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Original Contribution

CHANGES IN BLOOD ANTIOXIDANT STATUS OF HANOVERIAN HORSES DURING FOUR YEAR SEASONS

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ABSTRACT

The aim of the present study was to establish the influence of climatic changes on antioxidant status in the blood of Hanoverian horses during the four seasons of the year. The oxidant/antioxidant equilibrium of 20 healthy horses was assessed by blood antioxidant marker analyses, i.e. determination of malondialdehyde (MDA) blood concentrations, erythrocyte activity of the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT). The highest MDA values (3.106 ± 0.165 μM), combined with low SOD activity (1876.69 ± 146.5 U/gHb) and a compensatory increase of the CAT activity (34508.94 ± 1511.23 U/gHb) were detected in the spring. The results showed strong oxidative stress during the spring, resulting from the continuous influence of the low temperatures and humidity in the winter. Oxidative stress tended to increased in autumn (MDA 2.032 ± 0.132 mM, SOD 4095.414 ± 196.17 U/gHb and CAT 27410.75 ± 3225.06 U/gHb) although at an extent lower than the winter values. The beginning of summer could be accepted as the most appropriate time for physical training of horses because of the lowest MDA values (1.530 ± 0.047 mM) measured.

Key words: Hanoverian horses, seasons, oxidative stress, ecological oxidative balance, malondialdehyde, catalase, superoxide dismutase

INTRODUCTION

Reactive oxygen species (ROS), including superoxide radical ($O_2^{\bullet-}$), hydroxyl radical (HO•), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), etc., are produced in aerobes by oxidation-reduction biochemical reactions as part of the normal oxygen cellular metabolism (1-3). A delicate equilibrium between ROS production and their elimination by endogenous antioxidant defense systems exists under normal physiological conditions. ROS, at low concentrations, are essential for the normal course of physiological processes such as cell differentiation and proliferation, apoptosis, cell-mediated immunity, cellular defense against microbial pathogens, melanogenesis and ageing (4, 5). On the contrary, excessive ROS or their inadequate removal, by cellular defense mechanisms, when the rate of their production is higher than the rate of detoxification by cellular defense mechanisms, induce oxidative stress that is manifested by impaired function of pro- and antioxidant systems in the affected cell or organism (6, 7). Abnormal ROS quantities are disposed and/or eliminated by the endogenous antioxidant defense that consists of enzymatic antioxidant defense – superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (Gpx), and non-enzymatic defense: vitamin C, vitamin A, α-tocopherol (vitamin E), glutathione, β-carotene, etc. The balance between the activities and the intracellular levels of these antioxidant enzymes is vital for the normal systemic life functions (8-10).

Literature lacks sufficient scientific information about the environmental effect on oxidative stress induction in horses. The role of oxidative stress in this animal species has been studied in various aspects health status, reproduction, temperature, humidity, physical exercise, etc. (7, 11-14). Some of the main

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acknowledged sources of oxidative stress are bacterial and viral infections, mycotoxins, environmental pollutants, ultraviolet radiation, psychological stress, physical training, antioxidant-depleted feeds, etc. (12-15). There is no data available concerning the alterations in the antioxidant status in blood of Hanoverian horses during the four seasons (winter, spring, summer and autumn). Our previous studies observed impaired blood antioxidant status in broiler chickens infected with *Eimeria acervulina* (16) and *Eimeria tenella* (17).

The aim of this study was to establish the effect of climatic changes on the antioxidant status in blood of healthy Hanoverian horses during the four seasons of the year. The specific objectives of the present investigation were: (i) to determine blood plasma concentrations of malondialdehyde – a marker of lipid peroxidation; (ii) to determine erythrocyte activity of the antioxidant enzymes SOD and CAT in winter, spring, autumn and summer.

MATERIALS AND METHODS

Experimental animals

The study was carried out in the Experimental Equine Base at Trakia University, Stara Zagora, Bulgaria, with 20 Hanoverian horses. Blood samples were obtained in the four seasons – winter, spring, summer and autumn.

Biochemical investigations

The biochemical investigation were conducted in the "Oxidative Stress Laboratory", Medical Faculty, Trakia University, Stara Zagora, Bulgaria.

Blood samples have been taken the jugular vein. Ethylenediaminetetraacetic acid (EDTA) was used as an anticoagulant

- *Peripheral blood processing*

Collected blood was centrifuged at 3000 g for 15 min and plasma was separated. Then, the plasma was deproteinated with 25% trichloroacetic acid by continuous mixing for 5 min and centrifuged at 2000 g for 15 min.

