Shiraz University

IJVR

ISSN: 1728-1997 (Print)
ISSN: 2252-0589 (Online)

Vol. 21 No. 3 Ser. No. 72 2020

IRANIAN JOURNAL OF VETERINARY RESEARCH

Original Article

Evaluation of cardiac troponin I, atrial natriuretic peptide and some oxidative/antioxidative biomarkers in the serum and hemolysate of trained Arabian horses after exercise

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(Received 23 Mar 2020; revised version 30 May 2020; accepted 10 Jun 2020)

Abstract

Background: Limited information existed on performance tests in Iranian Arab horses. **Aims:** The objective of this study is to investigate time related changes of cardiac troponin I (cTnI), atrial natriuretic peptide (ANP) and oxidative/antioxidant biomarkers in the serum of Arabian horses before and after regular training. **Methods:** Blood samples were collected from jugular vein of 25 Arabian horses before exercise; 5 h and 18 h after exercise and used to measure the cTnI, ANP, malondialdehyde (MDA), ferric reducing antioxidant power (FRAP), glutathione peroxidase (GPX), and superoxide dismutase (SOD) concentrations. Data analysis was performed using SAS. **Results:** Significant time related changes were seen for cTnI, MDA, and GPX concentrations ($P<0.05$). There were no time significant variations in the concentrations of ANP, FRAP and SOD. The values of cTnI and MDA significantly increased after exercise. The amounts of GPX significantly increased 5 h after exercise and then decreased up to 18 h after exercise. **Conclusion:** The results of the present study can be used in future studies in evaluating the health status of Arabian horses. In addition, the present results can be used as primarily described data in the evaluation of Arabian horses.

Key words: Arabian horse, Blood sample, Cardiac biomarkers, Training schedule

Introduction

Cardiac abnormality is an important reason for the weak performance of race horses. Echocardiography is well-established as an examination in resting horses, but its application is limited in assessing the myocardial function during exercise and it can only be done after exercise while heart rate (HR) is high. The availability of equipment and expert user is another important issue in dealing with echocardiography. At present, cardiac troponin I (cTnI) is a cardiac biomarker which has been effectively employed in equine veterinary practice for assessment of myocardial damage (Van Der Vekens *et al*., 2012). In previous reports, comprehensive investigation was done in cTnI concentrations in clinically normal horses and horses with experimentally induced cardiac disease (Kraus *et al*., 2010). Further investigation in horses has revealed that the peak of a mild rise in plasma cTnI concentration was observed between 3 and 6 h after exercise (Durando *et al*., 2006).

Another cardiovascular biomarker is cardiac natriuretic peptides, which are a group of hormones composed of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) (Ruskoaho, 2003). During the exercise as well as in horses with heart diseases, ANP is stored in the equine atrial myocytes and is released during stretching of atrial tissue (Trachsel *et al*., 2011). Other studies in healthy horses after exercise have revealed an increase in ANP concentration due to physiological response to increased HR, increased atrial pressure, and/or changes in blood volume (Trachsel *et al*., 2013).

In horses, the occurrence of oxidative stress, and subsequent production of reactive oxygen substances (ROS) due to exercise has been well described (Smarsh and Williams, 2017). Although regular moderate training causes oxidative stress (OS) reduction, acute and strenuous aerobic and anaerobic exercise can overproduce ROS. It seems that chronic exposure to regular training exercise improves the antioxidant defense systems of the body (Kirschvink *et al*., 2006; Fazio *et al*., 2016). During intense exercise, erythrocytes seem to be more vulnerable to oxidative damage, because they are permanently faced with high oxygen fluxes, high concentrations of polyunsaturated fatty acids (PUFAs) and heme iron (Petibois and Déléris, 2005). When horses exercise, intravascular hemolysis is one of the most highlighted processes for the demolition of erythrocytes (Andriichuk and Tkachenko, 2017). Nevertheless, the erythrocytes contain enzymatic and non-enzymatic antioxidants defense against free radical-induced lipid peroxidation. New studies have suggested that by endurance training, erythrocyte susceptibility to oxidative stress decreases and the erythrocyte antioxidant

defense against oxidative stress increases. It should be noted that recent studies of equine exercise physiology have primarily been dedicated to identifying the advantages of biochemical parameters and to assess physiological capacity and adaptation to alleviating loads (Lamprecht and Williams, 2012; Andriichuk *et al*., 2016).

