Polish Journal of Veterinary Sciences Vol. 18, No. 2 (2015), 407–413

DOI 10.1515/pjvs-2015-0052

Original article

Transport induced inflammatory responses in horses

J. Wessely-Szponder[1], Z. Bełkot[2], R. Bobowiec[1], U. Kosior-Korzecka[1], M. Wójcik[1]

[1] Chair of Preclinical Veterinary Sciences, Department of Pathophysiology
[2]Department of Food Hygiene of Animal Origin Faculty of Veterinary Medicine, University of Life Sciences, ul. Akademicka 12, 20-033 Lublin, Poland

Abstract

Deleterious response to road transport is an important problem in equine practice. It determines different physiological, immunological and metabolic changes which lead to increased susceptibility to several disorders such as pneumonia, diarrhea, colics, laminitis, injuries and rhabdomyolisis. The aim of our study was to look for possible relationships between transportation of female young and older horses over a long and short distance and an inflammatory state reflected by an increase of acute phase protein concentration, oxidative stress and muscle injury. The study was conducted on 24 cold-blooded female horses divided into four groups. Six fillies aged 6-18 months and six mares aged 10-12 years were transported over the distance of about 550 km, six fillies aged 6-18 months and six mares aged 10-12 years were transported over the distance of about 50 km. Plasma and serum were obtained from blood samples taken before transportation (T0), immediately after transportation (T1) and at an abattoir during slaughter (T2). In these samples fibrinogen, MDA, AST and CK were assessed. Fibrinogen increased in all studied groups especially in fillies after long distance transportation, where it reached 205±7.07 mg/dl before transportation, 625±35.35 mg/dl after transportation, and 790±14.14 mg/dl during slaughter. MDA concentrations rose after transportation and reached the maximal level during slaughter. CK activity was more elevated after short transportation in younger horses, whereas initial activity of AST was higher in older horses. We estimated that intensified responses from acute phase, oxidative stress and muscle injury parameters indicated an inflammatory state.

Key words: horse, transportation stress, acute phase response, muscle injury

Introduction

Horses at transportation as well as when entering the slaughterhouse have to cope with several stressors. Before slaughter, during transportation, lairage and handling, these animals are exposed to various stressors that can trigger behavioral changes and several physiological responses, such as increased heart rate and concentrations of stress-related hormones which might lead to changes of biochemical, haematological and oxidative stress parameters (Nemec Svete et al. 2012).

Correspondence to: J. Wessely-Szponder, e-mail: joanna.wessely@up.lublin.pl

Transportation, with a variety of physical and psychological stimuli, is often considered one of the main causes of stress that disrupt homeostasis and metabolism of animals (Wernicki et al. 2006, Andronie et al. 2009, Omnaz et al. 2011). Furthermore, in horses stressed by transportation the reciprocal relationship between inflammation and oxidative stress may be exaggerated (Kumar et al. 2013, Cai et al. 2014). Earlier data highlighted the potential role of reactive oxidative species (ROS) as a signal inducing inflammatory responses from such cells as macrophages, smooth muscle cells and fibroblasts. As a consequence of this reaction, a large amount of pro-inflammatory molecules form these cells is released, including inflammatory cytokines and chemokines (Yuichi Uchino et al. 2012). These pro-inflammatory molecules are involved in a variety of horse diseases related to transportation, such as pneumonia, diarrhea, colics, laminitis, injuries and rhabdomyolisis, which is well documented (Onmaz et al. 2011). Overproduction of ROS resulting in oxidative stress is also involved in several respiratory inflammatory diseases, including recurrent airway obstruction (RAO)(Lee and Chang 2012).

The acute phase proteins (APP) were proposed recently as indicators of stress response in horses, especially during transport. In this species fibrinogen is among the most frequently measured APP (Casella et al. 2012). It is a soluble plasma glycoprotein synthesized by hepatocytes, which is involved in the clotting cascade and plays a key role in the inflammatory response.