- *Erythrocyte processing*

The erythrocyte pellet was washed thrice with saline, and the cell suspension was diluted with cold water to lyse the erythrocytes. To 0.2 mL lysate, 1.8 mL water and ethanol/chloroform (3:5⅟v:v) were then added to precipitate haemoglobin. The tubes were shaken vigorously for 5 min and centrifuged at 2500 g for 20 min. The supernatants were used for determine enzyme activity.

- *Determination of the end products of lipid peroxidation*

The deproteinized plasma was used for determining lipid peroxidation products spectrophotometrically using the thiobarbituric acid reactive substance (TBARS) method, and measurement of MDA at 532 nm (18). Results were expressed in μM.

- *Determination of superoxide dismutase activity*

Erythrocyte lysates were assayed for CuZn-SOD activity as described by Sun et al. (19) with minor modifications. Briefly, the xanthine / xanthine oxidase system was used to generate the superoxide anion $(O_2^{\bullet -})_x$. This anion reduced nitroblue tetrazolium (NBT) to formazan, which was monitored at 560 nm. SOD in the sample removes the $(O_2^{\bullet -})_x$ and inhibits the reduction. The level of this reduction is used as a measure of SOD activity. One unit of enzymatic activity is defined as the amount of enzyme causing 50% inhibition of the reduction of NBT to formazan. Results were expressed as units per gram haemoglobin (U/gHb).

- *Determination of catalase activity*

Catalase activity was assessed in the erythrocyte lysates by the method described by Beers and Sizer (20). Hydrogen peroxide was used as a substrate and the decrease in H_2O_2 concentration at 22 °C in phosphate buffer (pH = 7) was followed spectroscopically at 240 nm. One unit of CAT activity is defined as the amount of enzyme that degrades 1μM H_2O_2 per min. Results are presented as units per gram haemoglobin (U/gHb).

- *Haemoglobin concentrations*

Haemoglobin concentrations of lysates were analysed by the cyanmethaemoglobin method (21).

Statistical analysis

The data were statistically processed by two-way analysis of variance (ANOVA). All results are presented as mean ± SEM. The differences were considered as significant when P values were less than 0.05.

RESULTS

The data for blood MDA concentrations and the activities of the antioxidant enzymes SOD and CAT in erythrocyte lysate during the four seasons are presented on **Figures 1, 2 and 3.** The results found considerable changes in the MDA levels during the four seasons of the year **(Fig. 1)**.

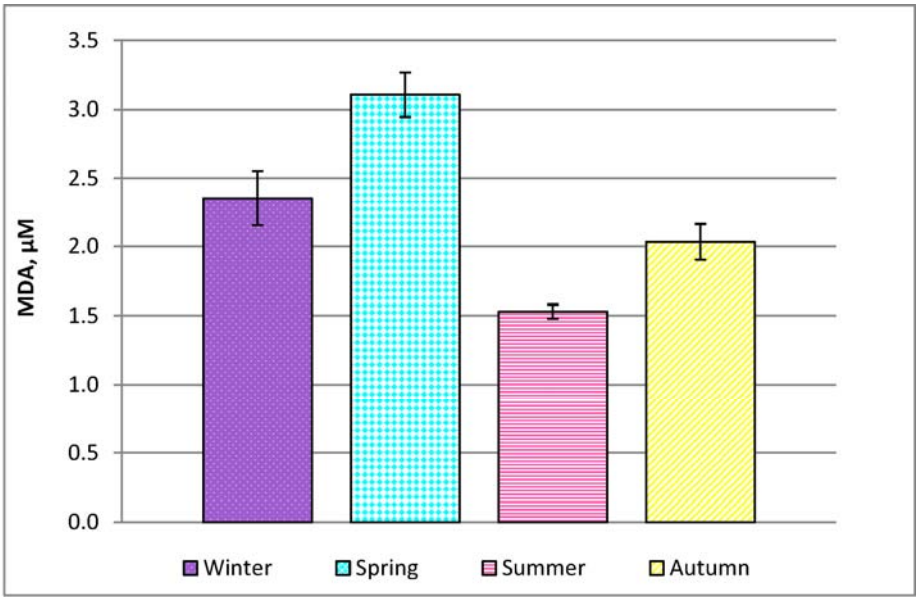

Fig. 1. Plasma MDA concentrations in Hanoverian horses during four seasons of the year.

The highest MDA concentrations were measured during the early spring – 3.106 ± 0.165 µM. These values were statistically significantly higher than the winter values (2.354 ± 0.197 µM), the summer concentrations (1.530 ± 0.047 µM) and those in the autumn (2.032 ± 0.132 µM), ($P < 0.0001$, Figure 1). At the same time, the blood MDA concentrations measured in Autumn were significantly higher than the Summer values ($P < 0.001$, **Fig. 1**).

