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Original article

The influence of selenium and vitamin E supplementation on cytological assessment of red blood cell line of bone marrow in fallow deer kept in captivity

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Abstract

Cytological evaluation of bone marrow smears stained by May-Grünwald Giemsa method was performed. The smears came from 20 fallow deer (*Dama dama*) 3 days old divided into 2 groups each consisting of 10 animals. The experimental group (E) received intramuscularly selenium and vitamin E at a dose of 3.0 ml (tocopherol acetate – 50 mg, sodium selenite – 0.5 mg, solvent - 1 ml) in the 3rd day of age. The control group (C) did not receive any supplementation or placebo. For hematological analyzes blood was collected three times: on 0, 15th and 25th day of the experiment. Serum concentration of selenium and vitamin E was determined using high performance liquid chromatography and glutathione peroxidase activity (GSH-Px) by kinetic method. On the 15th day after supplementation, a statistically significant increase in the percentage of erythroblastic cell line was observed in bone marrow smears. At that time, the increase in GSH-Px activity in the E group was also observed, reaching the value of 165.3 U/gHb, which was statistically significant. The percentage of proerythroblasts (8.23% in group E and 5.02% in group C) differed significantly between groups at the 25th day after supplementation. This study revealed that supplementation of selenium and vitamin E resulted in an increase in the number of erythrocytes to an average of 13.5 ($\times 10^{12}$/l) in the experimental group on 25th day with a significant increase in hemoglobin to 193 g/l in the experimental group.

Key words: bone marrow, hematopoiesis, erythroblast, selenium, vitamin E

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Introduction

Selenium is a trace element, which is quite poorly distributed in natural environment. Until recently, the role of selenium was mostly considered in perspective of its toxic properties, which manifest in: emaciation, hair loss, deformation of hoof and hoof diseases. Cardiac muscle atrophy, liver cirrhosis, microcytic anemia was also observed in deficient ruminants. As a further consequence, the animals with advanced stages of deficiency developed CNS disorders, problems with mobility, colic pain, dyspnea, optic nerve paralysis and death as a result of respiratory failure (Tinggi 2003). Single dose of Se in the range of 1-6 mg/kg of body weight induces lethal poisoning in most animal species (Whanger et al. 1996). Different chemical forms of selenium have different toxic potentials. In reports reviewed by Nuttal (2006) serum selenium concentrations span the following ranges: 400-30000 μg/L associated with acute toxicity, 500-1400 μg/L associated with chronic toxicity, and <1400 μg/L free of toxicity. In higher animals selenium is closely linked to vitamin E and sulfur-containing amino acids. Chemical compounds of this element have a great influence on processes of humoral immunity by increasing the level of IgM (Maggini et al. 2007). Adding selenium into feed increases the phagocytic activity of neutrophils and macrophages and the number of T lymphocytes (Hoffman 2007, Kamada et al. 2007). Selenium deficiency results in a decrease in the number of lymphocytes and NK cells, which contributes to the progression of inflammatory processes.

Inhibited neutrophil migration and reduced free radicals production, which are results of selenium deficiency, lower the ability to kill foreign and pathologically altered cells (Yang et al. 2004). Fluctuations in serum Se and GSH-Px levels are closely related to the occurrence of oxidative stress in humans and animals, leading to worsening of health. Especially in cattle and hens, there is a close relationship between the state of selenium deficiency in relation to the development and degree of hematopoiesis pathologies. This demonstrates the indisputably significant role of selenoproteins in the regulation of hematopoietic processes. High plasma level of GSH-Px and high number of platelets and erythrocytes stimulate the processes of erythropoiesis (Chow and Tappel 1974, Sakata et al. 1982). It is worth emphasizing that selenium and vitamin E deficiencies are highly conducive to the lysis of erythrocyte membranes and the formation of methemoglobin, which can be attributed to the increase of intracellular forms of oxygen (Nemoto and Finkel 2002). Studies in mice demonstrated the evidence of an unquestionable effect of selenium on the activity of erythropoiesis. Significant disturbances of hematopoietic processes due to deficiency states are characterized by hemoglobin denaturation, presence of methemoglobin, high number of Heinz bodies and decrease in the number of proerythroblasts.