Oxidative stress should also be taken into account during transportation. As estimated previously, in horses oxidative stress may play a role in pathogenesis of recurrent airway obstruction, joint disease, grass sickness, Cushing disease and urticaria (Omnaz et al. 2011). Under normal conditions natural antioxidant defense is sufficient to neutralize reactive oxygen species (ROS) (Soares et al. 2011). However, excessive production of ROS may cause oxidative stress, lipid peroxidation and oxidative damage of tissues, including muscles. During transportation of horses oxygen uptake by active muscles markedly increases and consequently generation of ROS rises (Omnaz et al. 2011), which may cause muscle injury (Marlin et al. 2002).

During exercise or transport muscles may undergo a micro-damage. These lesions may be measured by determining the serum activity of muscle enzymes creatine phosphokinase (CK) and aspartate aminotransferase (AST). CK has the function of phosphorylation of creatine and actively participates in energy metabolism in tissues. AST, in turn, is the enzyme which deaminates aspartate to oxaloacetate. The muscle enzyme activity is normally low in plasma, since they are within myocyte, however, after exercise or muscle injury their activity significantly rises due to increased cellular permeability, cell necrosis, deficient elimination or increased synthesis (Soares et al. 2011). Although some studies described the activity of these enzymes after exercises, there are few reports about their activity after transportation.

Interactions between acute phase, oxidative status and muscle injury were evaluated thoroughly in horses during exercise (Chiaradia et al. 1998, Linder and Hatzipanagiotou 1998, Hargreaves et al. 2002, Marlin et al. 2002, Cywińska et al. 2012). Furthermore, there are some reports documenting acute phase response during transport (Casella et al. 2012) as well as oxidative status (Ishida et al. 1999, Onmaz et al. 2011) and muscle injury (Stull and Rodiek 2000). Serum cortisol, haematological, biochemical and antioxidant enzyme variables of blood sampled in a slaughterhouse lairage, before stunning and during exsanguination were also studied (Nemec Svete et al. 2012). However, to date there have been no reports describing how these variables change in cold-blooded horses during and after long and short transport and during slaughter. Therefore, the aim of our study was to look for possible relationships between transportation of young and middle-aged female horses over a long and a short distance and APP concentration, oxidative stress, and muscle injury as indicators of inflammatory response during stress involved in transportation and slaughter.

Materials and Methods

The experiment was conducted on 24 cold-blooded female horses divided into four groups. Group 1 consisted of six fillies aged 6-18 months (13±4.05, mean ±SD) weighing 420±20.73 kg. Group 2 consisted of six mares aged 10-12 years (11±0.89) weighing 650±24.00 kg. Female horses from both groups were transported from 5 p.m. over a total distance of 550 km (about 12h) and then rested for about 24 hours before slaughter. Group 3 consisted of six fillies aged 6-18 months (14±4.24) weighing 400±19.23 kg and Group 4 consisted of six mares aged 10-12 years (11±1.1) weighing 600±26.00 kg. Animals from both groups were transported over a total distance of 50 km (about 1 h from 10 p.m.) and rested for about 6 hours before slaughter. The routes taken were secondary roads and expressways. Commercial vans designed to haul a maximum of 11 horses were used for the transportation. Horses were transported in accordance with Directive 1/2005

CEE (European Economic Community). All horses that were involved in this study were transported for the first time.

The values of rectal temperature, heart and respiratory rates before and after transportation varied over a wide range of physiological indices. Measurement of rectal temperature, heart rate and respiratory rate before and after transport showed an average increase of 0.35°C, 5 beats and 7 breaths respectively after transportation.

The horses were clinically examined before and after transportation. Blood samples from all horses were taken from the jugular vein at three different times: before transportation (time 0 – T0), immediately after transportation (time 1 – T1) and at an abattoir during slaughter (time 2 – T2). Blood was collected into the tubes containing EDTA as an anticoagulant and into tubes without any anticoagulant to be used for serum biochemistry assays. Obtained blood samples were centrifuged for 15 min at 1500 g and plasma or serum was harvested and stored at -70°C until it was used for analyses.

