

Effect of Different Doses of Organically Bound Selenium on Antioxidant Status and Levels of Metal Ions in Postpartum Sows

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Received: 24 February 2013 / Accepted: 9 April 2013 / Published: 1 May 2013

The study was focused on the effect of different doses of organically bound selenium on antioxidant status in postpartum sows. 24 sows, Large White (English Large White) breed were divided into three balanced groups. Animals were fed with the basic feed ration containing selenium in the dose of 0.05 mg/kg feed mixture (FM). The first group of sows (n = 8) were fed without the addition of selenium into the diet and served as a control group. The second one (n = 8) received 0.30 mg Se/kg FM in the diet. The third group of sows were fed by selenium in the dose of 0.55 mg Se/kg FM. Antioxidant status and concentration of heavy metals in erythrocytes were monitored. Selenium in a dose of 0.30 mg/kg FM increased activities of antioxidant enzymes glutathione peroxidase by 12.4 % (P < 0.05) and glutathione-S-transferase by 17.6 % (P < 0.05). Higher antioxidant activity measured by FRAP (increase by 12.4 % (P < 0.05)), FR (by 12.9 % (P < 0.05)) and ABTS (by 10.2 % (P < 0.05)) methods was found. Reduced form of glutathione was increased for 27.0 % (P < 0.001). Concentration of selenium was increased by 51.7 % (P < 0.001). Content of cadmium was reduced by (16.3 % (P < 0.05)), on the other hand, content of copper was increased by 32.4 % (P < 0.001) in comparison with control group. Selenium in a dose of 0.55 mg/kg FM increased activities of antioxidant enzymes glutathione peroxidase by 17.8 % (P < 0.01), superoxide dismutase by 11.8 % (P < 0.05) and glutathione-S-transferase by 25.3 % (P < 0.01). The increased antioxidant activity measured by different methods was found - FRAP method showed an increase by 17.8 % (P < 0.05), FR by 20.3 % (P < 0.05), and ABTS by 12.4 % (P < 0.05). Reduced form of glutathione was increased by 33.1 % (P < 0.001). Concentration of selenium was increased by 55.2 % (P < 0.001). Content of cadmium was

significantly reduced (by 12.8 % ($P < 0.05$)), on the other hand, content of copper was significantly increased (by 29.5 % ($P < 0.001$)) in comparison with control group of animals. The obtained results show that the addition of selenium in a dose of 0.30 and 0.55 mg/kg FM increased antioxidant status in postpartum sows. The results may show possible beneficial effect of selenium on health status in postpartum sows.

Keywords: Selenium; Antioxidant Status; Erythrocytes; Sows; Electrochemical Detection

LIST OF THE ABBREVIATIONS

ABTS – 2,2-azinobis(3-ethylbenzothiazolin-6-sulphonic acid); FM – feed mixture; FRAP – Ferric Reducing Antioxidant Power; FR – free radicals; GPx – glutathione peroxidase; GST – glutathione-S-transferase; GSH – reduced glutathione; MDA – malondialdehyde; Se0 – control group of sows, Se1 – the first experimental group of sows (0.30 mg Se/kg feed mixture; Se2 – the second experimental group of sows (0.55 mg Se/kg feed mixture)

1. INTRODUCTION

Selenium belongs in the nutrition of farm animals into the group essential trace elements. It is an integral part of the antioxidant capacity of the organism, where plays a major role in the protection against damage of tissues by free radicals as an integral part of enzyme glutathione peroxidase. Many years after its discovery, this element was considered toxic in overdoses. There is only a small limit between its essentiality and toxicity [1]. Supplementation of selenium during pregnancy increases the number of new-born calves and also their viability at a later age. Concentration of this element increases during lactation not only in blood, but also in the milk from which also passes into calves [2,3]. According to some authors selenium supplementation in the diet of animals increases the activity of superoxide dismutase, which, together with glutathione peroxidase, represents main defense mechanism against free radicals [4,5]. The supplementation of selenium in a dose of 0.3 mg/kg FM increases the weight of new-born piglets and increases antioxidant capacity in these animals (production of MDA, GPx, SOD, GSH) [6,7]. Free radicals are created in the body during normal physiological processes and also under stress situations as heat stress, childbirth, and excessive physical burden. Excessive production of free oxygen radicals can be caused by an excessive intake of mycotoxins [8], ions of zinc and cadmium [9,10], or lead [11,12]. When an imbalance between the production of free radicals and antioxidants available in the organism creates oxidative stress, oxidative damage of tissues, proteins, lipids, or DNA occur [4,11]. Oxidation stress may lead also to creation of a tumour disease [13].