Selenium is not stored in animal and human organisms and deficiency might easily develop from the reduced supply of this element, even with proper nutrition. The bioavailability of selenium depends on the diets, for example: diets rich in low-molecular-weight proteins, vitamin E, A and C increase significantly the bioavailability of this element. In combination with proteins, Se forms complexes called selenoproteins, which play important roles for the brain, internal secretion glands and reproductive system (Hollenbach et al. 2008). One of the most important selenoproteins is GSH-Px, found in erythrocytes and the liver, is responsible for protecting hemoglobin and fatty acids from oxidation. It also regulates the course of oxidative stress leading to cells death. The absence of GSH-Px in mice leads to anemia development due to the damage of precursor cells of the erythroblastic line (Marinkovic et al. 2007). These cells are equipped with antioxidant enzymes that support the processes of dismutation of peroxide radicals, hydrogen peroxide detoxification and consequently their role is reduced to maintaining a reducing intracellular environment. In studies dealing with hematopoietic activity, the influence of selenium and vitamin E compounds on the activity of the erythroblast line in mice has been repeatedly emphasized. Therefore, it is interesting to see whether therapeutic doses of selenium and vitamin E have an effect on the hematopoietic activity of the erythroblastic cell line in ruminants, in this case fallow deer. It is important to define the moment in the process of erythropoiesis when the progenitor cells of the bone marrow erythroblastic line are activated for increased activity and differentiation. It is worth emphasizing that this is the first study that describes this subject in ruminants. In addition, it is worth considering whether the administration of selenium and vitamin E in any way affects the morphology of cells from erythroblastic cell line.

Materials and Methods

The experiment was approved by the Local Ethics Commission for Animal Experiments (decision number 61/2011). Fallow deer of the Pilsko-Lithuanian line at about 3 days of age were divided into 2 groups each consisting of 10 individuals. The animals used in the experiment were clinically healthy, in good physical condition and properly nourished. The experimental group (E) was given intramuscularly selenium and vitamin E at a dose of 3.0 ml (tocopherol acetate – 50 mg,

sodium selenite – 0.5 mg, solvent – 1 ml) at 3 days of age. The control group (C) included animals that did not receive any supplementation. Approximately 1 ml of the bone marrow was collected three times with biopsy needle (MDG 18G, 63 mm long) from the 3rd-4th rib in the sternal region. All the animals were subjected to sedation using 0.9 mg/kg xylazine hydrochloride ("Rompun" Bayer, Leverkusen, Germany) and zilzetam with tiltamine at a dose of 1.1 mg/kg ("Zoletil", Virbac, France). The first sampling took place before selenium and vitamin E administration, in both groups 3 days after birth. The second and third sampling occurred 10 and 25 days after selenium and vitamin E supplementation, respectively. The biopsy site was prepared according to the standard surgical procedures. The bone marrow was collected into 1 ml test tubes without anticoagulant. Bone marrow smears were made on prepared and labeled glass slides (Marienfeld) immediately after sampling. The smears were stained using May-Grünwald Giemsa (MGG) method and cytologically evaluated. Hematological counter SH-96/24D by Alchem was used to count bone marrow cells. For hematological analysis, blood samples were taken, under standard conditions, from the external jugular vein into 2 ml tubes containing K_2EDTA. Blood in both groups was collected three times: before the administration of vitamin E and selenium, on the 10th and 25th day after supplementation. All samplings were followed by bone marrow collection. After collection, blood smears were stained using MGG method and then subjected to microscopic evaluation (Zeiss light microscope) for the cytological assessment of erythroblastic cell line. Time of bone marrow staining with May-Grünwald dye was 80 seconds and for Giemsa dye 5 minutes. In the cytological evaluation of bone marrow smears, 1000 cells of both cell lines were counted and the percentage of cells from the erythroblastic line was estimated. Giemsa dye was diluted with pH 7.2 phosphate buffer at 1:10 ratio. Blood for biochemical analyses was taken into tubes containing coagulation activator (Vacuette). Serum concentration of selenium was determined of animals of both groups. Serum samples (1 ml) were mineralized in a 3:1 mixture of nitric and perchloric acid. Mineralization was carried out in an electric aluminum heating block with temperature programming, which raised temperature in 2-3 hours from 120 to 200°C. After cooling concentrated hydrochloric acid was added to the mineralization mixture and heated at 80°C for 20 minutes to reduce Se^6 to Se^4. In parallel with the test samples, reagent samples and samples with addition of Se (0.050 and 0.100 μg/g of serum) were prepared. Similarly to Se, serum levels of vitamin E in animals of both groups were determined by high performance liquid chromatography (HPLC) with the HP-1050 chromatograph from Hewlett Packard. Clin-Rep ready-made Recipe Chemical was used, the flow rate was 1.5 ml/min. at a wavelength of 326 nm. The activity of GSH-Px was determined in full blood using kinetic method with cumene hydroxide and phosphate buffer (Ransel kit). Statistical calculations were performed using the Anova test for multiple independent trials. Peripheral blood morphology was determined using the Siemens diagnostic hematology analyzer ADVIA 2120i. The hematological parameters of peripheral blood included: hemoglobin concentration (Hb), number of red blood cells (RBC), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), reticulocyte percentage (RETIC%) and absolute reticulocyte count (RETIC $*10^9$). Reticulocytes staining was performed using toluidine blue (Kolchem) in order to demonstrate the presence of RNA residues in erythrocytes and consequently a proper evaluation of these cells.