Fibrinogen level was estimated using the heat precipitation method (Szponder and Wessely-Szponder 2010). Diluted plasma was clotted with thrombin and the fibrin formed by the action of thrombin on fibrinogen was hydrolysed by boiling in NaOH solution (10%), then its tyrosine content was measured at 720 nm with Folin-Ciocalteu's phenol reagent on the basis of standard curve.

One of the indicators of lipid peroxidation during oxidative stress is malondialdehyde (MDA) (Hargreaves et al. 2002). Plasma concentration of MDA was measured using a spectrophotometric method. The aliquots of 250 µl of plasma were mixed with 5 volumes of phosphate buffer and centrifuged 2000 x *g* for 15 minutes. Then, 0.5 ml of supernatant was mixed with 1.25 ml of trichloroacetic acid (TCA) and with 0.75ml of thiobarbituric acid and thereafter heated for 20 min in a boiling water bath. After cooling to the room temperature, 2 ml of n-butanol was added and the mixture was shaken vigorously for 3 min and centrifuged 10 min at 1,500 x g. After the transfer of upper n-butanol layer to a glass cuvette its absorbance was measured at 532 nm. Concentrations of MDA were read from a standard curve obtained by using malondialdehyde bis-dimethylacetal (Wójcik et al. 2010).

CK and AST activities were assessed using a kinetic method with Cobas 6000 (Roche Diagnostics) using commercially available tests of Roche Diagnostics.

Data were presented as mean±S.D. Statistical analysis was carried out using the Student's t test. The level of significance was set at $p<0.05$. The relations between MDA level and CK and AST activity were evaluated using a regression coefficient.

Results

We estimated a marked increase of plasma fibrinogen level at measurements after transportation (T1) and during slaughter (T2) in comparison with values before transportation (T0) in all examined horses. Plasma fibrinogen level in group 1 increased significantly ($p<0.05$) from 205±7.07 mg/dl before transportation (T0) to 625±35.35 mg/dl after transportation (T1), and to the value of 790±14.14 mg/dl during slaughter (T2). The changes in groups 2, 3, and 4 were similar but less pronounced (Fig. 1).

The MDA plasma value after transportation (T1) and during slaughter (T2) was elevated in all groups in comparison with the values before transportation (T0). However, statistical significance was only in the last measurement in all examined groups in comparison with the value before transportation and the greatest increase (up to the value of 15.25±2.0 nM/ml) was seen in group 2 in middle-aged mares after long distance transportation. Comparison between both groups after short distance transportation revealed a higher increase of MDA level in the group of older horses (Fig. 2).

Serum CK activity increased markedly ($p<0.05$) in groups of young horses (group 1 and 3). In group 1 enzyme activity rose from the value of 267.83±57.32 IU/l before transportation to 820.83±66.2 IU/l just after transportation and 732.00±47 IU/l during slaughter, whereas in group 3 the value during slaughter reached 1090.8±98 IU/l. Differences between measurements in groups of older mares (group 2 and 4) were less pronounced (Fig. 3).

The median values of serum activity of AST augmented after transportation and during slaughter, significant differences were noted between the values before transportation and during slaughter in groups of young horses ($p<0.05$), whereas higher initial level of AST was seen in older horses (Fig. 4).

We observed positive correlations between MDA and enzyme activities during slaughter. Serum CK activity correlated with MDA level in group 1 ($r=0.77$) and in group 4 ($r=0.90$), whereas AST activity correlated with MDA in group 2 ($r=0.91$) and 3 ($r=0.48$).

Discussion

In our study plasma fibrinogen level increased after transportation in all examined groups and the maximal response was seen in group 1 up to the value

