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Original Scientific Paper

ACTIVITY OF SELENENZYMES GPx-1 AND GPx-3 IN THE BLOOD OF WORKING HORSES IN THE TERRITORY OF CENTRAL SERBIA

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Summary

Glutathione peroxidase (GPx) is an enzyme that has 8 isoenzyme forms, of which GPx-1 is an obligatory intracellular enzyme, while GPx-3 is active in extracellular fluids, especially in blood plasma. The main role of GPx is the protection of cells from oxidative stress induced by free oxygen radicals. GPx activity is taken as a reliable indicator of selenium status in the human and animal organism. Selenium is introduced in organism mainly through the food chain. Ingested selenium is incorporated in the form of selenomethionine and selenocysteine into tissue proteins, i.e. enzymes. Detailed investigation related to the content of selenium in feed and domestic animals were carried out in Serbia, especially in economically significant species such as poultry, pigs, sheep and cattle. However, working cold-blooded horses, especially those that are fed exclusively with locally grown feed, or are on pasture, have not been examined in detail so far. Due to their specific way of breeding, they are ideal indicators of the selenium status of monogastric herbivores in a given locality.

The goal of our study was to determine the status of selenium based on the activity of GPx-1 and GPx-3 in blood samples of non-supplemented working horses in the territory of central Serbia.

In our study, a total of 12 samples of horse blood taken from the localities of the municipalities of Kraljevo, Zaječar, Valjevo and Dimitrovgrad were tested as follows: 12 samples of blood plasma and 12 samples of washed erythrocytes. Measurement of GPx-3 and GPx-1 activity was carried out using the Guncler method at a wavelength of 366 nm. For each animal, during sampling, data on gender, age, vaccination and deworming, composition and origin of the given nutrients were recorded.

The average activity of GPx-1 was 502.02 ± 91.77 µKat/l, and GPx-3 3.46 ± 1.02 µKat/l, which indicates the existence of a marginal selenium deficit in the population of unsupplemented working horses in the territory of central Serbia.

Keywords: GPx, horses, selenium, Serbia.

INTRODUCTION

The metabolism of selenium is very complex because this trace element is essential for the organism, but also potentially very toxic. Domestic and wild animals ingest selenium through food. The content of selenium in nutrients of plant origin depends on numerous factors, among which stand out: the composition and characteristics of the soil, the chemical form of selenium in the soil, the amount of selenium in the soil, the type of plant and the growth phase of the plant itself. Several selenium deficient areas have been defined in the world, and one of them is part of the Balkan Peninsula (Valčić et al., 2013).

The main role of selenium is the protection against oxidative stress and the neutralization of the generated free oxygen radicals through the action of GPx. GPx consists of four identical subunits whose individual molecular masses range from 18,000 to 23,000 with a stoichiometric ratio of four gram-atoms of selenium per mole of enzyme. GPx is very specific towards the hydrogen donor (reduced glutathione) and non-specific towards the reducing substrate. In addition to the decomposition of hydrogen peroxide, the enzyme participates in the reduction of hydroperoxides of various organic compounds (fatty acids, nucleic acids, thymine, prostaglandins, etc.) into the corresponding alcohols.

Physiological effectors of selenium function are selenoproteins, or selenoenzymes - glutathione peroxidase (GPx-, EC 1.11.19), and in addition to glutathione peroxidase, selenium is present in iodothyronine deiodinase, which plays a role in the conversion of thyroxine (T_4) into biologically active 3,3',5 - triiodothyronine (T_3). Myodegeneration known as White muscle disease (WMD) is the most common disease of domestic animals, including horses, which occurs as a result of a pronounced selenium deficiency. Marginal deficiency in horses leads to infertility in mares and slower growth in foals (Savage and Lewis, 2002).

Areas of pronounced deficit of this microelement have been described in the world and in Europe. It is known that the selenium deficit is most pronounced in China, the North-West and South-East of the USA, and in Serbia in the area of the Sjenica-Pešter Plateau. Research conducted in the previous two decades in Serbia (Valčić et al., 2013) showed that the average content of selenium in plant foods in the territory of the Republic of Serbia is in the interval from marginally deficient to deficient, where the situation is most favorable in Vojvodina.

Selenium status in domestic animals can be assessed based on the direct determination of selenium in blood samples, or indirectly based on the activity of the enzyme GPx.

