OXIDATIVE STRESS BIOMARKERS IN THE BLOOD OF HOLSTEINER HORSES DURING EXERCISE TRAINING*

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ABSTRACT

The aim of the present study was to investigate the exercise-induced changes of oxidative stress biomarkers and antioxidant defenses in well-trained Holsteiner horses. Seventeen Holsteiners (7 females and 10 males), 7-15 years old, were used. All horses were involved in equestrian show jumping. To assessed the exercise-induced changes of oxidative stress biomarkers and antioxidant enzymes activity was proposed common to all horses the training session of average intensity: walk – 5 min; trot – 10 min; walk – 5 min; trot – 10 min; walk – 10 min; gallop – 10 min; walk – 10 min. Duration of training was 1 hour. Lipid peroxidation and oxidatively modified protein level as oxidative stress biomarkers, antioxidant defenses such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GPx) activity, ceruloplasmin (CP) level as well as total antioxidant capacity in the blood of horses before and after training session was assayed. A decrease of lipid peroxidation biomarker in erythrocytes after training session was observed. Elevated level of oxidative modified protein in erythrocytes of Holsteiner horses indicate about exercise-induced oxidative stress. The correlation analysis between markers of lipid peroxidation (blood, plasma and erythrocytes TBARS levels) and carbonyl derivatives of protein oxidative modification content indicate about close relationship between lipid peroxidation and protein oxidation during exercise in sport horses. Moreover, correla-

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tive analysis between GPx, SOD and CAT activities and oxidative stress biomarkers (plasma TBARS level, carbonyl derivates of protein damage and plasma TAC level) suggest that these enzymes plays a pivotal role in the blood antioxidant defense of horses both at the resting period and after training session.

**Key words:** oxidative stress, antioxidant enzymes, blood, exercise training, Holsteiners

**INTRODUCTION**

The Holsteiner is a breed of horse originating 750 years ago in a region in northern Germany known as Schleswig-Holstein. Holsteiners are a dominant force of international show jumping, and are found at the top levels of dressage, combined driving, show hunters, and eventing. One of the reasons to the success of the Holsteiner in equestrian sport has been the consistent purity and high quality of the mares. Holsteiners make up only 6% of the European warmblood population. But there is a large number of Holsteiner succeeding in worldwide and international competition. For example, at the 2010 World Equestrian Games, of the 4 horses on the winning German team, 2 were Holsteiners and one was half Holsteiner. At the 2008 Olympic Games in Hong Kong, 9 medals went to Holsteiners: 4 gold, 1 silver and 4 bronze. The Holsteiner is dominating the sport of international show jumping (*American Holsteiner Horse Association*, www.breyerhorses.com).

Holsteiners are a large, deep bodied horse averaging between 16 and 17.1 hands high. They are well muscled with a graceful arching neck and a short flexible topline. They have strong well angled joints with the hind legs stepping deep under the body. Holsteiners have natural balance and elasticity (Fig. 1). They are of a warm blooded temperament,
being quiet and easy going and occasionally a little lazy. They come in all solid colors; however bay and chestnut are the most common (Dutson 2005, Clark 2007).

Good health of the sport horses is essential for optimal training and high levels of performance (Escribano et al. 2011). The adaptive responses induced by repetitive exercise act to reduce the effect of the strain induced by the physiologic stressors associated with exercise. The body acts to minimize the disruption to homeostasis induced by exercise by increasing the capacity of the system to deal with the work imposed by exercise. For example, the stress of increased force production by muscle during exercise stimulates changes in muscle structure and function that act to reduce the stress on individual muscle fibers, while increasing the overall capacity of the muscle. This phenomenon is common to many, but not all, body systems and the cumulative effect is a change in body composition and capacity for physical work (Hinchcliff and Geor 2008).

The athletic capacity of horses is attributable to a number of physiologic adaptations. In some cases these adaptations are not affected by training – for example, lung size, whereas others change in response to training – for example, blood volume. The superior athletic ability of horses is attributable to their high maximal aerobic capacity, large intramuscular stores of energy substrates and in particular glycogen, high mitochondrial volume in muscle, the ability to increase oxygen-carrying capacity of blood at the onset of exercise through splenic contraction, efficiency of gait, and efficient thermoregulation (Hinchcliff and Geor 2008).