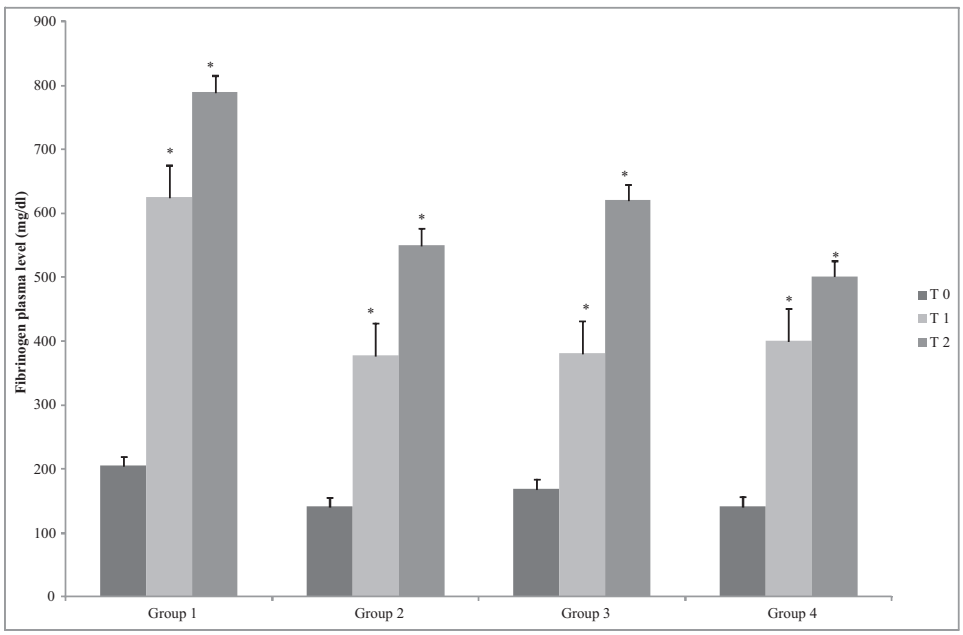

Fig. 1. Plasma fibrinogen level (in mg/dl) in horses before transportation (T0), after transportation (T1) and during slaughter. * p<0.05 compared to the value before transportation (T2) (mean ± SD).

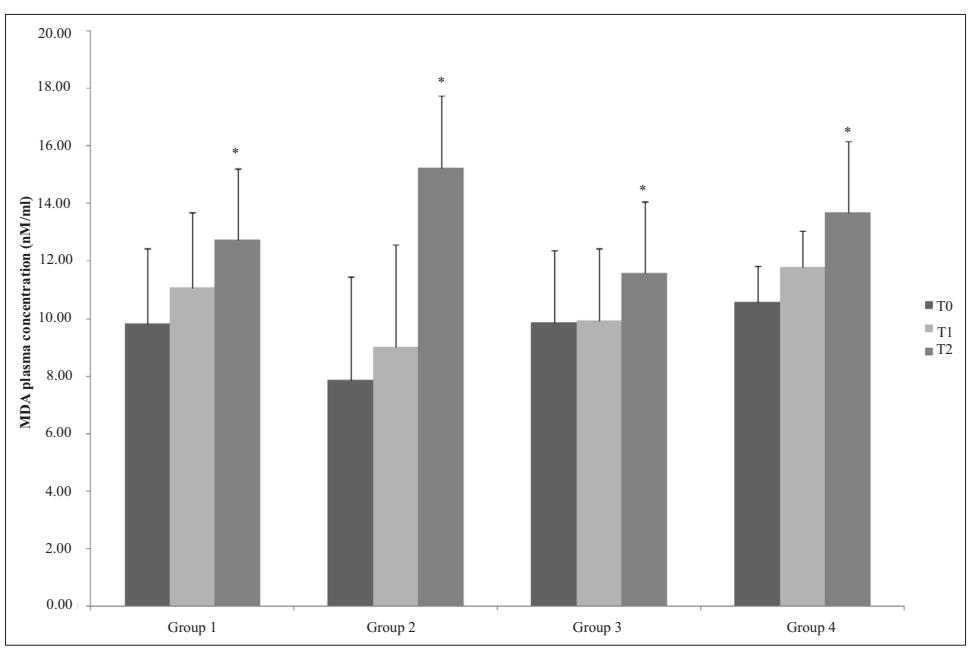

Fig. 2. MDA content (in nM/ml) in blood of horses before transportation (T0), after transportation (T1) and during slaughter (T2). *p<0.05 compared to the value before transportation (mean ± SD).