GPx-1 is an obligatory intracellular enzyme, while GPx-3 is present in the extracellular space, especially in the blood plasma. Numerous authors have established that the concentration of selenium in the blood and the activity of glutathione peroxidase in whole blood are highly correlated, ranging from r=0.70 to r=0.93, in all types of domestic animals fed with feed containing selenium in concentrations below toxic doses (Wolff et al., 2017; Pavlata et al., 2000; Maylin et al., 1980; Blackmore et al., 1982; Caple et al., 1978).

Assessment of selenium status of horses based on GPx activity in blood is considered a reliable and sensitive method. Selenium is incorporated into erythrocytes during erythropoiesis and GPx-1 activity is considered an indicator of long-term selenium intake, while plasmatic GPx-3 activity is primarily an indicator of short-term selenium status (Harris, 1998). Previous research by Maas et al. (1996) and Blackmore and Brobst (1981) state that physiological values of GPx activity in horse whole blood can be considered in the range of 300 to 600 µKat/l.

In the territory of Serbia, only sporadic tests of selenium status and GPx activity were performed in thoroughbred sports horses that are intensively bred (Mihailović et al., 1996). However, to date, no tests have been conducted on the selenium status of non-supplemented working horses.

This work represents the first preliminary examination of GPx-1 and GPx-3 activity in order to determine the selenium status of working, non-supplemented, cold-blooded horses in the territory of central Serbia.

MATERIALS AND METHODS

In the study, a total of 12 horse blood samples from central Serbia (Kraljevo, Zaječar, Valjevo and Dimitrovgrad) were tested, as follows: 12 blood plasma samples and 12 washed erythrocyte samples. A clinical examination was obtained before the blood sampling, so that only animals that were healthy and that had not been under therapy in the previous months were included in the study. At the same time, only those animals that did not receive vitamin-mineral supplements orally or parenterally in the past 12 months were selected, and were fed exclusively with locally grown feed or grazed on local pastures.

Blood plasma was separated by centrifugation of heparinized blood samples at 1000 x g for 20 minutes. Samples of washed erythrocytes were obtained from whole blood samples after triple washing of erythrocytes with physiological

solution and triple centrifugation. The samples were transported to the laboratory in a cold chain, and immediately stored in a freezer at -18°C.

In order to take blood samples, horses were subjected to venipuncture of the jugular vein with the owner's consent and in compliance with all measures of good veterinary practice.

The activity of cytosolic glutathione peroxidase (GPx1) and blood plasma glutathione peroxidase (GPx3) was determined by the method of Günzler et al. (1984) on a Cecil 2000 spectrophotometer, with a water bath and a thermostat that maintained a constant temperature of 37°C. The principle of this measurement is based on the spectrophotometric recording of NADPH consumption in the coupled enzyme system.

The composition and final concentrations of the reagents are shown in Table 1.

Table 1 Composition of reagents used for spectrophotometric determination of GPx activity

Reagents	**Volume (µl)**	**Final concentration**
Potassium phosphate buffer (400 mmol/L, pH 7)	500	100 mmol/L
GSH (604 mmol/L)	200	6 mmol/L
Glutathione reductase (GR)	50	0.375 IJ/mL
Blood plasma sample	20	
or		
Erythrocyte hemolysate sample*	10	
10 minutes preincubation on 37 ^{O}C		
NADPH 3 mmol/L in 0,1% $NaHCO_3$	200	0.3 mmol/L
TBH	550	1.575 mmol/L
Redistilled water	480 (for a plasma sample); 490 (for a erythrocyte sample)	-

* Hemolysate is prepared by adding 10 µl of washed erythrocytes to 200 µl of Drabkin's reagent.

MS Excel 2007 and GraphPadPrism5 programs were used for statistical data processing.

Results are presented using descriptive statistics parameters (Mean, SD and CV%).

RESULTS AND DISCUSSION

The activity of cytosolic glutathione peroxidase (GPx1) in the washed erythrocytes of the tested horses ranged from 288.48 to 632.12 µKat/l. Statistically, the examined group was homogeneous, given that the coefficient of variation was 21.7%. The average activity of GPx1 was Mean=502.02 µKat/l, and the standard deviation SD=108.96 (Table 2).