Spectrometric methods are mainly used in the analysis of antioxidant properties. However, these methods are dependent on many parameters, such as temperature, time of the analysis, character of a compound or mixture of compounds (extracts), concentration of antioxidants and prooxidants and many other substances [7,14-21]. Electrochemical methods used for the determination of antioxidant capacity have been still developing. These provide rapid, simple and sensitive alternative method in the analysis of bioactive compounds associated with the scavenging of the radicals as well as the antioxidant capacity itself. They are low-cost and usually do not require time consuming sample preparation. They are used both in stationary systems, where differential pulse polarography and voltamperometric methods belong to the most used techniques, and dynamic, flow, eventually

electromigration system, where the advantage of voltammetry and amperometry is considered. Dynamic systems are used and cover rapid and sensitive quantification of simple analytes; on the other hand, stationary systems are suitable for the quantification of limited amount of analytes and for studying formation of complexes of simple as well as more complicated biomolecules with target molecule(s). The development of the new instrumentation with the possibility of miniaturization in contrast to the conventional, routinely used systems is the main object of electrochemical techniques in the analysis of bioactive compounds [22-27].

The aim of this experiment was to determine the influence of different levels of selenium in the organic form on oxidative stress in postpartum sows using spectrophotometric and electrochemical techniques.

2. EXPERIMENTAL PART

2.1 Chemicals

HPLC-grade methanol (>99.9%; v/v) came from Merck (Dortmund, Germany). Other chemicals were purchased from Sigma-Aldrich (St. Louis, USA), unless noted otherwise. Stock standard solutions of reduced glutathione (1 mg/ml) were prepared with ACS water (Sigma-Aldrich) and stored in darkness at -20 °C. Working standard solutions were prepared daily by dilution of the stock solutions. All solutions were filtered through 0.45 µm nylon filter discs (Millipore, Billerica, MA., USA) prior to analysis using HPLC-ED. The pH was measured using WTW inoLab Level 3 with Level 3 terminal (Weilheim, Germany).

2.2 Experimental animals

Own experiment was carried out on sows farrowing in Hospodářské obchodní družstvo Jablůňov – Ruda (Czech Republic). In the experiment, 24 postpartum sows, which were divided into three groups according to age, were included. Age of sows ranged from 2 to 3 years. Large White (English Large White) breed (*Sus scrofa domestica*) was used in the experiment. Experimental animals were stabled individually in gestation (farrowing) crates and had access to water *ad libitum*. All animals were fed by 6.4 kg of (complete) basic feed mixture (Table 1) containing 0.05 mg Se/kg of feed mixture from native sources. Three groups of animals were included in the experiment. The first group (Se0) of sows (n = 8) served as a control and the intake of selenium was not increased by its supplementation. The second group (Se1) sows (n = 8) were fed with 0.25 mg Se/kg FM (total income was 0.30 mg Se/kg FM). The third group (Se2) sows (n = 8) were fed with 0.50 mg Se / kg FM (total income was 0.55 mg Se/kg FM). Sows were fed by premix in the morning feeding. To supplement selenium selenium-enriched yeasts (Se-methionin) were used (Sel-Plex - Alltech's, USA). The experiment lasted from mid-January to the end of March 2012. Due to the limited capacity, experimental animals were included in the experimental monitoring continuously.

Table 1. Composition of (complete) feed mixture for sows

Component	% representation
Wheat grains	58.80
Winter barley grains	20.00
Soybean meal	15.00
Mineral and vitamin premix	3.20
Oat grains	3.00

2.3 Blood sampling

Duration of the experiment was set for 20 days. Before the start of the experimental monitoring control sample of blood was obtained from all animals included in the experiment. Blood sample was subsequently collected at 10-day intervals (in the tenth and twentieth day of the experiment) from *vena jugularis externa* into plastic sample containers with coagulant heparin (Fig. 1 and 2). Blood sampling was always carried out three hours after feeding. Blood samples were transported in a cool box to the laboratory within three hours of the sampling. Erythrocytes were obtained by centrifugation (2300 r.p.m. for 20 min, MPW-350E, Brno, Czech Republic), the erythrocytes were subjected to the required analyses.

**Figure 1.** Collecting of blood sample.**Figure 2.** Blood sample.

2.3.1 Preparation of samples for electrochemical determination of metal (Zn, Cd, Cu) – microwave digestion

To 10 μL erythrocytes was added 500 μL of digestion mixture (350 μL HNO_3 + 150 μL H_2O_2). Samples were digested in MW Anton Paar, rotor MG-65. The program (SUP 6) begins and ends with the same ten-minute-long-step, beginning with the power of 50 W and ending with the power of 0 W. Microwave power was 100 W in the main part of the program (30 min.).

2.4 Description of spectrophotometric analysis

Spectrophotometric measurements were carried out using an automated chemical analyser BS-400 (Mindray, China). It is composed of cuvette space tempered to 37 ± 1 $^\circ\text{C}$, reagent space with a carousel for reagents (tempered to 4 ± 1 $^\circ\text{C}$), sample space with a carousel for preparation of samples and an optical detector. Transfer of samples and reagents is provided by robotic arm equipped with a dosing needle (error of dosage up to 5 % of volume). Cuvette contents are mixed by an automatic mixer including a stirrer immediately after addition of reagents or samples. Contamination is reduced due to its rinsing system, including rinsing of the dosing needle as well as the stirrer by MilliQ water. For detection itself, the following range of wavelengths can be used - 340, 380, 412, 450, 505, 546, 570, 605, 660, 700, 740 and 800 nm.