of 790±14.14 mg/dl during slaughter. As estimated in a report of Crisman et al (2008) the induction of fibrinogen production could be involved in increased release of IL-6. In our previous work we detected especially elevated IL-6 concentration in group of fillies after long distance transport during slaughter and also in this group elevated level of fibrinogen was noted (Wessely-Szponder et al. 2014). According to Foreman and Ferlazzo (1996), stress caused by transportation resulted in an increase of fibrinogen plasma level as a marker of inflammation in horses (Borges 2007). Studies of Piccione et al. (2014) showed that fibrionogen level is a parameter affected by physical activity. Elevated fibrinogen level was observed in athletic horses during the first week of intense training, then returned to baseline manifesting an adaptive response

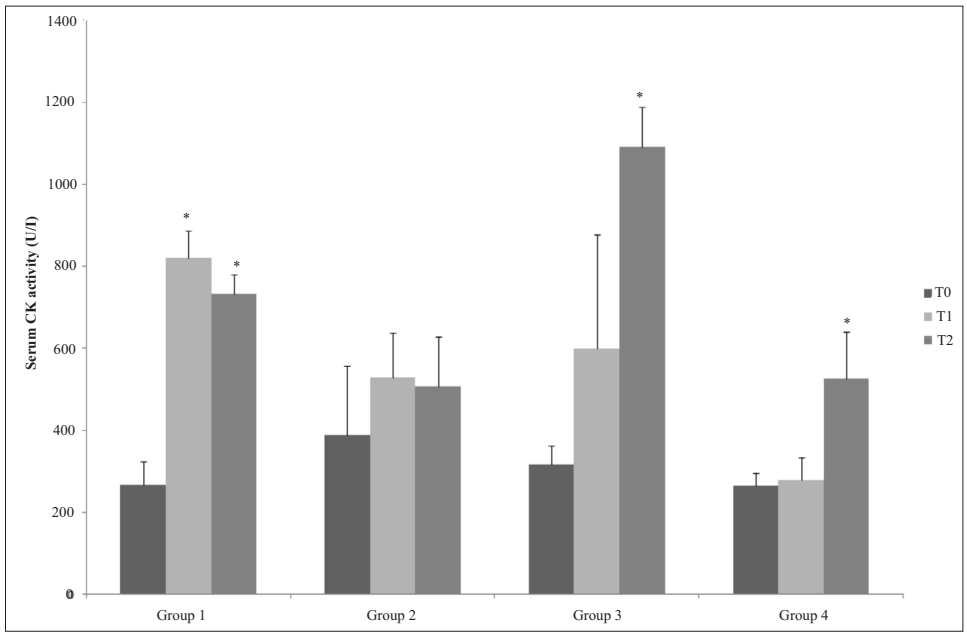

Fig. 3. Serum CK activity (in U/l) in horses before transportation (T0), after transportation (T1) and during slaughter (T2). * p<0.05 compared to the value before transportation (mean ± SD).