The objective of this study is to measure cardiovascular biomarkers, oxidative stress amounts and oxidant/antioxidant capacity in regularly trained Arabian horses before and after exercise in order to first recognize any changes in the amounts of measured variables during exercise; and second to specify the effects of sex and age groups of horses, and finally to determine the magnitude of changes in measured variables and to describe maximum concentrations compared to previous data. It is assumed that there is a correlation between the change in serum biochemical parameters and exercise that will help trainers to assess the health status of their horses as well as help them to set better training schedules.

Materials and Methods

Horses and training

In the present research study, a total of 25 Arabian (8 mares and 17 stallions) horses were selected from the same training center located in Yazd, Iran. The mean and median ages of the horses were 6.8 and 5 years, respectively. The mean, median, minimum and maximum ages for mares were 6.1, 4, 2 and 12 and for stallions were 7.1, 6.5, 2, and 20, respectively. The healthiness of the horses was previously confirmed by physical, hematological and biochemical examinations, cardiac auscultation for cardiac murmurs as well as electromyocardiography (ECG) performed by a local vet. Also, all horses were dewormed and vaccinated against common pathogens according to a similar program. The mares were not pregnant during the study. All the animals were housed in the same stable in individual boxes under natural summer photoperiod (sunrise at 05:00 h, sunset at 19:00 h) with 22-25°C indoor temperature. Diet consisted of dry alfalfa hay (about 6 kg/day), source of energy (mixture of the barley, corn, wheat, and molasses) and about 5 kg/day concentrates (dry matter (DM), 92%; crude protein, 13%; Zn, 0.4%; Cu, 0.15%; Fe, 0.35%; Mn, 0.42%; Se, 0.0015%; Ca, 1.22%; lysine, 0.88% and methionine, 1.35%). Salt and water were available *ad libitum*.

All horses underwent training 6 days per week with one day of resting on Saturdays. Training started at 6:00 a.m. every day and continued for 4 h. Almost all of the horses participated in a standard daily training protocol which included warming up (20 min), walking (1 h), trotting and galloping (10 min). According to the performance, individual abilities and the age of each horse, the trainer may have slightly changed the standard procedure for some horses. It should be mentioned that the intensity and distance of training was approximately similar in all of the horses.

Blood samples

All of the procedures and the experiments were done according to the ethical standards in the Helsinki Declaration of 1975, as revised in 2000 and 2008, which is approved by the national law as well (ethical permission No.: 41419).

Blood was drawn from the jugular vein of each horse 3 times: immediately before exercise, 5 h and 18 h after exercise. Times of the sampling were selected based on previous reports to reach higher cTnI values and also based on daily exercise of the horses. Since some of the measured parameters were unstable, sampling was done within three days and all the measurements were obtained in less than 1 month.

For serum preparation, the blood was taken into tubes without anticoagulant and centrifuged at 1800 g for 15 min. Then, the serum was removed and stored at -80°C. No hemolysis was seen in serum samples. A portion of blood was also taken into the tubes with potassium ethylene diamine tetra acetic acid (K2-EDTA) to be used for hemolysate preparation. After primary centrifugation (1800 g for 15 min), plasma was removed and the remaining packed erythrocyte was washed three times with normal saline. Eventually four volumes of cold distilled water were added to one part of the washed packed erythrocyte; after being shaken and after final centrifugation, the supernatant hemolysate was aliquoted and stored at -80°C. The serum was used to measure cTnI, ANP, malondialdehyde (MDA) and ferric reducing antioxidant power (FRAP) concentrations. Hemolysate samples were used for the measurement of glutathione peroxidase (GPX), and superoxide dismutase (SOD) amounts.