2.4.1 Determination of Glutathione-S-Transferase (GST)

The method is based on glutathione-S-transferase (GST)-catalyzed reaction between reduced glutathione (GSH) and GST substrate, 1-chloro-2,4-dinitrobenzene (CDNB), which has the broadest range of isoenzyme detectability (e.g., alpha-, mu-, pi- and other GST isoforms). Under certain conditions, the interaction between glutathione and CDNB is dependent on the presence of active GST. The GST-catalyzed formation of GS-DNB produces a dinitrophenylthioether, which can be detected spectrophotometrically at 340 nm [28]. A 180 μL volume of reactants consisting of 2 mM CDNB and PBS (1.4 mM NaH_2PO_4 , and 4.3 mM Na_2HPO_4 , pH 7.4) - (1:19, v/v, 37 $^\circ\text{C}$) was added to sample in a plastic microtube. Further, 12.5 mM GSH (30 μL) in 0.1 M phosphate buffer (pH 7.4) was added. A wavelength of 340 nm was used for determination of GST activity [7].

2.4.2 Determination of glutathione peroxidase (GPx)

Glutathione Peroxidase Cellular Activity Assay Kit (CGP1, Sigma Aldrich, USA) was used for GPx assay. A 260 μL volume of reagent R1 (0.3 mM NADPH reagent in GPx buffer) was pipetted into a plastic cuvette with subsequent addition of 10 μL of sample and after mixing, a 30 μL volume of reagent R2 (3 mM tert-butyl hydroperoxide) is added to the cuvette which starts the reaction. The decrease in absorbance is measured at 340 nm using kinetic program for 126 seconds. The spectrophotometer calculates GPx activity according to a calibration curve.

2.4.3 Determination of superoxide dismutase (SOD)

Kit 19160 SOD (Sigma Aldrich, USA) was used for assay of superoxide dismutase (SOD, EC 1.15.1.1.). A 200 μL volume of reagent R1 (WTS solution diluted 20 times with buffer) was pipetted into a plastic cuvette and agent was incubated at 37 °C for 108 s. Afterwards, a 20 μL volume of sample was pipetted and in 378 s, the reaction was started by adding a 20 μL volume of reagent R2 (enzyme solution 167 times diluted with buffer). It was incubated for 72 s and then absorbance was measured at $\lambda = 450$ nm. Kinetic reaction was measured for 180 s and absorbance was read every 9 s.

2.4.4 Determination of antioxidant activity by ABTS test

The ABTS radical method is one of the most used assays for the determination of the concentration of free radicals. It is based on the neutralization of a radical-cation arising from the one-electron oxidation of the synthetic chromophore 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS): $\text{ABTS}^{\bullet} - e^- \rightarrow \text{ABTS}^{++}$. This reaction is monitored spectrophotometrically by the change of the absorption value. A 150 μL volume of reagent (7 mM ABTS $^{\bullet}$ (2,2'-azinobis 3-ethylbenzothiazoline-6-sulfonic acid and 4.95 mM potassium peroxodisulphate)) is poured with 3 μL of a sample. Absorbance is measured at 660 nm. For calculating of the antioxidant activity, difference between absorbance at the last (12th) minute and second minute of the assay procedure was used [7].

2.4.5 Determination of antioxidant activity by FRAP method

The FRAP method (Ferric Reducing Antioxidant Power) is based on the reduction of complexes of 2, 4, 6-tripyridyl-*s*-triazine (TPTZ) with ferric chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), which are almost colourless, and eventually slightly brownish. This chemical forms blue ferrous complexes after its reduction. Reagent preparation: Solution 1: 10 $\text{mmol} \cdot \text{L}^{-1}$ solution of TPTZ in 40 $\text{mmol} \cdot \text{L}^{-1}$ of hydrochloric acid. Solution 2: 20 $\text{mmol} \cdot \text{L}^{-1}$ solution of ferric chloride hexahydrate in ACS water. Solution 3: 20 $\text{mmol} \cdot \text{L}^{-1}$ acetate buffer, pH 3.6. These three solutions (TPTZ, FeCl_3 , acetate buffer) are mixed in a 1:1:10 rate. A 150 μL volume of reagent is injected into a plastic cuvette with subsequent addition of a 3 μL sample. Absorbance is measured at 605 nm for 12 minutes. Difference between absorbance at the last (12th) minute and second minute of the assay procedure was used for calculating of the antioxidant activity.

2.4.6 Determination of antioxidant activity by Free Radicals method

This method is based on ability of chlorophyllin (the sodium-copper salt of chlorophyll) to accept and donate electrons with a stable change of maximum absorption. This effect is conditioned by an alkaline environment and the addition of catalyst. A 150 μL volume of reagent is injected into a plastic cuvette with subsequent addition of a 6 μL sample. Absorbance is measured at 450 nm in the

second minute of assay and the last (12th) minute. Difference between these two values is considered as an outputting value [7].

