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Original scientific paper

THE EFFECTS OF DIFFERENT STORING TEMPERATURES ON THE ACTIVITY OF SHEEP BLOOD AND PLASMA GLUTATHIONE PEROXIDASE*

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Abstract: The aim of the study was to determine the stability of the activity of glutathione peroxidases 1 and 3 during storage at +4^{0}C and -18^{0}C. Blood samples were taken from eight sheep and the activity of the enzyme was determined in the plasma (GPx3) and erythrocytes (GPx1) on the first, third, fifth and seventh day in samples stored at +4^{0}C and after one and three months in samples stored at -18^{0}C. GPx3 activity decreased significantly during storage at both temperatures, while GPx1 remained steady even after three months of storage at -18^{0}C. Obtained results indicate that GPx3 activity has to be determined in fresh sheep plasma samples, while the activity of sheep erythrocyte GPx1 can be determined even after 3 months of storage at -18^{0}C.

Key words: activity, glutathione peroxidase (GPx), sheep, selenoproteins

INTRODUCTION

Selenium metabolism is very complex because this microelement is essential for the organism but at the same time potentially very toxic. Domestic and wild animals take selenium through food. The level of selenium in plant nutrients depends on several factors: the chemical form of selenium in the soil, the quantities in the soil, the plant species and the growth stage 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).

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Selenium is an essential microelement with multiple and very important roles in the body. Its physiological roles are directly related to the functions of the protein and it is incorporated in protein composition through atypical amino acid of selenocysteine.

26 selenoproteins have so far been identified in mammals and their physiological function is known only for some of them. The first protein that has been proven to have selenocysteine in its composition is glutathione peroxidase (GPx) (Rotruck et al., 1973; Flohe et al., 1973). So far, 5 isoenzymatic GPx formats have been identified that reduce hydrogen peroxide and organic hydroperoxes, protecting cells from oxidative damage. This group of selenoenzymes includes cytosolic glutathione peroxidase (GPx1), gastrointestinal glutathione peroxidase (GPx2), plasma (extracellular) glutathione peroxidase (GPx3), phospholipid glutathione peroxidase (GPx4) and new-found glutathione peroxidase 6 from olfactory epithelium and embryonic tissue.

There are strict mechanisms for regulation of selenium levels necessary for optimum expression of all selenoproteins. The selenoprotein synthesis is regulated by the level of selenium in the food, but it also depends on the tissue and the type of selenoprotein. It is not clear how the cells and tissues give priority to its incorporation into certain selenoproteins.

Glutathione peroxidase 1 (GPx1) is an important cytoplasmic enzyme that reduces hydrogen peroxide and peroxides of free fatty acids, diminishing the possibility of damage to the cell due to oxidative stress. It is very sensitive to changes in selenium status and the level of informational iRNA and the protein itself decreases in individuals with selen deficiency. Since glutathione peroxidase is seleno-enzyme and its activity to a certain extent linearly depends on the amount of selenium in the food, the activity of this enzyme can be a good indicator of the status of selenium in the body. In addition, GPx1 erythrocyte is a good parameter for assessing the status of selenium for a longer period of time because the enzyme is synthesized only during the erythropoiesis.

Glutathione peroxidase 3 (GPx3) is the only enzyme in the glutathione peroxidase family that secretes into the intercellular space. Hydrogen peroxide, hydroperoxides of fatty acids, phospholipid hydroperoxides are substrates for this enzyme are, and it plays a significant role in antioxidative processes in the blood plasma (Brigelius-Flohe, 1999). The main source of extracellular GPx3 are kidneys, and the epithelial cells of proximal tubules of nephron and parietal cells of the Bouman capsule (Yoshimura et al., 1991) are synthesized. As the activity of this enzyme decreases to 99% in the case of selenium deficiency, GPx3 is widely used in the evaluation of selenium status. Also, the activity of this enzyme is used to evaluate selenium status over a short period of time because the cells synthesizing this enzyme very quickly

stop the synthesis in the conditions of the deficit.

Therefore, GPx1 and GPx3 activities are excellent biomarkers for assessing the status of selenium. However, it is sometimes impossible to determine their activity in newly sampled blood samples, especially if individuals originate from different locality. Therefore we considered it necessary to establish an adequate way of storing samples until determining the activity.

MATERIAL AND METHODS

In order to investigate the activity of GPx from blood plasma (GPx3) and erythrocyte cytosol (GPx1), blood was taken by venipuncture of v. jugularis sheep. The blood was taken from eight sheep, both sexes, and they were kept under farm conditions. Heparin (12 IU / ml blood) was used as an anticoagulation agent. The blood was centrifuged for 15 minutes on 1000 x g to separate blood plasma. After separation of blood plasma, the erythrocytes were washed with a physiological solution and centrifuged for 3 times. Blood plasma samples and erythrocyte samples were stored at +4 0C for seven days and at -18 0C for three months. The activity of glutathione peroxidase (GPx1 and GPx3) was measured on the first, third, fifth and seventh days after storage at a temperature of +4 0C, and again after a month and three months storage at -18 0C.

Determination of glutathione peroxidase activity

The activity of cytosolic glutathione peroxidase (GPx1) and glutathione peroxidase of blood plasma (GPx3) was determined by the method according to Günzler et al. (1974) on the Cecil 2000 spectrophotometer, with a water bath and a thermostat that maintained a constant temperature of 37 oC. The principle of this measurement is based on the spectrophotometric registering of NADPH consumption in the coupled enzymatic system.