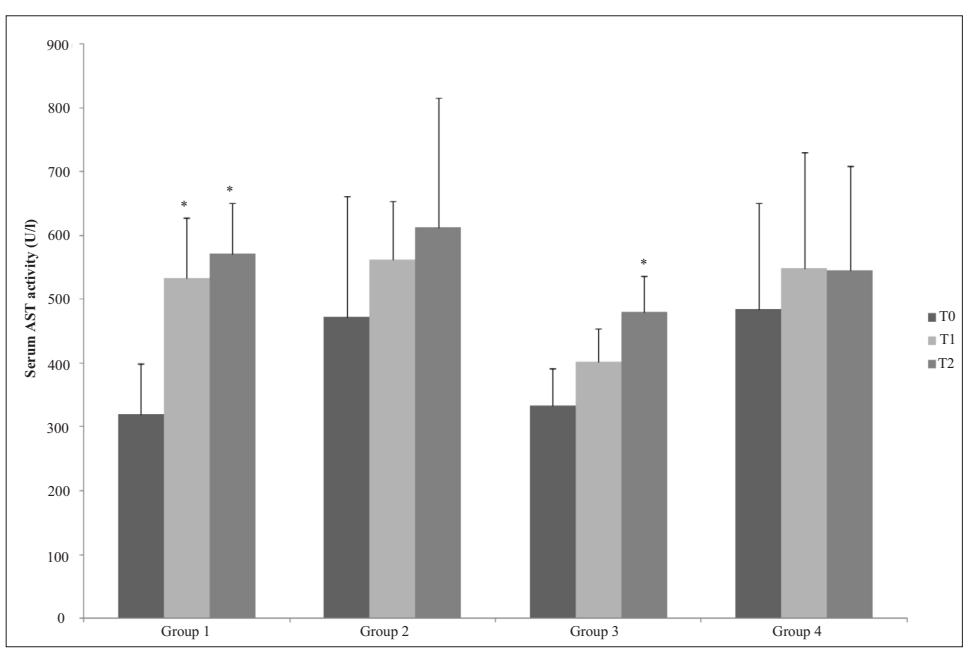

Fig. 4. Serum AST activity (in U/l) in horses before transportation (T0), after transportation (T1) and during slaughter (T2). * p<0.05 compared to the value before transportation (mean ± SD).

(Piccione et al. 2014). Also in the report of Coyne et al. (1990) fibrinogen level increased during acute running exercise. Contrary to these observations, in the report of Casella et al. (2012) road transport did not influence fibrinogen level, however, all horses involved in the study had previous experience of transportation.

We estimated an increased level of plasma MDA in all groups after transportation and during slaughter. The maximal response was noted in group 2 (15.25±2.0 nM/ml) during slaughter after 12 h transportation. Obtained results were consistent with the previous report where MDA dramatically increased after a 12 h transport (Omnaz et al. 2011). Also Ishida et al. (1999) discovered that MDA level rose over the time of transport with the maximal value after 23 h. According to these authors stress caused by transport evokes a response that essentially depends on its dur-

ation. In our study higher values of MDA were estimated in groups of older horses. This relation to age was discovered in human study of Bouzid et al. (2014) who detected higher oxidative stress in older patients in response to exhaustive exercise.

As estimated previously (Kirschvink et al. 2008), in horses in some pathological processes including RAO, grass sickness and equine motor neuron disease an inflammatory process was correlated with oxidative stress. Under these conditions MDA was used as a marker of such inflammatory status. Moreover, in humans MDA is a sensitive marker of inflammation in patients with rheumatoid arthritis (Kalavacherla et al. 1994).

During oxidative stress, damage to cell membranes contributes to muscle injury, fatigue and several pathological conditions (Omnaz et al. 2011). The activities of CK and AST in plasma are commonly used as indicators of skeletal muscle damage in horses (Linder and Hatzipanagiotou 1998). Thus, some studies described changes in these enzymes after exercise (Soares et al. 2011), when elevated AST and CK activities demonstrated muscle cell damage and leakage of these enzymes into the circulation. Fewer reports concern changes in enzymes caused by transportation of horses.

Our study revealed that changes in muscle enzymes were related with the age of horses and the distance of transportation. Serum CK activity elevated more in groups of young horses just after transportation (T1) and during slaughter (T2) compared to the value before transportation (T0). The highest response was in younger horses which were transported over a short distance and rested for 6 h. In groups of middle-aged horses the changes were less clear. Our results are in accordance with the report of Soares et al. (2011), where CK after a muscle injury was rapidly released into circulation and its activity peaked 4-12 hours later. Moreover, the 8-hour road transport resulted in an increase of both enzymes CK and AST in cold-blooded horses (Niedźwiedź et al. 2012). In another, previously conducted study, CK activity was higher in younger horses than in older ones (Linder and Hatzipanagiotou 1998).

Similarly to CK, AST activity rose after transportation and during slaughter. However, a significant difference was between T0 and T2 only in groups of young horses ($p<0.05$), whereas the initial level of serum AST was higher in groups of older horses. As estimated, AST was released more slowly than CK and peaked 24 hours after stress (Soares et al. 2011). In the study by Linder and Hatzipanagiotou (1998), values of AST activity were higher in older horses in comparison with two-year-old ones.