2.4.7 Photometric determination of malondialdehyde

The principle of malondialdehyde determination is reaction between malondialdehyde (MDA) with thiobarbituric acid (TBA) under formation of TBA-MDA-TBA adduct that absorbs strongly at 535 nm. Trichloroacetic acid (TCA) is added to the sample because of its ability to precipitate proteins, bilirubin, unsaturated fatty acids and lipoproteins. A 300 μL sample of blood plasma was mixed with 10 μL 0.5 M solution of butylated hydroxytoluene (BHT) in 96 % ethanol (*v/v*) and 310 μL 20 % TCA (*v/v*) prepared in 0.6 M HCl. After 20 min incubation on ice mixture was centrifuged at 11,000 r.p.m. for 15 min. Subsequently, 400 μL of supernatant was mixed with 800 μL of 30 mM TBA and mixture was incubated in a thermomixer Comfort (Eppendorf, Germany) at 90 °C for 30 min. After cooling in ice MDA absorbance was measured using a spectrophotometer at 535 nm and the concentration was subtracted from the calibration curve [29].

2.4.8 Determination of sulfhydryl groups (SH groups)

Ellman's spectrophotometric method was used for determination of sulfhydryl (-SH) moieties [30]. Ellman's reagent (277 μL , R1, 2 mM 5.5'-dithiobis(2-nitrobenzoic) acid (DTNB) in 50 mM sodium acetate CH_3COONa) was mixed with sample (45 μL). Further, reagent R2 (33 μL , 1 M Trizma base: CH_3COOH) was added. Mixture was incubated at 37 °C for 10 min. Absorbance was measured at 412 nm. Values of absorbance of reagent R1 itself (blank) and mixture after 10 min. long incubation were used for determination of total-SH content.

2.5 Determination of reduced glutathione

Determination of reduced glutathione (GSH) was carried out using high-performance liquid chromatography with electrochemical detection (HPLC-ED). HPLC-ED system consisted of two chromatographic pumps, Model 582 ESA (ESA Inc., Chelmsford, MA) (working range 0.001 - 9.999 $\text{mL}\cdot\text{min}^{-1}$) and a chromatographic column with a reverse phase Zorbax Eclipse AAA C18 (150 \times 4.6; 3.5 μm particle size, Agilent Technologies, USA) and twelve-channel CoulArray electrochemical detector (Model 5600, ESA, USA). The detector is composed of three analytical flow cells (Model 6210, ESA, USA). Each cell contains four analytical cells. Each cell contains two reference electrodes (hydrogen palladium), two auxiliary electrodes and a porous graphite working electrode. Electrochemical detector is placed in the control module; its whole space is thermostatically controlled. The sample (20 μL) was injected automatically by the autosampler (Model 542, ESA, USA), which has integrated a thermostated space for column. Samples were stored in a carousel at 8 °C during the analysis. The column was thermostated at 32 °C. Flow rate of mobile phase was 1 $\text{mL}\cdot\text{min}^{-1}$. Mobile phase consisted of A: trifluoroacetic acid (80 mM) and B: 100 % Met-OH. Analytes were eluted by a linearly increasing gradient: 0-1 min (3 % B), 1-2 min (10 % B), 2-5 min (30 % B),

and 5-6 min (98 % B). Detection of separated compounds was performed at applied potential of 900 mV [31-39]. Time of analysis was 20 min.

2.6 Electrochemical determination of Se

Determination of selenium by differential pulse voltammetry was performed using a 797 VA Computrace instrument connected to 889 IC Sample Center (Metrohm, Switzerland) with a standard cell with three electrodes. A hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm^2 was the working electrode. An Ag/AgCl/3 M KCl electrode was the reference and platinum electrode was auxiliary electrode. The analyzed samples were deoxygenated prior to measurements by purging with argon (99.999 %). 0.128 M ammonium sulphate, 0.123 mM copper sulphate and sulphuric acid (to adjust pH to 2.2) were used as a supporting electrolyte. The supporting electrolyte was exchanged after each analysis. The parameters of the measurement were as follows: initial potential of -0.4 V, end potential -0.9 V, deoxygenating with argon 120 s, accumulation time 200 s, deposition potential -0.6 V, time interval 0.05 s, voltage step 6 mV, pulse amplitude 30 mV, volume of injected sample: 20 μL , volume of measurement cell 2 mL (20 μL of sample + 1980 μL electrolyte).