Cardiac troponin I and ANP were measured using company validated horse specific enzyme linked immunosorbent assay (ELISA) kits (Shanghai Crystal Day Biotech Co., Ltd., Shanghai, China, with an analytical sensitivity of 2.46 ng/l and 0.43 ng/l, respectively) by ELISA Reader (Biotek, Elx 50, USA). The performance of kits was evaluated by the manufacturer and reported as 10% and 12% for Intra-Assay and Inter-Assay coefficient of the variations respectively for both cTnI and ANP. Also, the lower limits for cTnI and ANP were 0.005 ng/ml and 1 pg/ml, respectively. Glutathione peroxidase and SOD amounts were measured using Randox commercial kits (Ransel and Ransod, Randox Laboratories Ltd., Ardmore, UK) by Auto Analyzer (Mindry, BS-200E, Shenzhen, China).

Thiobarbituric acid reactive substances (TBARS) assay

The level of lipid peroxidation was determined by TBARS assay. To sum up, 100 µL of serum was added to 250 µL of Trichloroacetic acid (TCA, 10%w/v) in glass tubes. The tubes were boiled in a water bath for 15 min and then centrifuged at 1800 g for 10 min. Next, the supernatant was taken and mixed with 25 µL of

Thiobarbituric acid (TBA) solution (0.67% w/v). Finally, the tubes were boiled again in water bath for 15 min and the absorbance of the cooled sample was read at 490 nm. The blank is composed of reagents and distilled water.

FRAP assay

The antioxidant capacity of serum samples was determined by the colorimetric method (Benzie and Strain, 1996). Based on this method, Fe^{3+}-tripyridyltriazine (Fe (III)-TPTZ) complex was converted to Fe^{2+}-tripyridyltriazine (Fe (II)-TPTZ) complex by non-enzymatic antioxidants that existed in the serum sample. As a result, the color of the mixture changed to blue which can be detected at the wavelength of 593 nm.

Statistical analysis

Data analysis was performed using SAS (version 9.2, SAS Institute, Cary, NC). Because serum metabolites were measured over time, a repeated measure ANOVA (PROC MIXED) was used. All outcome variables were screened for normality by visual assessment of the distributions and calculation of kurtosis and skewness. The distributions of ANP, SOD, and FRAP were normal. Serum concentrations of cTnI, GPX, and MDA were skewed to the right. Therefore, a logarithmic transformation of variables was considered in the model. Variables considered in the model include time of sampling (immediately before exercise, 5 h after exercise and 18 h after exercise), gender (male and female) and age (younger than 5 years as age group 1 and older than 5 years as age group 2). All variables were offered to the model and then removed in a backward stepwise elimination approach. Interactions between intervention and the significant covariates were tested and included in the general model if significant. The horses were considered as the random effect and error term. The mathematical model of the statistical analysis is defined by the following equation:

$$Y_{ijkm} = \mu + T_i + A_j + G_k + TA_{ij} + TG_{ik} + H_m + e_{ijkm}$$

Where,

i: The time of sampling
j: Age independent variable
k: Sex independent variable
m: Individual horses
Y_{ijkm}: The output variable depending on μ (overall mean)
T_i: Time of sampling effect: i = 1 for immediately before exercise, i = 2 for 5 h after exercise, and i = 3 for 18 h after exercise
A_j: Age effect: j = 1 for horses younger than 5 years, and j = 2 for horses older than 5 years
G_k: Gender effect: k = 1 for male, and k = 2 for female
TA_{ij}: Interaction between time and age
TG_{ik}: Interaction between time and gender
H_m: Random horse effect
e_{ijkm}: Random error term

All values were reported as least squares means (LSM) and standard error (SE). In all analysis the value of $P \leq 0.05$ was considered as significant.

Results

The variation of cTnI, ANP, GPX, SOD, MDA, and FRAP amounts with respect to the time of sampling are presented in Table 1.

The results reported in Table 1 clearly showed that cTnI concentrations significantly increased in post-exercise samples. There were significant differences between pre-exercise with 5 h and 18 h post-exercise values (P=0.0003), while no significant difference was observed between 5 h and 18 h post-exercise samples. A significant effect of gender on cTnI concentrations was also observed; cTnI values in mares (0.183 ± 0.014 ng/ml) were significantly higher than in stallions (0.137 ± 0.008 ng/ml) since P-value was less than 0.05. Also, no significant age effect and interaction were observed on cTnI values.

Atrial natriuretic peptide results (Table 1) suggested no significant increase during the sampling time (P=0.0586), although there were significant differences between pre-exercise and 18 h post-exercise as well as between 5 h and 18 h post-exercise values.