The activity of glutathione peroxidase (GPx3) in the blood plasma of the examined horses was in the interval from 2.42 to 5.35 µKat/l. Statistically, the examined group was homogeneous considering that the coefficient of variation was <30%. The average activity of GPx3 was Mean=3.46 µKat/l, and the standard deviation SD=1.02 (Table 2).

GPx3 activity is an indicator of the status of short-term selenium intake in the organism. In addition to selenoenzymes, more than 20 different selenoproteins are present in the plasma (Kryukov et al., 2003), which, according to their function can be antioxidants, regulators of redox reactions, participants in the metabolism of thyroid hormones, transport proteins, etc. In domestic animals, more than 98% of GPx activity is located in erythrocytes, which is consistent with our results.

Table 2 Activity of GPx-1 and GPx-3 in blood samples of working horses in central Serbia

	Mean	**SD**	**CV%**	**Iv**
GPx-1 (µKat/l) (n=12)	502.02	108.96	21.70	288.48-632.12
GPx- 3 (µKat/l) (n=12)	3.46	1.02	29.50	2.42-5.35

To date, precise reference values for GPx activity in animal blood have not been established. Namely, the literature indicates wide intervals of GPx activity in horse blood, as Maas et al. (1996) gave values in the interval from 80 to 500 µKat/l, while Blood and Radostits (1989) believe that the reference physiological values are significantly higher and amount to 500 to 2500 µKat/l. An additional problem when defining the recommended reference values of GPx activity is the lack of conformity of the units through which the activity of this selenoenzyme is determined, so that GPx activity is often expressed through IU (international units), µmol/ml/min, Ug/Hb, Ug/prot, etc., which further complicates the inter-laboratory comparison of the obtained values.

When interpreting the values obtained in our study, it should be considered that GPx-1 activity was determined in washed erythrocyte samples, not in whole blood samples as is often shown in the literature, and it is necessary to extrapolate the results to whole blood values based on blood hematocrit in a horse. This approach is fully justified if we understand that the participation of blood plasma in GPx

activity is extremely low (<1%) and that it has no significant effect on the overall activity of GPx in the blood. All the animals that were included in our study were clinically healthy, without signs of anemia, dehydration, endoparasitosis and other health problems that could indicate the possible existence of hematocrit disorders (hemorrhages, dehydration, etc.). Taking into account that the average physiological hematocrit of the blood of adult horses is 0.35 (Calamari et al., 2009), we can conclude, based on the set mathematical proportion, that GPx values in the whole blood of horses in central Serbia range from 101 to 221 µKat/l, which clearly indicates that working horses in the area of central Serbia are in the zone of marginal deficit of this microelement.

Similar results were recorded in the territory of the Czech Republic, where the GPx activity measured in whole blood samples of 159 adult horses was 286.43 µKat/l (Ludvikova et al., 2005). In the aforementioned study, the authors determined the high correlation coefficient (r= 0.84) between measured GPx activity and blood selenium concentration, which further confirms the validity of measuring GPx activity in order to determine the selenium status of horses. At the same time, the aforementioned group of authors examined the linearity of the relationship and the correlation between GPx activity and selenium concentration in order to determine the limit values of activity that indicate the status of selenium deficiency. They determined that GPx activity values exceeding 200 µKat/l can be considered adequate, as well as that they are achieved with selenium content in whole blood >75 µg/l. According to those authors, values in the interval from 100 to 200 µKat/l are marginal, and GPx activity values <100 µKat/l are inadequate, i.e. deficient.

It is interesting to note that all the horses that were included in our study were non-supplemented working horses that were regularly exposed to a certain degree of physical effort, either in work under saddle, or as recreational or harness horses. Numerous authors (Ott et al., 2022; Mami et al., 2019; Gondim et al., 2009) point out that physical effort is one of the triggers of oxidative stress, especially in horses that are exposed to intense effort during endurance competitions i.e. endurance races. One of the main advantages of our study is the fact that we examined the status of GPx in a category of horses that has not been studied so far in our climate, namely non-supplemented working horses that are fed exclusively with locally grown feed, which allows us to understand the real situation in the field. Certainly, the tests should be extended to other areas in order to provide more reliable data on the basis of which we will be able to make recommendations on possible selenium supplementation of working horses on the territory of the Republic of Serbia.

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