2.7 Electrochemical determination of Zn, Cd, and Cu

Determination of zinc, cadmium and copper by differential pulse voltammetry were performed with the 797 VA Computrace instrument connected to 813 Compact Autosampler (Metrohm, Switzerland) using a standard cell with three electrodes. A hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm^2 was the working electrode. An Ag/AgCl/3M KCl electrode was the reference and platinum electrode was auxiliary. For data processing, 797 VA Computrace software by Metrohm CH was employed. The analysed samples were deoxygenated by purging with argon (99.999 %) prior to measurements. Acetate buffer (0.2 M CH_3COONa + 0.2 M CH_3COOH , pH 5) as a supporting electrolyte was used. The supporting electrolyte was exchanged after each analysis. The parameters of the measurement were as follows: initial potential of -1.2 V, end potential 0.2 V, deoxygenating with argon 90 s, deposition 420s, time interval 0.04 s, step potential 5 mV, modulation amplitude -25 mV, adsorption potential -1.2 V, volume of injected sample: 15 μL , volume of measurement cell 2 mL (15 μL of sample + 1985 μL acetate buffer).

2.8 Statistics

Data were processed using MICROSOFT EXCEL® (USA) and STATISTICA.CZ Version 8.0 (Czech Republic). Results are expressed as mean \pm standard deviation (S.D.) unless noted otherwise (EXCEL®). Statistical significances of the differences between GPx; SOD; GST; FRAP; ABTS; FR; MDA; GSH; SH groups; levels Se; Zn; Cu and Cd were determined using STATISTICA.CZ. Differences with $p < 0.05$ were considered significant and were determined by Student t-test, which was applied for means comparison. The values were based on the differences between the groups

supplemented with the selenium dose of 0.55 and 0.30 mg (values in the group 0.55 minus the values in the group 0.30). Values were recalculated to relative percentages with the first sample representing 100 %; other samples were related to this.

3. RESULTS

3.1 Antioxidant activity

Primarily, we evaluated the health status. The experimental animals showed no health problems during the experiment.

