxidant capacity (TAC, %), ketonic derivatives of oxidatively modified proteins (OMP₄₃₀, nmol/ml), and SOD activity (U/ml) (A), between ketonic derivatives of oxidatively modified proteins (OMP₄₃₀, nmol/ml), total antioxidant capacity (TAC, %), and catalase activity (µmol/min · l) (B), between GR activity (nmol/min · ml), aldehyde (OMP₃₇₀, nmol/ml) and ketonic derivatives of oxidatively modified proteins (OMP₄₃₀, nmol/ml) (C), and between total antioxidant capacity (TAC, %), catalase (µmol/min · l), and GR activity (nmol/min · ml) (D) in the blood of jumping horses before exercise

Source: own research

Post-exercise high level of TAC in the erythrocytes of jumping horses is determined by GR activity (r = 0.731, p = 0.025) (Fig. 7B). CAT activity is in synergic relation with GR activity (r = 0.944, p = 0.0001) and causes to decrease of SOD activity (r = -0.708, p = 0.033) (Fig. 7A).
CONCLUSION

• No significant differences in alkyl hydroperoxide and middle molecules contents in the blood of jumping horses before and after exercise were observed. TBARS level in the plasma of horses showed a significant decrease immediately after exercise as compared with the resting period. Our results suggest that training session results in different consequences on oxidative stress biomarkers in the plasma and erythrocytes of horses. This difference in TBARS level between resting and training periods most likely is a consequence of differing levels of oxidative stress occurring in the tissues and erythrocytes. The decrease in plasma TBARS level in jumping horses after exercise could be attributed as adaptive mechanism to exercise training with activation of the antioxidant defense.

• Significant decrease of ketonic derivatives in the erythrocytes of horses after training session is result of exercise-induced adaptation. This is an important adaptive response because the induction of a repair mechanism – proteasome complex – is regarded as a repair enzyme. This could be due with decrease in the accumulation of potentially harmful post-translationally modified proteins. Exercise can induce the activity of the proteasome complex involved significantly in the degradation of oxidatively modified proteins.

• Our results showed that regular physical training of jumping horses is associated with beneficial adaptive responses preventing the decline of total antioxidant
capacity following the exercise. There were no statistically significant alterations in the activities of antioxidant defenses excepted catalase and glutathione reductase activity in the blood of horses after exercise. It can be hypothesized that significant increase of catalase and glutathione reductase activity in horses after exercise is a manifestation of the compensatory and adaptive reaction of the organism to training session.

• High level of total antioxidant capacity is determined by superoxide dismutase, catalase, glutathione reductase activity and causes to decrease of ketonic derivatives of oxidatively modified proteins with increase of catalase and glutathione reductase activity in the blood of horses before exercise. High level of total antioxidant capacity in the erythrocytes of jumping horses is determined by glutathione reductase activity. Catalase activity is in synergic relation with glutathione reductase activity and causes to decrease of superoxide dismutase activity in the blood of horses after exercise.

REFERENCES


SUMMARY

High performance equestrian sport like show jumping demanding regular intensive training which may induce to accelerated generation of lipid peroxidation and protein oxidation which induces adverse effects on the health status and performance of horses (Muñoz-Escassi et al. 2006, Krumrych 2010; Soares et al. 2011). After intense exercise in horses oxidative stress can occur and lead to cellular and muscular damage (Art and Lekeux 2005, Kirschvink et al. 2008, Fazio et al. 2014). However, some controversy exists in terms of poorly reproducible and even contradictory results regarding to consequences of regular intense physical activity on transitory effect of oxidative stress and enhanced ability of adaptation to increasing loads in jumpers horses. Therefore, the aim of the present study was to investigate the effect of intensive exercise on levels of oxidative stress biomarkers (alkyl hydroperoxides, middle molecules, 2-thiobarbituric acid reactive substances, the carbonyl derivatives of oxidatively modification of proteins) and antioxidant defenses (superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase activity, ceruloplasmin level, total antioxidant capacity) in well-trained Ukrainian warmblood horses involved in equestrian show jumping. Nine well-trained Ukrainian Warmblood horses (3 mares, 4 geldings, 2 stallions) aged 8.3 ± 1.6 years; body weight 484.10 ± 27.96 kg; mean withers height, 166.44 ± 4.27 cm; mean chest volume, 188.55 ± 3.57 cm; mean body length, 161.88 ± 2.60 cm; mean body condition score, 3.5) were used in this study. All horses performed regularly in national jumping competitions and had been in regular training for several years. All horses were participated in the same daily training program.
They had three times per week a higher-level exercise bout consisted of the warm-up (10 min of the walk, 30 min of the trot, and 10 min of the gallop) and series of jumping over obstacles with an average height of 110 ± 10 cm. No significant differences in alkyl hydroperoxide and middle molecules contents in the blood of jumping horses before and after exercise were observed. TBARS level in the plasma of horses showed a significant decrease immediately after exercise as compared with the resting period. Our results suggest that training session results in different consequences on oxidative stress biomarkers in the plasma and erythrocytes of horses. This difference in TBARS level between resting and training periods most likely is a consequence of differing levels of oxidative stress occurring in the tissues and erythrocytes. The decrease in plasma TBARS level in jumping horses after exercise could be attributed as adaptive mechanism to regular exercise with activation of the antioxidant defense. Significant decrease of ketonic derivatives in the erythrocytes of horses after training session is result of exercise-induced adaptation. This is an important adaptive response because the induction of a repair mechanism – proteasome complex – is regarded as a repair enzyme. This could be due with decrease in the accumulation of potentially harmful post-translationally modified proteins. Exercise can induce the activity of the proteasome complex involved significantly in the degradation of oxidatively modified proteins. Our results showed that regular physical training of jumping horses is associated with beneficial adaptive responses preventing the decline of total antioxidant capacity following the exercise. There were no statistically significant alterations in the activities of antioxidant defenses excepted catalase and glutathione reductase activity in the blood of horses during exercise. It can be hypothesized that significant increase of catalase and glutathione reductase activity in horses after exercise is a manifestation of the compensatory and adaptive reaction of the organism to training session. High level of total antioxidant capacity is determined by superoxide dismutase, catalase, glutathione reductase activity and causes to decrease of ketonic derivatives of oxidatively modified proteins with increase of catalase and glutathione reductase activity in the blood of horses before exercise. High level of total antioxidant capacity in the erythrocytes of jumping horses is determined by glutathione reductase activity. Catalase activity is in synergic relation with glutathione reductase activity and causes to decrease of superoxide dismutase activity in the blood of horses after exercise.