Results

The results are presented with numerical values in a tabular form. Table 1 shows the percentage distribution of erythroblastic cell line in evaluated bone marrow smears. In group E, a statistically significant increase in the percentage of proerythroblasts (7.58%), basophilic erythroblasts (10.63%), polychromatic erythroblasts (18.78%) and orthochromatic erythroblasts (12.67%) on the 15th day after supplementation was observed. The percentage of reticulocytes in group E was markedly high (0.97%) and the absolute number of reticulocytes was also elevated (38.4×10^9/l) on 15th day (Table 2). Significant increase ($p<0.05$) in hemoglobin level, and number of erythrocytes in the experimental group (Table 2) was determined. The percentage distribution of RDW was significantly higher ($p<0.05$) on the 15th (20.1%) and 25th day after supplementation (24%). Activity of GSH-Px differed significantly between the groups before and 25 days after the supplementation ($p<0.05$) (Table 3). The selenium content on the 15th day increased to 0.442 μmol/l, which was also a statistically significant difference (Table 3). Activity of GSH-Px in group E on the 15th day reached 165.3 U/gHb, which was a statistically significant difference. The presence of Heinz bodies in erythrocytes was found in the cytological evaluation of peripheral blood smears in both groups (Fig. 1 and 2).

Table 1. Percentage distributions of erythrocytes in evaluations of the bone marrow sampled from fallow deer.

			PROERBL %	BASO ERBL %	POLY ERBL %	ORTO ERBL %
DAY 0	Control group	Mean	4.89	7.65	15.1	9.01
		SD	0.99	1.0	2.35	1.85
	Experimental group	Mean	4.25	6.93	14.76	9.35
		SD	1.54	2.63	1.23	1.76
DAY 15	Control group	Mean	5.27^{y}	8.21^{y}	15.25^{y}	9.63^{y}
		SD	2.54	1.98	0.78	1.16
	Experimental group	Mean	7.58^{x}	10.63^{x}	18.78^{x}	12.67^{x}
		SD	2.72	1.63	1.52	0.98
DAY 25	Control group	Mean	5.02^{y}	7.11^{y}	16.11	9.42^{y}
		SD	2.42	1.67	0.89	1.96
	Experimental group	Mean	8.23^{xA}	11.89^{x}	19.95	14.56^{xA}
		SD	2.52	0.87	0.98	1.87

Explanations: PROERBL – proerythroblast, BASO ERBL – basophilic erythroblast, POLY ERBL – polychromatic erythroblast, ORTO ERBL – orthochromatic erythroblast

^{A} – statistically significant difference at $p \leq 0.05$ between day 0 and 25th

^{x} – statistically significant difference at $p \leq 0.05$ between groups

^{y} – statistically significant difference at $p \leq 0.05$ between groups

Table 2. Hematologic parameters of peripheral blood in fallow deer.

			RBC ($\times 10^{12}$/l)	HGB (g/l)	MCH (pg)	MCV (fl)	MCHC (g/l)	RDW (%)	RETIC (%)	RETIC ($\times 10^{9}$)
DAY 0	Control group	Mean	8.8	186	13.8	41.1	335	19.1	0.87	32.1
		SD	1.13	13.8	0.51	2.03	12.71	0.87	0.25	1.21
	Experimental group	Mean	8.9	178	12.1	42.14	327	18.5	0.98	32.3
		SD	1.12	14.2	0.78	2.04	12.8	0.75	0.21	1.31
DAY 15	Control group	Mean	11.8^{y}	185	13.1	40.8	330	18.51^{y}	0.82^{y}	33.8^{y}
		SD	1.13	14.3	0.42	1.87	11.6	0.81	0.21	1.5
	Experimental group	Mean	13.3^{x}	191	13.6	42.4	336	20.1^{x}	0.97^{x}	38.4^{x}
		SD	1.15	12.5	0.48	2.01	11.8	0.91	0.22	1.61
DAY 25	Control group	Mean	11.7	184	12.9	42.1	333	18.9^{y}	0.86^{y}	34.5^{y}
		SD	0.12	17.5	0.48	1.87	11.9	0.79	0.23	1.31
	Experimental group	Mean	13.5	193	13.9	43.4	337	24^{x}	0.94^{x}	38.7^{x}
		SD	1.41	12.6	0.51	1.94	11.7	0.96	0.26	1.62