The elevated metabolic rate associated with physical exercises can increase mitochondrial \( \text{O}_2 \) consumption in muscle tissue and, consequently, mitochondrial reactive oxygen species (ROS) generation (Ji and Leichtweis 1997, Deaton and Marlin 2003). The main sources of ROS that are generated during exercise are the mitochondria (respiratory chain), although activated phagocytes (respiratory burst) and several enzymes, i.e. oxidases, might also contribute to an increased ROS release (Packer et al. 2008). Other sources of oxidative stress during physical exercise are inflammatory responses mediated by neutrophils (Escribano et al. 2005), the interaction of metmyoglobin and methemoglobin with lipid peroxides (Vollaard et al. 2005), and the activity of xanthine oxidase (Cooper et al. 2002), possibly within an ischemia-reperfusion model (Koyama et al. 1999).

Numerous studies have shown that exercise-induced oxidant/antioxidant changes in trained horses vary with regard to exercise type (race, standardized treadmill exercise, standardized race track exercise, endurance). It is generally agreed that exercise induces significant alterations of the circulatory oxidant-antioxidant balance (Kirschvink et al. 2008). Exhausting or moderate exercise in horses may increase ROS production exceeding the capacity of antioxidant defences (Chiaradia et al. 1998, Kinnunen et al. 2005a, b). Oxidative stress is the imbalance of pro- and antioxidants in favor of the former. Oxidative stress may result in the damage of biological components, e.g. lipids, proteins and generic material, and could be associated with a number of pathologic conditions, including muscle and fatigue injury (Halliwell and Gutteridge 1999, Chiaradia et al. 1998, 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). Among athletic/sports animals, the horse has a unique ability to increase its oxygen uptake by a factor of 60 during heavy exercise. This is achieved by physiological adaptations of all links in the oxygen chain. Ventilation is increased by 30-fold (Art and Lekeux 2005). However, in animals and humans clearly undergo significant adaptive responses to regular, moderate endurance exercise that involve greatly increased endurance capacity, permitted by dramatic mitochondrial biogenesis and significantly increased number of muscle mitochondria (Packer et al. 2008). Adaptive mechanisms seem to decrease oxidative stress and they encompass increased antioxidant defense, reduced basal production of oxidants, and reduction of radical leak during oxidative phosphorylation (Ji et al. 2006, Radák et al. 2008).

The factors limiting exercise capacity of horses vary with the type and duration of exercise. However, an understanding of what is likely to limit performance for each breed and use of horse is important not only in understanding the physiology of that form of exercise, but also in determining the likely causes of poor performance in animals with clinical disease (Hinchcliff and Geor 2008). In our previous studies we demonstrated different responses of oxidative stress biomarkers as well as antioxidant defenses in the blood of well-trained horses (Andriichuk et al. 2012, 2013a, b, c, d, 2014). Given the fact that in the modern equestrian sport used athletic horses over a several years and they involved in regular training, the aim of the present study was to investigate the exercise-induced changes of oxidative stress biomarkers and antioxidant defenses in well-trained Holsteiner horses.

**MATERIALS AND METHODS**

**Horses and training.** Seventeen Holsteiners (7 females and 10 males), 7-15 years old, were used. All horses were involved in equestrian show jumping. Horses were housed in the same environment. They had the same vaccinate status and no signs of clinical disease. The females were non-pregnant. The diets were composed of grass hay and concentrated forage, fed three times per day. All horses were thoroughly examined clinically and screened for hematological, biochemical and vital parameters, which were within reference ranges. To assessed the exercise-induced changes of oxidative stress biomarkers and antioxidant enzymes activity was proposed common to all horses the training session of average intensity: walk – 5 min; trot – 10 min; walk – 5 min; trot – 10 min; walk – 10 min; gallop – 10 min; walk – 10 min. Duration of training was 1 hour. Animal care and experimental procedure were in accordance with the Guide for the Care and Use of Laboratory Animals.

**Blood samples.** Blood samples were obtained by jugular venipuncture, in the morning, 90 minutes after feeding while horses were in stalls, and immediately after training session. Samples were aspirated into 10 ml syringe and immediately transferred into sterile EDTA tubes. Samples were kept in a chilled cooler before transport to the laboratory. The plasma separation was performed immediately after blood collection. For isolation of erythrocyte suspension, blood samples were centrifuged at 3,000 rpm for 10 min. The erythrocytes were washed three times with five vol-
Vumes of saline solution and centrifuged at 3,000 rpm for 10 min. After centrifugation, plasma samples were transferred to other tubes, frozen at -20°C and stored until analysis.