The composition as well as the final reagent concentrations 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	4 mmol/L
Glutathione reductase (GR)	50	6 mmol/L

Blood plasma; hemolyzed erythrocytes (разбл. 21x)	20; 10	0,375 IJ/mL
NADPH	200	0,3 mmol/L
TBH	550	1,575 mmol/L
Redistilled water	480; 490	

Statistical data processing

For statistical data processing, MS Excel 2007 and GraphPadPrism5 programs were used. The results are presented using the descriptive statistics parameters (Xsr, SD and KV). The statistical significance of the difference in the obtained values between individual groups was determined by Student's t-test, for levels of significance $p < 0.05$, $p < 0.01$, and $p < 0.001$.

RESULTS

By determination of GPx3 blood plasma activity of the sheep, on the third day after storage at a temperature of 4 oC, a statistically significant decline in activity was determined as shown in Table 2. The storage of blood plasma samples at -18 oC led to a statistically significant enzyme activity decline, and after 3 months no activity was recorded in the samples (Table 4).

Table 2. Activity of GPx3 from sheep plasma after 1,3,5 and 7 days of storage at +4 oC (µKat)

	Days			
	1	**3**	**5**	**7**
Xm±SD	**5,57**±0,52	**1,39**±0,38**	**0,89**±0,39**	**0,55**±0,27**
CV	9,38	27,57	43,36	49,23

** p<0,01 in relation to activity determined on Day 1

Xm- arithmetic mean

SD- standard deviation

CV- coefficient of variation

The activity of cytosolic GPx1 in erythrocytes during storage at +4 oC increased over time and at the beginning of the observed period it was 1370 µKat, and after seven days 2370 µKat (Table 3). For storage at -18 oC, during the observed period of three months, there was not statistically significant change in activity (Table 4).

Table 3. Activity of GPx1 from sheep erythrocytes after 1,3,5 and 7 days after storage at +4 oC temperature (µKat)

	1	3	5	7
Xm±SD	**1369**±367	**1771**±265**	**1863**±247**	**2369**±308**
CV	26,85	14,98	13,26	13,00

** p<0,01
Xm- arithmetic mean
SD- standard deviation
CV- coefficient of variation

Table 4. Activity of GPx3 blood plasma and GPx1 from sheep erythrocyte after 1 and 3 months of storage at -18 oC (µKat)

	GPx3		GPx1	
	1month	3 months	1 month	3 months
Xm±SD	**2,69**±1,14	/	**1388**±337	**1334**±141
CV	42,30	/	24,32	10,58

** p<0,01
Xm- arithmetic mean
SD- standard deviation
CV- coefficient of variation

DISCUSSION AND CONCLUSION

Determination of the activity of glutathione peroxidase enzyme is important for assessing the status of selenium in individuals, which is of particular importance to individuals who are extensively cultivated, and especially in areas that are poor in selenium, such as some areas of the Balkan Peninsula (Valčić and sar). Sampling of materials to investigate the activity of this enzyme often involves a period of several months, with the impossibility of determining the enzymatic activity immediately after sampling, or immediately after storaging the samples in the laboratory. Different temperatures can be used for storing biological material. The storage efficiency will depend on several factors, such as storage temperature, the type of biological material and the nature

of the substance to be tested. Reduced temperatures are also used for the storage of samples in which enzyme activity can be investigated after a certain time. The aim of this paper was to determine if there is a change in the activity of GPx3 and GPx1 during storage of blood plasma and erythrocytes of sheep at a temperature of + 4 ° C for a week, and after one and three months at a temperature of -18 ° C. The activity of GPx3 in sheep has dropped dramatically after 3 days. There are few results to be compared to our results. Studies were mainly done on human material. The activity of GPx3 from human plasma decreases only after 8 days (Ogunro et al., 2010). Several authors state that GPx1 activity declines after 7 days of storage (Deuhim et al., 2014; Marjani et al., 2007; Aslan et al., 1997; Jozwik et al., 1997). According to our results, the activity of glutathione peroxidase in sheep erythrocytes (GPx1) does not decline significantly during the first 7 days of storage, moreover, in our samples, the activity of GPx1 increased in accordance with the results of Harapin et al. (2008). As we didn't aliquot the erythrocytes immediately after rinsing, but we left them in the tubes from which we took a sample every two days, it is possible that this phenomenon is a consequence of the erythrocyte sedimentation during storage, and that in subsequent studies of the same samples there would be more erythrocytes in the same volume sample, and therefore more enzymes. Shepard and Millar (1981) had a decrease in activity in erythrocyte hemolytes during storage at + 4 ° C, while the erythrocyte activity increased, and our results in this respect are consistent with their results. Freezing to -18 ° C affected the activity of GPx3 and GPx1 differently. GPx1 activity was maintained after three months of storage while GPx3 activity after the same period was immeasurable. Harapin et al. (2008) recommend that the peroxidase activity should be determined in fresh samples, after two days at the latest or at a storage temperature of -18 ° C. Based on the performed studies, it can be concluded that the activity of GPx3 from sheep plasma significantly decreases during storage at +4 °C for 7 days and must be determined within the first 24 hours of blood extraction. The erythrocyte samples should be instantly aliquotted to a precisely determined volume that would be used for the enzyme activity after storage. In this way, the effect of sedimentation of erythrocytes will be avoided.

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