Explanations: hemoglobin concentration (Hb), number of red blood cells (RBC), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), reticulocyte percentage (RETIC%), absolute reticulocyte count (RETIC*10^{9})

^{x} – statistically significant difference at $p \leq 0.05$ between groups

^{y} – statistically significant difference at $p \leq 0.05$ between groups

Discussion

The present results have been compared to the findings obtained by other researchers in other ruminants, because there are no developed reference values for the fallow deer. In sheep, normal and sufficient serum concentration of selenium is considered at approximately 0.89 μmol/l, low at 0.25-0.63 μmol/l and defi-

Table 3. Selenium and vitamin E concentration, and activity of glutathione peroxidase in fallow deer.

		Se (μmol/l)		Vit. E (μg/ml)		GSH-Px (U/gHb)	
		Control group	Experimental group	Control group	Experimental group	Control group	Experimental group
DAY 0	Mean	0.195	0.265	2.5	3.25	28.2	34.1^{AB}
	SD	0.012	0.025	1.20	1.14	2.76	3.2
DAY 15	Mean	0.197^{y}	0.442^{x}	2.63^{y}	3.66^{x}	25.4^{y}	165.3^{xA}
	SD	0.012	0.03	0.85	1.28	2.11	14.68
DAY 25	Mean	0.183^{y}	0.412^{x}	2.31^{y}	3.56^{x}	18.8^{y}	198.5^{xB}
	SD	0.011	0.029	1.11	1.25	2.10	20.86

^{A} – statistically significant difference at $p \leq 0.05$ between day 0 and 15th
^{B} – statistically significant difference at $p \leq 0.05$ between day 0 and 25th
^{x} – statistically significant difference at $p \leq 0.05$ between groups
^{y} – statistically significant difference at $p \leq 0.05$ between groups

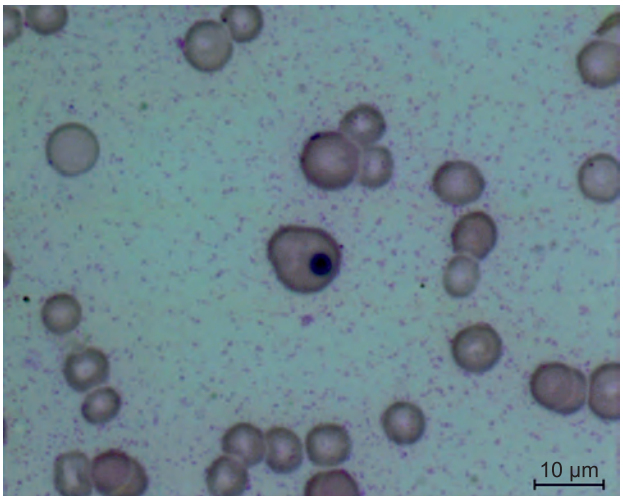

Fig. 1. Erythrocyte with visible Heinz body (group E, 15th day), MGG staining method ×1000

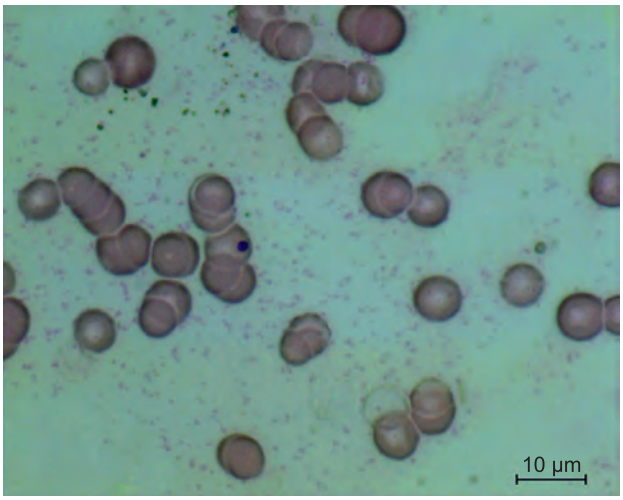

Fig. 2. Erythrocyte with visible Heinz body (group C, 15th day), MGG staining method ×1000.