**Biomarker analysis.** 2-Thiobarbituric acid (TBA), oxidized and reduced glutathione (GSSG and GSH), NADPH, and 5,5-dithiobi 2-nitrobenzoic acid (DTNB), ethylene diaminetetraacetic acid (EDTA), quercetin, tetrathylenediamine (TEMED), hydrogen peroxide, ammonium molybdate, sodium azide, 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 Kamyshnikov (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 heptan 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 A233 per ml. In blank, mixture with distilled water was used.

**Thiobarbituric acid reactive substances (TBARS) assay.** The level of lipid peroxidation was determined by quantifying the concentration of 2-thiobarbituric acid reacting substances (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 (blood, 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 1.56·10⁻⁵ mm⁻¹ cm⁻¹ as extinction coefficient.

**The carbonyl derivatives content of protein oxidative modification (OMP) assay.** The rate of protein oxidative destruction was estimated from the reaction of the resultant carbonyl derivatives of amino acid reaction with DNFH 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 0.1m DNPH (dissolved in 2m 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 2m 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 8m 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 derivates, OMP$^{370}$) and 430 nm (ketonic derivates, 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 0.1m K, Na-phosphate buffer, pH 7.8 and 0.08m 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 µm 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 H$_2$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% H$_2$O$_2$ solution) and to 1.0 ml of 4% ammonium molybdate dissolved in 12.5 mm H$_2$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 H$_2$SO$_4$ solution to incubation medium and 1 ml of 125 mm H$_2$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 µmol H$_2$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$^{-1}$·cm$^{-1}$ of NADPH. The GR 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 hemolyzed erythrocytes (1:20), 1 ml of 20 mm 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 µmol 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 % 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 Fe²⁺/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 FeSO₄, 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·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**

Lipid peroxidation markers include lipid hydroperoxides, conjugated dienes, malondialdehyde (MDA), thiobarbituric acid reactive substances (TBARS), and isoprostanes, with the level of F2-isoprostanes in blood or urine to be widely regarded as the reference marker for the assessment of oxidative stress (Nikolaides et al. 2011). 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. 2005a, b, 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. 2000). The exercised-induced changes of lipid hydroperoxides level are shown in Fig. 2.

![Fig. 2](image)

Fig. 2. Lipid hydroperoxides level (A233/μl) in the blood of stallions (n = 10) and mares (n = 7) of Holsteiner horses at the resting period and after exercise training (n = 17).

There were no significant differences in plasma LOOH level between resting period and after exercise training of Holsteiner horses (Fig. 2). LOOH are a biomarker of lipid peroxidation because LOOH contain a conjugated diene structure (Han et al. 2000). Conjugated dienes have been used to assess low-density lipoprotein oxidation in vitro. Exercise has been shown to increase plasma conjugated dienes (Marzatico et al. 1997, Balakrishnan and Anuradha 1998), and to increase conjugated dienes in an in vitro determination of low density lipoprotein susceptibility to oxidation (Liu et al. 1999). However, not all studies found that conjugated dienes increased in response to exercise. Duthie et al. (1990) reported no change in plasma conjugated dienes in subjects who performed a half-marathon. Our results consistent with Kinunen et al. (2005a, b) which is not revealed significant changes in antioxidants or oxidative stress marker lipid hydroperoxides (LPO) after prolonged exercise of endurance horses.

Aldehydes, especially MDA, have been frequently used as markers of oxidative stress in response to exercise (Urso and Clarkson 2003) presents the chain of chemical reactions leading to MDA, which can be measured by spectrophotometry or spectrofluorescence (Han et al. 2000). The most common method used to assess changes in MDA during exercise training is the 2-thiobarbituric acid reactive substances (TBARS) assay. However, the content of MDA, one of most important end-products of lipid peroxidation, in the tissues is usually accepted as an index of lipid peroxidation intensity (Chiaradia et al. 1998). The lipid peroxide during exercise training session was measured through analysis of the TBARS level and shown in
Fig. 3. There were no significant differences in the blood and plasma TBARS level between the resting period and after exercise training in Holsteiners. In contrary, TBARS level in the erythrocytes’ suspension showed a significant decrease by 31.6% (p = 0.002) immediately after exercise training as compared with the resting period.

![Graph showing TBARS levels in blood, erythrocytes, and plasma before and after exercise training](image)

* The level of significance is set at p < 0.05 compared between before and after exercise training (paired samples by Wilcoxon signed-rank test).