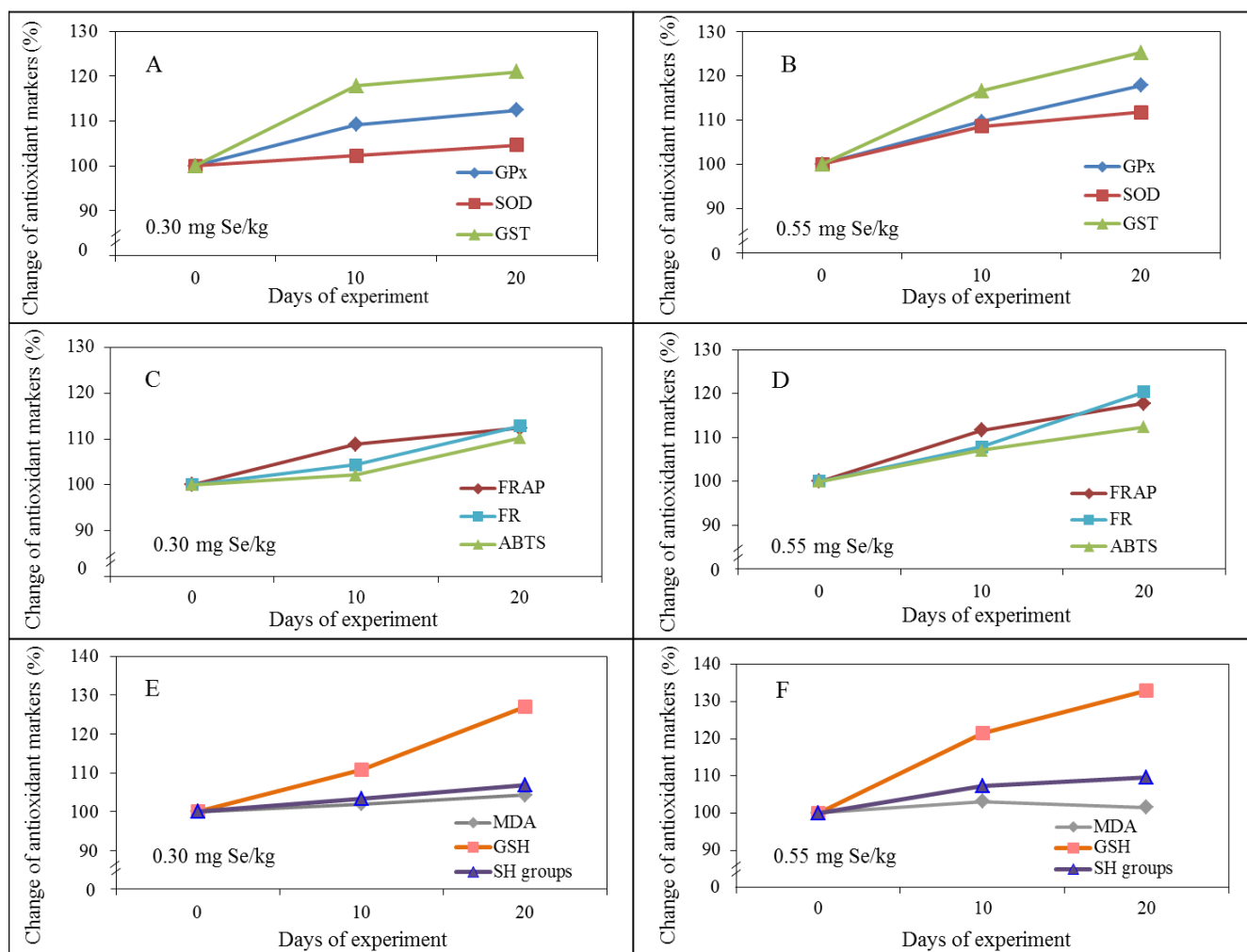


Figure 3. Effect of selenium on: (A and B) GPx, SOD and GST; (C and D) FRAP, FR and ABTS; (E and F) MDA, GSH and SH- groups. GPx - glutathione peroxidase; SOD - superoxide dismutase; GST – glutathione-S-transferase; GSH – reduced glutathione; FR – free radicals method; ABTS - 2,2-azinobis(3-ethylbenzothiazolin-6-sulphonic acid method; FRAP - Ferric Reducing Antioxidant Power method; MDA – malondialdehyde.

Therefore, we further aimed out attention on the evaluation of the effect of supplementation of different levels of selenium in the organic form on the antioxidant status of postpartum breeding sows.

Enzyme glutathione peroxidase (GPx) is the basic antioxidant that is directly related to the concentration of selenium in the organism. GPx values ranged from 1075 to 1789 U/mL (Figs. 3A and 3B). GPx activity was increased in the group of sows Se1 throughout the experiment compared with the control group. At the end of the experimental monitoring this difference was 12.4 % ($P < 0.05$), which corresponds to an increase in GPx activity by about 5.2 U/mL/day. Compared with control animals, sows of the Se2 group showed GPx activity increased by 9.7 % ($P < 0.01$, 10th experimental day) and 17.8 % ($P < 0.01$, 20th experimental day), respectively. This increase corresponded to 14.8 U/mL/day. Activity of superoxide dismutase (SOD) ranged from 91.2 to 114.9 U/mL (Figs. 3A and 3B). There were no significant differences in SOD activity in sows of the Se1 group compared to the control animals. Sows of the Se2 experimental group showed SOD activity increased by 11.8 % ($P < 0.05$) at the 20th experimental day compared with the control group of sows. Glutathione-S-transferase (GST) had in both experimental groups of sows (both the Se1 and Se2) similar trend (Figs. 3A and 3B). GST activity in the Se1 group of sows was at the 10th, respectively 20th experimental day increased by 17.6 % ($P < 0.05$) and 21.0 % ($P < 0.01$), respectively. When evaluating the activity of GST in sows of the Se2 group we determined its increase by 16.6 % ($P < 0.05$) at the 10th day and by about 25.3 % ($P < 0.01$) at the 20th day of experiment.

When evaluating the antioxidant capacity by FRAP, the Se1 group of sows showed by 12.4 % ($P < 0.05$) higher antioxidant activity compared to the control group at the 20th day of the experiment (Figs. 3C and 3D). In the group of sows of the Se2, the antioxidant activity measured by FRAP method was by 11.6 % higher ($P < 0.05$) at the 10th day of the experiment; similar trend was observed at the 20th day of the experiment, where an increase in antioxidant activity by 17.8 % ($P < 0.05$) compared with the control group sows was determined. FRAP values ranged from 6.2 to 13.8 $\mu\text{g/L}$ of trolox equivalents. Antioxidant capacity evaluated by FR method was in the Se1 group sows increased by 4.4 % at the 10th day of the experiment; at the end of the experiment (20th day) the antioxidant activity was by 12.9 % higher ($P < 0.05$) in comparison with the control sows (Figs. 3C and 3D). Also in the Se2 group of sows the antioxidant activity measured FR method was increased by 20.3 % ($P < 0.05$) in comparison with control animals at the 20th day of the experiment. FR values ranged from 87.5 to 166.0 $\mu\text{g/L}$ of trolox equivalents. When evaluating the antioxidant capacity by ABTS method, the Se1 group of sows showed significantly higher antioxidant activity (increase by 10.2% ($P < 0.05$)) determined at the end of the experiment compared with the control group (Figs. 3C and 3D). The Se2 group of sows had similar trend in the development of antioxidant activity measured by ABTS. At the 10th and 20th day of the experiment, this value was increased by 7.1 % ($P < 0.05$) and 12.4 % ($P < 0.05$), respectively. Levels of ABTS ranged within the interval from 366.1 to 470.6 $\mu\text{g/L}$ of trolox equivalents.