cient at less than 0.25 μmol/l. The commonly used method for selenium deficiency diagnosis is to determine the activity of glutathione peroxidase in erythrocytes. The highest number of pathologies related to Se and vitamin E deficiency in goat kids was recorded with selenium concentrations of about 0.38 μmol/l and peroxidase activity below 25 U/gHb (Bickhardt et al. 1999). This study did not determine such low concentrations of investigated parameters in fallow deer. On the 15th day after supplementation, a statistically significant increase in the percentage of all cells from the erythroblastic line was observed in group E. The number of reticulocytes in the experimental group was significantly higher, indicating an effect of selenium and vitamin E on process of erythropoiesis occurring in the bone marrow. Barić Rafaj et al. (2011) have found, that RDW in farmed red deer represents rather high value. In our experiment RDW value differed in the experimental group differed significantly from that found in the control group on the 25th day after supplementation. To the best of our knowledge, some hematological parameters, like RDW, have not been evaluated in studies of fallow deer during the selenium supplementation.

Our results correspond to those obtained by Marinkovic et al. (2007), who have confirmed the key role of selenoproteins in erythropoietic processes. It involves incorporating selenium into erythrocytes followed by selenocysteine biosynthesis (Arthur 2000). This research shows that supplementation with selenium and vitamin E increases the number of erythrocytes and promotes the growth of glutathione peroxidase activity after supplementation. In newborn ruminants, the number of erythrocytes in peripheral blood is lower than that in the adult animals. Administration of selenium with vitamin E clearly stimulates processes of erythropoiesis, as demonstrated in this study. Semba et al. (2009) reported tremendous impact of selenium deficiency in the development of anemia. This fact is

explained by the lysis of erythrocyte membranes as a result of lowering concentration of selenoproteins which determine erythrocytes resistance to oxidative stress. Interestingly, this study has demonstrated the relationship between low selenium concentrations and increased reticulocyte counts. Mostert et al. (2003) have demonstrated the role that selenium plays in the process of erythropoiesis. The results obtained by these authors confirm the role of selenium in the increase of hematopoietic bone marrow activity, especially the red blood cell line, by increasing the percentage of this line in the cytological evaluation of the bone marrow. Tron et al. (2005) have found that the possible mechanism by which selenium could participate in the pathogenesis of anemia is an increase in heme oxygenase-1 activity. Low levels of selenium may increase hepatic heme oxygenase-1 activity, which catalyses the initial stage of heme catabolism, and reduces the ratio of heme to bivaridine, carbon monoxide and free bivalent iron that prevent destructive changes in cells of the red blood cell line. The study by Canli et al. (2015) indicates a significant effect of selenium deficiencies on the development of maturation disorders affecting erythroblast progenitor cells in mice and the presence of Heinz bodies in many erythrocytes. These authors have observed the presence of a large number of Heinz bodies in peripheral blood smears in control and experimental group. Additionally, many Heinz bodies observed in peripheral blood smears before and after supplementation suggest that this might be a feature characteristic of the fallow deer. Study by Kaushal et al. (2011) has clearly demonstrated that state of selenium deficiency can indicate so-called stress erythropoiesis, which results in a short-lived increase in the number of erythrocytes with shortened survival that does not generate increase in hematocrit in the peripheral blood samples tested. The presented results allow us to conclude that selenium and vitamin E supplementation results in a very rapid response of the red blood cell line in the bone marrow to increase the number of these cells. It is worth emphasizing that anemia occurring in mice (Kaushal et al. 2011) was not observed in fallow deer. Studies performed on mice have revealed that in case of occurrence of the selenium deficiency leading to anemia, several drops of denatured hemoglobin are observed in erythrocytes. The cytological evaluation of the bone marrow in fallow deer provides the possibility for correct interpretation of hematopoietic processes in ruminants. According to our knowledge, this is the first ever study dealing with erythropoiesis in fallow deer kept in captivity after supplementation with selenium and vitamin E. The results obtained seem to justify the opinion that this interesting subject should be further explored.

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