We can explain these responses by a multitude of changes in the body at cellular, tissue, organ, and whole organism levels during exercise training. At the most fundamental level, training occurs through increased production of proteins, both structural and functional proteins. Accumulation of metabolites and waste products is believed to induce increased transcription of DNA specific for proteins, including enzymes, that control rate limiting functions associated with these metabolites. Increased transcription, if associated with increased translation of mRNA to protein and appropriate posttranslational events, results in production of more protein. The increased quantity or activity of the enzymes then results in an increase in the maximal rate at which the metabolites can be processed or waste products eliminated. At an organ level these changes result in an increase in function, usually associated with increases in organ size (Hinchcliff and Geor 2008).

Regular physical activity has been reported to be the most effective nonpharmacological intervention to enhance endogenous antioxidant capacity and alleviate oxidative stress-induced tissue damage as a result of adaptive responses to exercise-induced oxidative stress (Ji and Leichtweis 1997, Leeuwenburgh and Heinecke 2001, Packer et al. 2008). The majority of studies have reported an increase in oxidative stress, evident by increased lipid peroxidation, protein oxidation, and changes in glutathione redox status, despite a few studies noting null findings for each (lipid, protein, glutathione) (Fisher-Wellman and Bloomer 2009). It has been suggested that
acute and irregular exercise training have negative effects, whereas regular physical activity creates an advanced antioxidant system and decreases oxidative damage. Trained individuals have a higher antioxidant capacity and lower levels of oxidative damage than sedentary individuals in resting conditions (Ortenblad et al. 1997, Sełlamoglu et al. 2000). Leelarugrayub et al. (2005) concluded that athletes who had regular exercise showed the benefit of exercise by gaining a higher total antioxidant capacity, lower oxidation on protein and lipid. No increase in lipid peroxides after maximal bicycle exercise in either trained or untrained human subjects was observed (Kretzschmar et al. 1995), while the concentration of erythrocyte’s TBARS was increased in untrained but not trained rats after exhaustive exercise (Sen 1995). In the present study, the decrease in erythrocytes’ TBARS level in horses after training session could be attributed as adaptive mechanism to exercise training with activation of the antioxidant defense in erythrocytes (Table 1).

Oxidative damage to proteins can occur directly by interaction of the protein with ROS or indirectly by interaction of the protein with a secondary product (resulting from interaction of radical with lipid or sugar molecule) (DallełDonne et al. 2006, FisherłWellman and Bloomer 2009). Modification of a protein under conditions of oxidative stress can occur via peptide backbone cleavage, cross-linking, and/or modification of the side chain of virtually every amino acid (DallełDonne et al. 2006, FisherłWellman and Bloomer 2009). Moreover, most protein damage is irreparable and oxidative modification of the protein structure can lead to loss of enzymatic, contractile, or structural function in the affected proteins, thus making them increasingly susceptible to proteolytic degradation (Levine and Stadtman 2001, FisherłWellman and Bloomer 2009). ROS induced oxidation of arginine, lysine, threonine, or proline amino acid residues generates reactive carbonyl derivatives (RCD), which can be readily measured by reaction with 2,4-dinitrophenyl hydrazine (Radák et al. 2000). Protein RCD are used very often as a marker of oxidative modification of proteins (Radák et al. 2000, 2008). The increase of exercise intensity mediated by accumulation of oxidative protein damage in plasma could be due to the increased ROS production and/or the poor proteolytic breakdown of oxidatively modified proteins. Despite this, in general, oxidative modifications of proteins activate the proteolytic system.

The effect of training session on protein carbonyl contents in the erythrocyte suspension and plasma samples of horses are shown in Fig. 4. There were no statistically significant changes in the derivates of protein destruction in the plasma of trained Holsteiner horses during exercise training. Interestingly, statistically significantly increase of ketonic derivates (by 56%, p < 0.05) in erythrocyte suspension was noted.