When evaluating the reduced glutathione (GSH), the value was for 10.7 % higher ($P < 0.001$) in the Se1 group at the 10th day of the experiment in comparison with the control group animals (Figs. 3E and 3F). The increase in GSH content in this group continued also at the 20th day of the experiment, when it increase for about 27.0 % ($P < 0.001$) compared with the control group was determined. A similar trend in levels of GSH as in the Se1 group of sows was well evident in the Se2 group. At the first sampling (day 10), the difference between the control group and the Se2 group was 21.4 % ($P < 0.001$). At the end of the experiment (20th day) this difference was almost 33.1 % ($P < 0.001$). During

the experiment, the GSH levels ranged from 0.11 to 0.29 $\mu\text{g/mL}$ (Figs. 3E and 3F). Level of malondialdehyde (MDA) was an indicator of the level of lipid peroxidation (Figs. 3E and 3F). There were no significant differences between the control and experimental (Se1, Se2) groups of animals. MDA value ranged between 0.3 and 0.5 $\mu\text{g/mL}$. Not in the values of SH- groups was observed no significant difference between the experimental groups (Se1 and Se2) and the control group of sows (Figs. 3E and 3F). In the Se1 group of animals, this value was by 6.9 % higher in comparison with the control group of sows at the end of the experiment. In the second experimental group (Se2), the amount of SH- groups was increased by 9.6% in comparison with the control group at the 20th day of the experiment. Values of SH- groups ranged from 134.3 to 161.2 $\mu\text{g/L}$ of cysteine.

3.2 Metal ions' content

Further we aimed our attention on metal ions content in samples of interest. The calibration curves for selenium, cadmium, zinc and copper are shown in Figs. 4A, 4B, 4C and 4D, respectively. Differential pulse voltammograms of the metals of interest are shown in Figs. 5A, 5B, 5C and 5D, respectively. Concentrations of ions of selenium in erythrocytes in sows of the control group of animals did not change significantly (Fig. 6A).

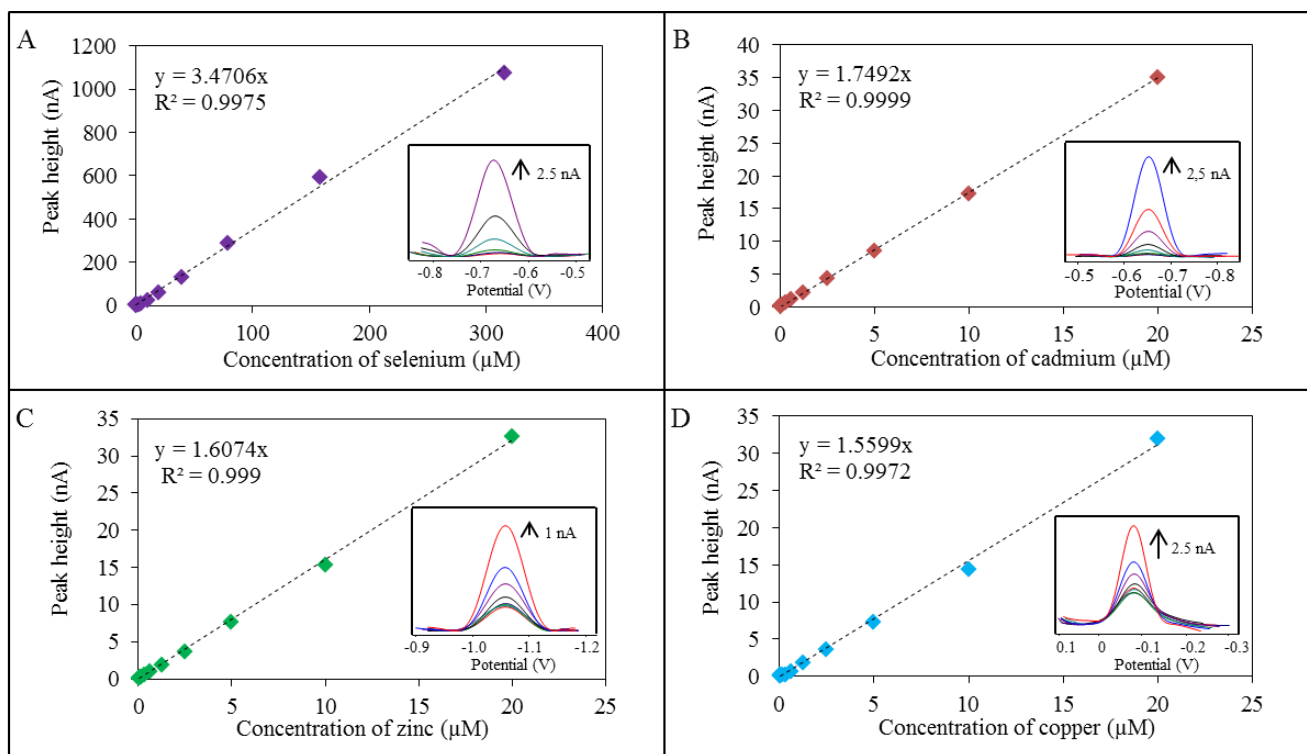


Figure 4. Calibration curves of (A) Se, (B) Cd, (C) Zn and (D) Cu. Acetate buffer (pH=5) was used as a supporting electrolyte. For detection of Se, 0.128 M ammonium sulphate, 0.123 mM copper sulphate and sulphuric acid (to adjust pH to 2.2) as supporting electrolyte were used. The DPV method was used for measurement.