In our experiment serum CK and serum AST activity positively correlated with MDA level in measurement during slaughter. This relationship between muscle enzyme release and biomarkers of oxidative stress was identified in a rat model of muscle injury and resulted from an increase in membrane permeability due to lipid peroxidation (Guozhen et al. 2005), whereas the relation between oxidative stress and muscle injury in transported cold-blooded horses has not been determined to date.

Our results revealed that changes of the acute phase, oxidative stress and muscle injury parameters indicated an inflammatory state that made these horses prone to more specific pathologies. These changes depended on the age of horses and the distance of transport. Increase of fibrinogen was higher in younger horses, CK activity was higher after short transportation in younger horses, whereas in older horses MDA level was more elevated, especially during slaughter. These differences of the studied variables contributed to knowledge in the use of fibrinogen concentration, oxidative stress, and muscle injury as biomarkers of physiological responses to transportation and slaughter in cold-blooded horses.

References

Andronie I, Parvu M, Andronie V, Ciurea A (**2009**) Effects of transportation stress on some physiological indicators in sport horses. Zootehnie si Biotehnologii 42: 379-384.

Borges AS, Divers TJ, Stokol T, Mohammed OH (**2007**) Serum iron and plasma fibrinogen concentrations as indicators of systemic inflammatory diseases in horses. J Vet Intern Med 21: 489-494.

Bouzid MA, Hammouda O, Matran R, Robin S, Fabre C (**2014**) Changes in oxidative stress markers and biological markers of muscle injury with aging at rest and in response to an exhaustive exercise. PloS One 9:e 90420.

Cai L, Yi F, Dai Z, Huang X, Zhao Y D, Mirza MK, Xu J, Vogel SM, Zhao YY (**2014**) Loss of caveolin-1 and adiponectin induces severe inflammatory lung injury following LPS challenge through excessive oxidative/nitrative stress. Am J Physiol Lung Cell Mol Physiol 306: L566-L573.

Casella S, Fazio F, Giannetto C, Giudice E, Piccione G (**2012**) Influence of transportation on serum concentrations of acute phase proteins in horse. Res Vet Sci 93: 914-917.

Chiaradia E, Avellini L, Rueca F, Spaterna A, Porciello F, Antonioni MT, Gaiti A (**1998**) Physical exercise, oxidative stress and muscle damage in racehorses. Comp Biochem Physiol B 119: 833-836.

Coyne CP, Carlson GP, Spensley MS, Smith J (**1990**) Preliminary investigation of alterations in blood viscosity, cellular composition, and electrophoresis plasma protein fraction profile after competitive racing activity in Thoroughbred horses. Am J Vet Res 51: 1956-1963.

Crisman MV, Scarratt WK, Zimmerman KL (**2008**) Blood proteins and inflammation in the horse. Vet Clin Equine 24: 285-297.

Cywińska A, Szarska E, Górecka R, Witkowski L, Hecold M, Bereznowski A, Schollenberger A, Winnicka A (**2012**) Acute phase protein concentrations after limited distance and long distance endurance rides in horses. Res Vet Sci 93: 1402-1406.

Foreman JH, Ferlazzo A (**1996**) Physiological responses to stress in the horse. Pferdeheilkunde 12: 401-404.

Guozhen L, Feng X, Wang S (**2005**) Effects of Cu/Zn superoxide dismutase on strain injury-induced oxidative damage to skeletal muscle in rats. Physiol Res 54: 193-199.

Hargreaves BJ, Kronfeld DS, Waldron JN, Lopes MA, Gay LS, Saker KE, Cooper WL, Sklan DJ, Harris PA (**2002**) Antioxidant status and muscle cell leakage during endurance exercise. Equine Vet J Suppl 34: 116-121.

Ishida N, Hobo S, Takahashi T, Nanbo Y, Sato F, Hasegawa T, Mukoyama H (**1999**) Chronological changes in superoxide-scavenging ability and lipid peroxide concentration of equine serum due to stress from exercise and transport. Equine Vet J Suppl 30: 430-433.