The present study revealed that SOD activities measured in the Winter (24097 ± 568.583) were statistically significantly higher, compared to the Spring, Summer and Autumn values (1877 ± 146, 4561.26±233.70 U/gHb, and 4095.41 ± 196.17 U/gHb, respectively), ($P = 0.00001$, **Figure 2**). A significant difference between the spring and autumn SOD activities was not observed ($P > 0.05$, **Fig. 2**). The lowest erythrocyte SOD activities in Hanoverian horses were found in the Spring.

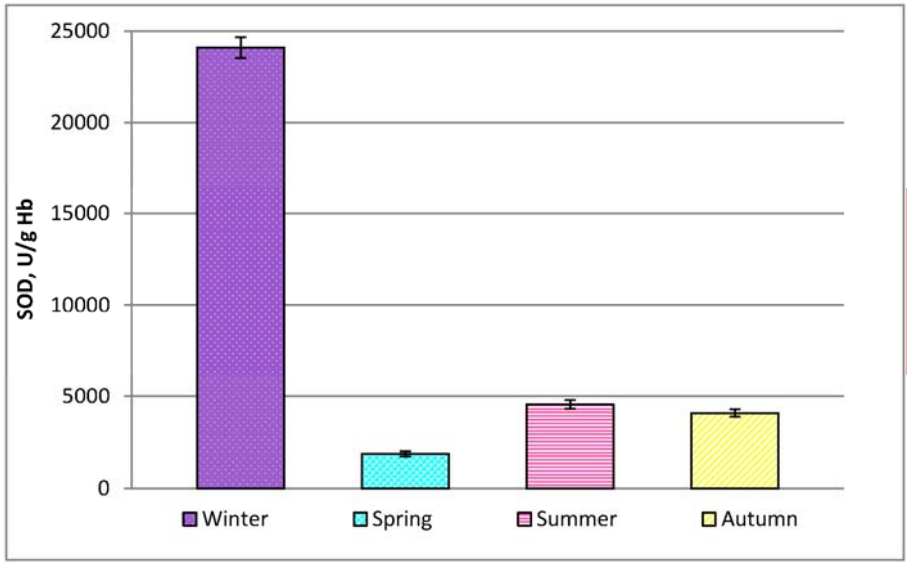

Fig. 2. Activity of Cu/Zn SOD in erythrocytes of Hanoverian horses during four seasons of the year.

The lowest CAT antioxidant enzymatic levels were measured in the Summer. Moreover, they turned out to be significantly different, in comparison to the values detected in the Autumn, Winter and Spring (27410 ± 3225 U/gHb, vs 29786 ± 1346 U/gHb and 34509 ± 1511 U/gHb, respectively; $P < 0.0001$, **Fig. 3**). There was also a considerable difference between spring and autumn CAT activities ($P < 0.05$, **Fig. 3**).

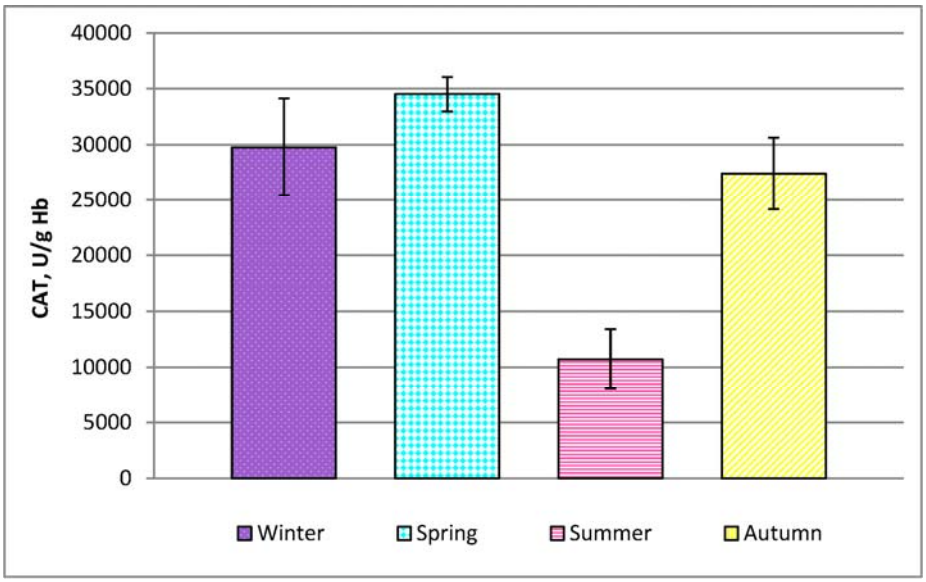

Fig. 3. Activity of CAT in erythrocytes of Hanoverian horses during four seasons of the year.

DISCUSSION

The current study presents for the first time information about seasonal variations in plasma malondialdehyde (MDA) concentrations – one of the end products of lipid peroxidation, and about the erythrocyte activity of the endogenous antioxidant enzymes: superoxide dismutase (SOD) and catalase (CAT), in Hanoverian horses.