Our results are in agreement with results of other researchers. An increase of protein carbonyls in blood has been reported after aerobic exercise (Alessio 2000). It is well known that the oxidative modification of proteins takes place in a selective manner during mild oxidative stress. Iron-containing proteins are especially prone to oxidative damage (Radák et al. 2000, Vollaard et al. 2005). Heme proteins like hemoglobin (Hb) and myoglobin (Mb) contain redox-active transition metal iron that makes them susceptible to causing oxidative damage (Vollaard et al. 2005). Due to their high ferrous ion concentration, erythrocytes might be exposed to risks of in-
Fig. 4. Effect of training on the aldehyde (A) and ketonic derivates (B) of oxidatively modified protein levels (nmol/ml) in the erythrocytes and plasma of Holstein horses (n = 17).
* The level of significance is set at p < 0.05 compared between before and after exercise training (paired samples by Wilcoxon signed-rank test)

decreased oxidative stress, mainly through the formation of ferryl hemoglobin (Devi et al. 2005), and in part, through the Fenton reaction of hydrogen peroxide (H$_2$O$_2$) with ferrous ion (Fe$^{2+}$) of Hb, that generates the powerful hydroxyl radical. Erythrocyte membrane proteins are susceptible to covalent damage, including cross-linking and aggregation by free radical-induced lipid peroxidation products (Devi et al. 2005). Although the structure of the globin chain allows heme to bind oxygen with minimal oxidation of ferrous to ferric iron, autoxidation is not entirely prevented; low concentrations of ferric heme are normally present in vivo (methemoglobin, metmyoglobin). These can then react with the peroxides formed during the autoxidation
process itself or elsewhere in the protein’s vicinity (Vollaard et al. 2005). Our data reveals that exercise causes oxidative stress and increase of oxidatively modified of protein in erythrocytes of horses of Holstein breed (Fig. 4B). These data are consistent with findings of Vollaard et al (2005) which have shown that oxidative modification of hemoglobin is a normal occurrence in vivo, and is enhanced by exercise.

In response to conditions of physical work the body’s antioxidant capacity may be temporarily decreased as its components are used to quench the harmful radicals produced (Fisher-Wellman and Bloomer 2009). Enzymatic antioxidants catalyze reactions to remove ROS or to regenerate (reduce) oxidized antioxidants (Halliwell and Gutteridge 1999). Antioxidant defense in the blood of well-trained horses of Holstein breeds before and after training session are shown in Table 1.

Table 1

<table>
<thead>
<tr>
<th>Antioxidant defense</th>
<th>Before exercise training (n = 17)</th>
<th>After exercise training (n = 17)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD, U/ml</td>
<td>15.90 ± 1.24</td>
<td>24.64 ± 1.61*</td>
<td>0.005</td>
</tr>
<tr>
<td>CAT, µmol/min·l</td>
<td>1.72 ± 0.27</td>
<td>2.0 ± 0.29</td>
<td>0.298</td>
</tr>
<tr>
<td>GR, nmol/min·ml</td>
<td>2.79 ± 0.38</td>
<td>1.48 ± 0.17*</td>
<td>0.013</td>
</tr>
<tr>
<td>GPx, µmol/min·ml</td>
<td>1.92 ± 0.31</td>
<td>1.43 ± 0.31</td>
<td>0.052</td>
</tr>
<tr>
<td>CP, mg/l</td>
<td>22.29 ± 3.48</td>
<td>22.93 ± 5.26</td>
<td>0.492</td>
</tr>
</tbody>
</table>

* The level of significance is set at p < 0.05 compared between before and after exercise training (paired samples by Wilcoxon signed-rank test)

There were no statistically significant changes in the activities of antioxidant defenses instead SOD and GR activity in the blood of well-trained Holsteiners (Table 1). SOD activity was higher by 55% (p < 0.05) after training session compared to the resting period. The GR activity after exercise was decreased by 47% (p < 0.05) compared to the resting period.

Superoxide dismutase, the first line of defense against oxygen-derived free radicals, catalyses the dismutation of the superoxide anion into hydrogen peroxide ($H_2O_2$). $H_2O_2$ can be transformed into $H_2O$ and $O_2$ by catalase, which is present in peroxisomes of eukaryotic cells (Ji and Leichtweis 1997). Glutathione-dependent antioxidant system consisting of reduced glutathione (GSH) and an array of functionally related enzymes plays a fundamental role in cellular defense against ROS and other oxidant species (Sen 1995, Ji and Leichtweis 1997). Among these enzymes, glutathione peroxidase (GPx) is a selenoprotein that reduces hydroperoxides as well as $H_2O_2$. Reduction of oxidized glutathione (GSSG) to GSH is mediated by the widely distributed enzyme glutathione reductase (GR) that uses NADPH as the reductant (Sen 1995, Ji and Leichtweis 1997).