The results presented in Table 1 suggest that GPX values significantly increased from before exercise to 5 h after exercise (P=0.0001) and then insignificantly decreased from 5 h to 18 h after exercise.

The time-related changes of SOD amounts (Table 1) were not significant (P=0.47).

The time-related changes of MDA amounts presented in Table 1, show that MDA concentrations increased significantly from pre-exercise to 5 h and 18 h after exercise (P=0.049).

The changes in the concentration of FRAP during the study (Table 1) show that the amounts of FRAP increased up to 5 h post-exercise and then decreased to the base line value after 18 h post-exercise without any significant changes (P=0.168).

The effects of gender and age were also evaluated in SOD, GPX, MDA, FRAP, and ANP and no significant effects were observed.

Discussion

The results of the present study suggested that

Table 1: The concentrations of measured variables (LSM±SE) in serum and hemolysat of Arabian horses before and after exercise

Parameter	Unit	Before exercise	5 h after exercise	18 h after exercise	P-value
cTnI	ng/ml	0.139 ± 0.008^{a}	0.156 ± 0.008^{b}	0.161 ± 0.008^{b}	0.0003
ANP	pg/ml	37.15 ± 1.99^{a}	37.57 ± 2.01^{a}	40.87 ± 2.19^{b}	0.0586
MDA	nmol/ml	1.02 ± 0.028^{a}	1.05 ± 0.029^{ab}	1.07 ± 0.029^{b}	0.0499
FRAP	µmol/L	590.6 ± 18.1	616.3 ± 18.1	592.5 ± 18.1	0.1681
GPX	U/g Hb	50 ± 1.1^{a}	59 ± 1.4^{b}	56 ± 1.3^{b}	0.0001
SOD	U/g Hb	1757.0 ± 134.2	1907.1 ± 134.2	1974.9 ± 134.2	0.4701

cTnI: Cardiac troponin I, ANP: Atrial natriuretic peptide, MDA: Malondialdehyde, FRAP: Ferric reducing antioxidant power, GPX: Glutathione peroxidase, and SOD: Superoxide dismutase. In each row, values with different superscript ($^{a, b}$) were significantly different ($P \leq 0.05$)

exercise triggered some degree of myocardial stress in Arabian horses. We also showed that in some horses, the concentrations of cTnI increased from 5 to 18 h after exercise. The results of a similar study (Nostell and Häggström, 2008) showed that horses generally have low cTnI values at rest, but intense short-term exercise induces mild increase in cTnI concentrations up to 10-14 h after exercise. The authors stated that the reason behind this increase is not clear. It cannot be concluded from the results of the current study whether these elevated cTnI concentrations are normal or due to myocardial disease. However, since all horses in this study had an acceptable performance and no previous diseases were reported based on their history and performed physical and laboratory examinations, it is improbable that they have cardiac disease. It has been reported that cTnI levels of clinically healthy horses are less than 0.2 ng/ml. Also, horses with cTnI values ranging between 0.2 and 0.3 ng/ml are in the gray zone, and those with cTnI greater than 0.3 ng/ml are considered to be abnormal (Walton, 2014). Twenty-four out of 25 horses used in the current study had cTnI values less than 0.2 ng/ml, which is different from cTnI values in horses with clinical sign of cardiac disease. In conclusion, the main reason of difference between long-term cTnI concentrations in the current research and other mentioned studies is not clear. Therefore, further research with more sampling and less sampling time intervals is suggested.

In the work done by Durando *et al*. (2006), cTnI concentrations of 4 horses were higher than normal values at 1, 3, 6, and 9 h. Among them, cTnI values of three horses remained higher than the normal values even 12 and 24 h after exercise. Normal physiologic phenomenon due to exercise is their justification. They have mentioned that it is less likely to be due to cardiac disease and myocardial damage.

In the research carried out by Trachsel *et al*. (2013), cTnI concentrations measured at rest and after a short-time exercise were lower than the detection limit of the assay in most of the samples; only a few samples reached higher values in post exercise.

Overall, the general inclination is toward mild increase of cTn due to prolonged endurance exercise, but the prevalence of cTn serum values vary considerably for unknown reasons. Probable descriptions for these variations include the difference in fitness levels of horses, the type and duration of exercise, the timing of the post-exercise sample, nutritional conditions, and the troponin assay adopted for the measurement and the detection limit of the assays (Lippi *et al*., 2008; Shave *et al*., 2012).