Lipid peroxidation is a degradation process that affects structural components of the biological membranes and is among the best markers of the extent of ROS-induced biological damage (4). MDA, one of the end products of lipid peroxidation, is isolated in tissues, blood and urine, and utilized as a biomarker of radical-induced damage (22). Recent investigations proved an enhanced effect of low temperatures (t) and high humidity on oxidative stress induction. The latter dependence was observed during the cold and humid winter periods (ambient t = 1-2°C and air humidity 64%) and early spring (t = 1-8°C and air humidity 72%), when the highest blood MDA concentrations **(Fig. 1)** and the lowest SOD activities **(Fig. 2)** were achieved. The increased plasma MDA concentrations measured in clinically healthy Hanoverian horses by the end of the winter and the beginning of the spring **(Fig. 1)** were probably related to abnormal ROS production, consequently to the prolonged effect of low temperatures and humidity. ROS accumulation is known to induce lipid peroxidation and oxidative stress (23). On the other hand, such an increase in MDA levels could be provoked by the reduced antioxidant SOD activity during the spring **(Fig. 2)**. The present study revealed that SOD activities measured in the Winter were statistically significantly higher, compared to the Spring, Summer and Autumn values, most probably resulting from oxidative stress and impaired EOB due to the cold. Superoxide dismutase is the first enzyme of endogenous antioxidant defense system that neutralizes the formation of toxic radicals $(O_2^{\bullet -})_x$ and converts them to H_2O_2 (2,24). The reduced Cu/Zn-SOD activity in the erythrocytes of the studied Hanoverian horses by the beginning of the spring (March) could be a result of excessive ROS production that has to be detoxified by SOD. The observed compensatory elevation of the antioxidant CAT activity resulted from the low SOD activity, **(Fig. 3)** in response to H_2O_2 accumulation during the winter (January and February) and early spring (March). Although CAT is not considered important for cells under normal conditions, it plays a primary role in their adaptation to oxidative stress and protects cells by degradation of reactive H_2O_2 to water and molecular oxygen (25). The obtained results support the latter hypothesis exhibiting a compensatory increase in the erythrocyte CAT activities in the beginning of the spring - March **(Fig. 3)**, coinciding with the highest oxidative stress levels measured up. The organisms possess complex and precise antioxidant defense mechanisms that prevent ROS formation and/or control their toxic effect (26). The equilibrium between antioxidant enzyme activities and the rate of production of ROS is essential for the survival of living organisms and their health (4). The state of balance between ROS generation and the

detoxifying potential of the endogenous antioxidant systemic defense is called ecological oxidative balance (EOB) of biological systems (27). The biological systems of aerobic organisms are maximally protected against the toxic ROS in a state of EOB (27). The observed deviations in blood SOD and CAT activities could exert a remarkable effect on the cell resistance to ROS-induced cell genome damage and cell death (25,28). The increased MDA concentrations combined with the deviations in erythrocyte SOD (Fig. 2) and CAT **(Fig. 3)** activities, established in the spring, could be attributed to disturbed EOB in horses, manifested through an impaired balance between prooxidant and antioxidant systems following uncontrolled toxic ROS production, i.e. oxidative stress. Consequently, biological systems are not protected against the oxidative radical challenge that could result in toxic damage of the organism in a state of impaired EOB and oxidative stress (27).

According to the present study, both the marked reduction of blood MDA concentrations was observed by the beginning of the summer (June), most probably resulting from EOB normalization in horses due to the warm and dry weather (t = 25.4°C; air humidity 42%). Thus, biological systems in aerobic organisms are maximally protected against the toxic effects of ROS in a state of EOB (27).

Lipid peroxidation tended to increase in autumn, when ambient temperatures were low (4.8°C) and humidity – high (90%), possibly as a result of the prolonged effects of high summer temperatures. The cold and humid autumn conditions may exhibit a variable effect on both studied antioxidant enzymes SOD and CAT. Foster and Cunningham (29), Williams et al. (13), Williams et al. (14), Marlin et al. (30), etc., also reported a relationship between ambient temperature, humidity, and oxidative stress in horses but combined with physical exercise of variable intensity. Therefore, the data obtained in the present investigations could not be interpreted fully in reference to the above literature results.

CONCLUSION

The present study provided evidence of a marked oxidative stress by the beginning of spring in Hanoverian horses, which is supported by high blood MDA concentrations, low SOD activity and compensatory high CAT activities in the erythrocyte lysate. The comparative analyses of the results obtained proved that the beginning of summer could be the most appropriate time for physical training of horses, because of the lowest established blood levels of oxidative stress biomarker MDA.

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