In our study, the enhancement of SOD activity in horses suggest that its activation probably is result of exercise-linked mitochondrial superoxide radicals ($O_2^••$) production. As SOD provides the first line of defense against produced mitochondrial superoxide radicals, the increase of SOD activity shown in well-trained horses could reduce the exposure to superoxide and even to the hydroxyl radicals formed via the Haber–Weiss reaction (Halliwell and Gutteridge 1999). Our results are in agreement with results of other researchers. It also has been shown that training causes the increased SOD activity in the
muscle and erythrocytes of students, swimmers, cyclists, and volleyball players (Deaton and Marlin 2003). Increased SOD activities have been reported by treadmill training of 8 weeks in male (Husain 2003) and 10 weeks in female (Somani et al. 1995). The total mitochondrial SOD activities was increased in male Wistar rats by 24-week treadmill training (Moran et al. 2004), and in female rats treadmill-trained for 10 weeks (Ramires and Ji 2001). Swim training for 4 weeks has also increased superoxide dismutase (Mn-SOD) activity in the left and right ventricles in male rats (Ravi Kiran et al. 2004). In relation to acute exercise, erythrocyte SOD and GPx activities were not changed in pentathlon horses following show jumping (Deaton and Marlin 2003). Increase in different antioxidants activity, such as, GPx and SOD, has been observed in Standardbreds after a 12-week period of aerobic and anaerobic training, while the increase in oxygen consumption (measured as change of VO$_2$ max) was positively correlated with the increase of erythrocyte SOD activity (Kirschvink et al. 2008). Sachdev and Davies (2008) reported about the adaptive response to free radicals in exercise, and showed that SOD expression is regulated by the redox-sensitive transcription nuclear factor kB (NF-kB). Hydrogen peroxide or another ROS could activate NF-kB in the cytosol, which translocates to the nucleus, resulting in increased expression of antioxidant enzymes. In addition, NF-kB has been shown as a broad antioxidant transcription factor regulating the expression of GR, GPx, peroxiredoxin, thioredoxin, thioredoxin reductase, CAT, and Cu/Zn-SOD (Osburn and Kensler 2008).

GR is not considered as a primary detoxificant of ROS, however GR recycles oxidised glutathione to its reduced form and has a central role in the glutathione dependent antioxidant protection (Sen 1995, Ji and Leichtweis 1997, Kinnunen et al. 2005a). GR activity of Holsteiner horses after training session was decreased compared to the resting period (Table 1). Reduced GR activity in Holsteiners may be caused by its use in elimination of ROS production during exercise training.

The total antioxidant capacity (TAC) in erythrocyte suspension and plasma is shown in Fig. 5. No significant variations in TAC during exercise training were

![Fig. 5. Effect of exercise training on the total antioxidant capacity in the erythrocytes and plasma of Holsteiner horses (n = 17)]
found. Trend to increase of TAC in erythrocytes compared to plasma was demonstrated. In several studies was detected the increase of TAC after exercise (Ginsburg et al. 2001, Santos-Silva et al. 2001). However, Alessio et al. (1997) found that plasma TAC did not increase in response to a 30 min exercise, despite an increase in TBARS level.

There were no significant changes in CAT activities in the blood of Holsteiners during training session. Similar Gul et al. (2006) noted that CAT activities were not affected in heart tissues of male rats under the influence of acute exhaustive exercise. Moreover, correlative analysis between of GPx, SOD and CAT activities and oxidative stress biomarkers (plasma TBARS level, protein derivates and plasma TAC level) suggest that these enzymes play a central role in the antioxidant defense in the blood of horses both at the resting period and after training session (Figs 6 and 7).

Serum antioxidant activity of humans and animals attributed to the presence of transferrin and ceruloplasmin (CP) (Kim 2008). Ceruloplasmin is a copper-containing glucoprotein with multiple physiological functions, including ferroxidase and oxidase activity (Harma et al. 2006). It has been suggested that CP might be the major antioxidant in plasma as a scavenger of oxygen radicals. CP being an acute phase reactant protein, its level rise immediately after cellular damage. It acts as an antioxidant through ferroxidase activity, and it also scavenges superoxide anion. Physiological factors like cancer, exercise, chronic inflammation, pregnancy increases CP level (Harma et al. 2006). The correlative analysis between CP content and oxidative stress biomarkers (erythrocytes and blood TBARS levels, plasma TAC) during the training session in horses suggest that CP also plays a pivotal role in antioxidant defenses in the blood of horses both at the resting period and after training session (Figs 6 and 7).