It has been reported that gender affects the antioxidant capacity change adaptation, and mares and stallions react differently to the exercise induced ROS production (Ginsburg *et al*., 2001; Ilhan *et al*., 2004). In a study by Andriichuk *et al*. (2014), a reduction in lipid peroxidation followed by a significant increase in glutathione reductase (GR) in stallions and an increase in SOD activity in mares were observed. All other differences in their study were insignificant. In this study, a significant effect of gender has been observed, i.e. the increase of cTnI values in mares is more than that of stallions. This could be due to an increase in O_2 consumption of cardiac muscles and insufficient antioxidant defense. It should be noted that SOD values insignificantly increased after exercise and also oxidative stress levels remained high after exercise.

In the current study, non-significant elevation of ANP, 5 h and 18 h after exercise was observed. The primary elevation is considered as a physiological response to the increase of HR, increase of atrial pressure, and/or change in blood volume. In addition, other factors can contribute to the response of ANP release, such as expression of ANP by remodeled left ventricular myocytes, activation of the renin-angiotensin system and sympathetic stimulation (Ruskoaho, 2003). However, the reason for long-term increase of ANP remains unknown. Some researchers have shown that a short-term mild exercise in horses induces a 1.5-3 fold increase in plasma atrial peptides caused by neuroendocrine reaction and acceleration of HR.

Based on the results of the study proposed by Trachsel *et al*. (2011), an increase in the amounts of ANP is observed in horses with cardiac disease, which suggests that ANP is directly correlated with the degree of left atrial enlargement.

In the current study, the significant increase in MDA and GPX values 5 h after exercise was clearly observed. From 5 h to 18 h after exercise, the values of GPX decreased, while MDA values remained high. The variations of FRAP and SOD were observed to be the same as GPX and MDA, respectively, but their changes were not significant.

The variation of GPX and MDA 5 h after exercise can be justified by insufficient antioxidant elevation. In this situation, horses undergo higher oxidative damage (Gondim *et al*., 2009). An increase in oxygen consumption throughout exercise activates the enzyme GPX to eliminate hydrogen peroxide and organic hydro peroxides from the cell. In the other hand, the tendency for GPX activity to decrease from 5 h to 18 h, may denote the effect of competition-inducing oxidative stress on horses. Furthermore, as it has been previously indicated, the observed increase of plasma lipid peroxides levels in sport horses after competition is an indicator of competition-induced lipid peroxidation (Muñoz-Escassi *et al*., 2006).

Lipid peroxides and MDA level are highly dependent on the antioxidants in the diet, because scavenging mechanism by antioxidants in the body will define the level of oxidative damage (Soares *et al*., 2007). Unfortunately, there is no provided information regarding dietary supplementation with antioxidants in the present study.

In the study done by Muñoz-Escassi *et al*. (2006), the increase of lipid peroxides and the decrease of GPX after exercise have been observed. The GPX reduction in some studies indicates a decrease in enzymatic antioxidant activity in RBCs.

The activation of antioxidant enzymes depends on the

specific tissues influenced by oxidative stress as well as the inherent ability of antioxidant defense (Andriichuk *et al*., 2016).

The results of the current research on 25 Arabian horses indicated that the amount of cTnI significantly increased after exercise. The cTnI and ANP values remained higher than their initial values from 5 h to 18 h after exercise. It cannot be inferred from the results that these long-term increases are normal or due to myocardial disease. Mild increase of cTnI cannot solely lead to an accurate diagnosis, and this mild increase along with clinical examinations and findings on electromyocardiogram and echocardiography are considered important in detecting cardiac disease in horses with poor performance. Although all horses in this study were performing well, further studies are required to publish reference ranges of cTnI and ANP in Arabian horses after exercise to evaluate their amounts in horses with weak performance. Also, more sampling after training is recommended for further analyses.

The presented data can be used to evaluate the response of cTnI, ANP, and some oxidative/antioxidative biomarkers due to exercise for future health and performance studies in Arabian horses.

Conflict of interest

The authors confirm that they have no conflict of interest to declare.

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