Kalavacherla US, Ishaq M, Rao UR, Sachindranath A, Hepsiba T (**1994**) Malondialdehyde as a sensitive marker of inflammation in patients with rheumatoid arthritis. J Assoc Physicians India 42: 775-776.

Kirschvink N, de Moffarts B, Lekeux P (**2008**) The oxidant/antioxidant equilibrium in horses. Vet J 177: 178-191.

Kumar KJ, Yang HL, Tsai YC, Hung PC, Chang SH, Lo HW, Shen PC, Chen SC, Wang HM, Wang SY, Chou CW, Hseu YC (**2013**) Lucidone protects human skin keratinocytes against free radical-induced oxidative damage and inflammation through the up-regulation of HO-1/Nrf2 antioxidant genes and down-regulation of NF-κB signaling pathway Food Chem Toxicol 59: 55-66.

Lee IT, Yang CM (**2012**) Role of NADPH oxidase/ROS in pro-inflammatory mediators-induced airway and pulmonary diseases. Biochem Pharmacol 84: 581-90.

Lindner A, Hatzipanagiotou A (**1998**) Effect of age and of performance parameters on CK, LDH and AST activities in plasma of Standardbred horses during exercise. Pferdeheilkunde 14: 456-460.

Marlin DJ, Fenn K, Smith N, Deaton CD, Roberts CA, Harris PA, Dunster C, Kelly FJ (**2002**) Changes in circulatory antioxidant status in horses during prolonged exercise. J Nutr 132: 1622S-1627S.

Niedźwiedź A, Zawadzki M, Filipowski H, Nicpoń J (**2012**) Influence of 8-hour transportation on selected physiological parameters in horses. Bull Vet Inst Pulawy 56: 193-197.

Nemec Svete A, Ćebulj-Kadunc N, Frangeź R, Kruljc P (**2012**) Serum cortisol and haematological, biochemical and antioxidant enzyme variables in horse blood sampled in a slaughterhouse lairage, immediately before stunning and during exsanguination. Animal 6: 1300-1306.

Onmaz AC , van den Hoven R, Gunes V, Cinar M, Kucuk O (**2011**) Oxidative stress in horses after a 12-hours transport period. Revue Med Vet 162: 213-217.

Piccione G, Bazzano M, Giannetto C, Marafioti S, Fazio F (**2014**) Training-induced changes in clotting parameters of athletic horses. J Vet Sci 15: 45-49.

Soares JC, Zanella R, Bondan C, Alves LP, de Lima MR, da Motta AC, Zanella EL (**2011**) Biochemical and antioxidant changes in plasma, serum, and erythrocytes of horses before and after a jumping competition. J Equine Vet Sci 31: 357-360.

Stull CL, Rodiek AV (**2000**) Physiological responses of horses to 24 hours of transportation using a commercial van during summer conditions. J Anim Sci 78: 1458-1466.

Szponder T, Wessely-Szponder J (**2010**) Plasma level of Protein C, fibrinogen concentration and platelet number in dogs with Legg-Calve-Perthes disease and osteochondrosis. Preliminary studies. Bull Vet Inst Pulawy 54: 433-436.

Uchino Y, Kawakita T, Miyazawa M, Ishii T, Onouchi H, Yasuda K, Ogawa Y, Shimmura S, Ishii N, Tsubota K (**2012**) Oxidative stress induced inflammation initiates functional decline of tear production. PLoS One 7.

Wessely-Szponder J, Bełkot Z, Bobowiec R, Wójcik M, Kosior-Korzecka U (**2014**) Crosstalk between adiponectin and cytokines (IL-6 and IL-8) during transportation stress in horses Med Weter 70: 546-549.

Wernicki A, Urban-Chmiel R, Kankofer M , Mikucki P, Puchalski A, Tokarzewski S (**2006**) Evaluation of plasma cortisol and TBARS levels in calves after short-term transportation. Revue Med Vet 157: 30-34.

Wójcik M, Kosior-Korzecka U, Bobowiec R (**2010**) Cytoprotective action of 17β-oestradiol against iron-induced hepatic oxidative stress in vitro. Bull Vet Inst Pulawy 54: 259-263.