![Graph showing correlation between TBARS and TAC](image)

A. TBARS (erythrocytes) : TBARS (blood) \( y = 1.25 + 0.75x; r = 0.764; p = 0.000; r^2 = 0.584 \)

TBARS (erythrocytes) : TAC (plasma) \( y = 57.29 + 0.38x; r = 0.552; p = 0.022; r^2 = 0.304, n = 17 \)
B. TBARS (plasma) : OMP\textsubscript{370} (plasma) $y = 9.43 + 0.51x; r = 0.624; p = 0.008; r^2 = 0.389$

TBARS (plasma) : OMP\textsubscript{430} (plasma) $y = 12.96 + 0.73x; r = 0.751; p = 0.000; r^2 = 0.565, n = 17$

C. TBARS (plasma) : GPx $y = 5.37 - 0.85x; r = -0.553; p = 0.021; r^2 = 0.305$

GPx : SOD $y = 10.98 + 2.39x; r = 0.602; p = 0.011; r^2 = 0.363, n = 17$
D. TBARS (erythrocytes): CP $y = 55.09 - 0.94x; r = -0.683; p = 0.003; r^2 = 0.466$

TBARS (erythrocytes): OMP$_{430}$ (plasma) $y = 21.02 - 0.15x; r = -0.820; p = 0.000; r^2 = 0.673, n = 17$

Fig. 6. Correlative dependences between oxidative stress biomarkers in the blood of Holsteiners at the resting period

A. TBARS (blood): CAT $y = 4.03 - 0.07x; r = -0.604; p = 0.010; r^2 = 0.365$

TBARS (blood): CP $y = 66.24 - 1.57x; r = -0.719; p = 0.001; r^2 = 0.517, n = 17$
B. TAC (plasma) : OMP₃₇₀ (plasma) $y = 14.13 - 0.04x; r = -0.547; p = 0.023; r^2 = 0.299$
TAC (plasma) : CAT $y = 6.48 - 0.06x; r = -0.626; p = 0.007; r^2 = 0.392$, n = 17

C. OMP₃₇₀ (plasma) : TBARS (plasma) $y = -32.75 + 3.25x; r = 0.775; p = 0.000; r^2 = 0.601$
OMP₃₇₀ (plasma) : OMP₄₃₀ (plasma) $y = -3.34 + 1.67x; r = 0.842; p = 0.000; r^2 = 0.709$, n = 17
Correlative analysis between oxidative stress biomarkers and antioxidant defense in the blood of Holsteiner horses at the resting period and after training session are presented in Figs 6 and 7. There were a positive correlation between erythrocytes and blood TBARS levels ($R = 0.764, p = 0.000$), as well as plasma erythrocytes TBARS level and plasma TAC ($R = 0.552, p = 0.022$) in the blood of Holsteiners at the rest period (Fig. 6A). At the same time, plasma TBARS level correlated positively with aldehyde ($R = 0.624, p = 0.008$) and ketone derivates of protein oxidation in the plasma ($R = 0.751, p = 0.000$) (Fig. 6B). Similarly, reverse correlation between plasma TBARS level and GPx activity ($R = -0.553, p = 0.021$) and positive correlation between SOD and GPX activity ($R = 0.602, p = 0.011$) was noted (Fig. 6C). There were a reverse correlation between erythrocyte TBARS level and CP content ($R = -0.683, p = 0.003$), as well as erythrocyte TBARS level and ketonic derivates of protein oxidation in plasma ($R = -0.820, p = 0.000$) (Fig. 6D).

After training session, blood TBARS level correlated reversely with CAT activity ($R = -0.604, p = 0.010$) and CP level ($R = -0.719, p = 0.001$) (Fig. 7A). Plasma TAC level reversely correlated with aldehyde derivates of protein oxidation in plasma ($R = -0.547, p = 0.023$) and CAT activity ($R = -0.626, p = 0.007$) (Fig. 7B). Aldehyde derivates of protein oxidation in plasma correlated positively with plasma TBARS level ($R = 0.775, p = 0.000$) and ketonic derivates of protein oxidation ($R = 0.842, p = 0.000$) (Fig. 7C). Significant correlation between plasma

D. TAC (plasma) : TBARS (blood) $y = -5.41 + 0.47x; r = 0.562; p = 0.019; r^2 = 0.316$

TAC (plasma) : CP $y = 92.06-0.98x; r = -0.540; p = 0.025; r^2 = 0.291, n = 17$

Fig. 7. Correlative dependences between oxidative stress biomarkers in the blood of Holsteiners after training session
TAC level and blood TBARS level ($R = 0.562$, $p = 0.019$), as well as plasma TAC and CP levels ($R = -0.540$, $p = 0.025$) was noted (Fig. 7D).

The correlation analysis between markers of lipid peroxidation (blood, plasma and erythrocytes TBARS levels) and carbonyl derivatives contents of protein oxidative modification indicates to the close relationship between oxidation of lipids and proteins during exercise in sport horses. A correlation between the oxidative stress biomarkers and antioxidant defenses in the horses after training session was also observed. Its indicates about a pivotal role of CAT and CP against exercise-induced oxidative stress in Holsteiners.

CONCLUSIONS

The results of present study showed an increase of antioxidant enzymes activity and attenuate on exercise-induced lipid peroxidation in the blood of Holsteiners after training session. It has been observed a decrease of lipid peroxidation biomarker in erythrocytes after training session, while it did not change in the plasma and blood. 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. Elevated level of oxidative modified protein in erythrocytes of Holsteiner horses indicate about exercise-induced oxidative stress. The correlation analysis between markers of lipid peroxidation (blood, plasma and erythrocytes TBARS levels) and carbonyl derivatives of protein oxidative modification content indicate about close relationship between lipid peroxidation and protein oxidation during exercise in sport horses. Moreover, correlative analysis between GPx, SOD and CAT activities and oxidative stress biomarkers (plasma TBARS level, carbonyl derivates of protein damage and plasma TAC level) suggest that these enzymes play a pivotal role in the blood antioxidant defense of horses both at the resting period and after training session. A correlation between the oxidative stress biomarkers and antioxidant defenses in the horses after training session was observed, which may indicate a protective response of CAT and CP activity against exercise-induced oxidative stress in Holsteiners. The knowledge of oxidative stress mechanisms and antioxidant defense response in athlete horses can allow us to plan an appropriate and high-grade training to obtain better performance and to preserve horse welfare.

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SUMMARY

Exercise-induced oxidant/antioxidant changes in trained horses vary with regard to exercise type (race, standardized treadmill exercise, standardized race track exercise, endurance). It is generally agreed that exercise induces significant alterations of the circulatory oxidant-antioxidant balance (Kirschvink et al. 2008). Given the fact that in the modern equestrian sport used athletic horses over a several years and they involved in regular training, the aim of the present study was to investigate the exercise-induced changes of oxidative stress biomarkers and antioxidant defenses in well-trained Holsteiner horses. Seventeen Holsteiners (7 females and 10 males), 7-15 years old, were used. All horses were involved in equestrian show jumping. To assessed the exercise-induced changes of oxidative stress biomarkers and antioxidant enzymes activity was proposed common to all horses the training session of average intensity: walk – 5 min; trot – 10 min; walk – 5 min; trot – 10 min; walk – 10 min; gallop – 10 min; walk – 10 min.
Duration of training was 1 hour. Lipid peroxidation and oxidatively modified protein level as oxidative stress biomarkers, antioxidant defenses such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GPx) activity, ceruloplasmin (CP) level as well as total antioxidant capacity in the blood of horses before and after training session was assayed. An increase of antioxidant enzymes activity (SOD and GR) and attenuate on exercise-induced lipid peroxidation in the blood of Holsteiners after training session was noted. It has been observed a decrease of lipid peroxidation biomarker in erythrocytes after training session, while its did not change in the plasma and blood. 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. Elevated level of oxidative modified protein in erythrocytes of Holsteiner horses indicate about exercise-induced oxidative stress. The correlation analysis between markers of lipid peroxidation (blood, plasma and erythrocytes TBARS levels) and carbonyl derivatives of protein oxidative modification content indicate about close relationship between lipid peroxidation and protein oxidation during exercise in sport horses. Moreover, correlative analysis between GPx, SOD and CAT activities and oxidative stress biomarkers (plasma TBARS level, carbonyl derivates of protein damage and plasma TAC level) suggest that these enzymes plays a pivotal role in the blood antioxidant defense of horses both at the resting period and after training session. A correlation between the oxidative stress biomarkers and antioxidant defenses in the horses after training session was observed, which may indicate a protective response of CAT and CP activity against exercise-induced oxidative stress in Holsteiners. The knowledge of oxidative stress mechanisms and antioxidant defense response in athlete horses can allow us to plan an appropriate and high-grade training to obtain better performance and to preserve horse welfare.