At the 10th and in the 20th day of the experiment, the Se1 group of sows demonstrated an increase in selenium concentration in erythrocytes by 51.7 % ($P < 0.001$) and 49.0 % ($P < 0.001$), respectively in comparison with the control animals. Similar trend in selenium concentration in erythrocytes was observed in the Se2 group of sows. In the 10th and 20th day of the experiment, level of selenium was increased by 47.9 % ($P < 0.001$) and 55.2 % ($P < 0.001$), respectively compared with the control group sows.

During the experiment, concentration of zinc, cadmium and copper ions in erythrocytes of experimental animals were also determined (Figs. 6B, 6C and 6D, respectively). There were observed significant changes in levels of zinc ions between control and experimental (Se1 and Se2 groups) animals (Fig. 6B). The level of zinc ions in erythrocytes ranged from 196 to 818 $\mu\text{g/l}$. Concentration of cadmium ions in erythrocytes of sows of the Se1 group was by 16.3 % lower ($P < 0.05$) and in the Se2 group by 12.8 % lower ($P < 0.05$) in comparison with control animals at the end of the experiment (day 20, Fig. 6C). The levels of copper ions in erythrocytes were not significantly changed during the first ten days of the experiment in all experimental groups of sows (Fig. 6D). In the third sampling day (day 20), increased concentration of copper ions in the Se1 group (by 32.4 % ($P < 0.001$)) in comparison with the control group was determined. Similar results were determined also in the Se2 group in comparison with the control animals. Increase in copper ions in erythrocytes by 29.5 % ($P < 0.001$) was recorded.

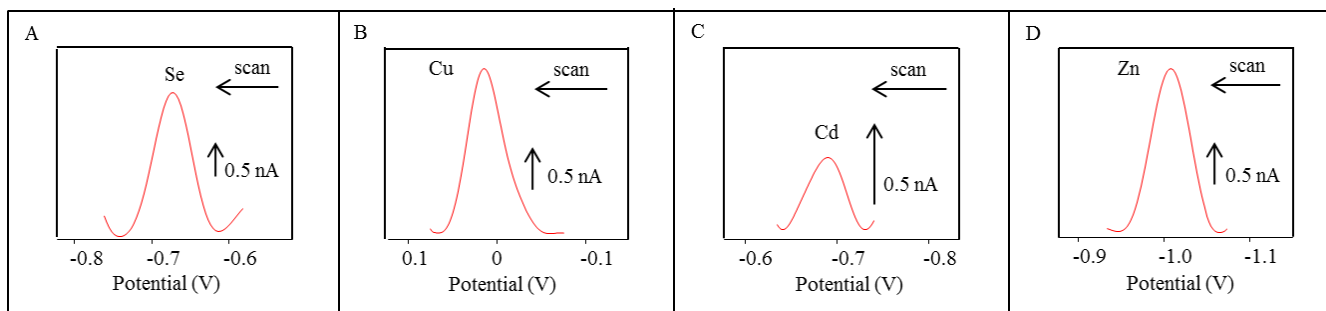


Figure 5. Electrochemical voltammograms of (A) Se, (B) Cu, (C) Cd and (D) Zn. These signals were measured in a blood sample of sow No. 13 using the DPV method.

4. DISCUSSION

It has been found in experiments performed by Hu et al. [40] and Zhan et al. [2] that the supplementation of 0.3 mg Se/kg FM in organic form (for 90 days) leads to a significant increase in the concentration of selenium ($P < 0.05$), GSH ($P < 0.05$), SOD ($P < 0.05$) and GPx ($P < 0.05$) in milk. We recorded similar results in our experiment. On the other hand, content of MDA decreased ($P < 0.01$) in comparison with group supplemented by selenium in inorganic form at the same dose of 0.3 mg Se/kg FM. In the experiment, which was carried out with rabbits, supplementation with selenium in organic form at a dose of 0.08, 0.24, 0.41, 0.59 and 0.70 mg Se/kg FM significantly increased GPx activity and selenium content in the liver with increasing intake of selenium. Level of SOD was not significantly affected in any of the experimental groups [41]. These conclusions were confirmed also in our

experiments. However, in contrast to these authors, SOD activity determined in our experiment was significantly increased ($P < 0.05$) in the Se2 group of sows. Breeding boars were supplemented by 0.3 or 0.6 mg Se/kg FM in organic or inorganic form in the experiment, which lasted for four months. Boars that received 0.6 mg of Se in organic form had significantly higher ($P < 0.01$) levels of selenium in the ejaculate. The level of antioxidant enzymes (GPx and SOD) was also increased ($P < 0.01$). Antioxidant capacity measured using the FR and ABTS methods was significantly increased ($P < 0.05$) in comparison with the group of boars supplemented with 0.3 mg Se/kg FM. Selenium did not affect the amount of zinc in the ejaculate of any of the experimental groups of animals [42]. These results correlate fully with our conclusions.

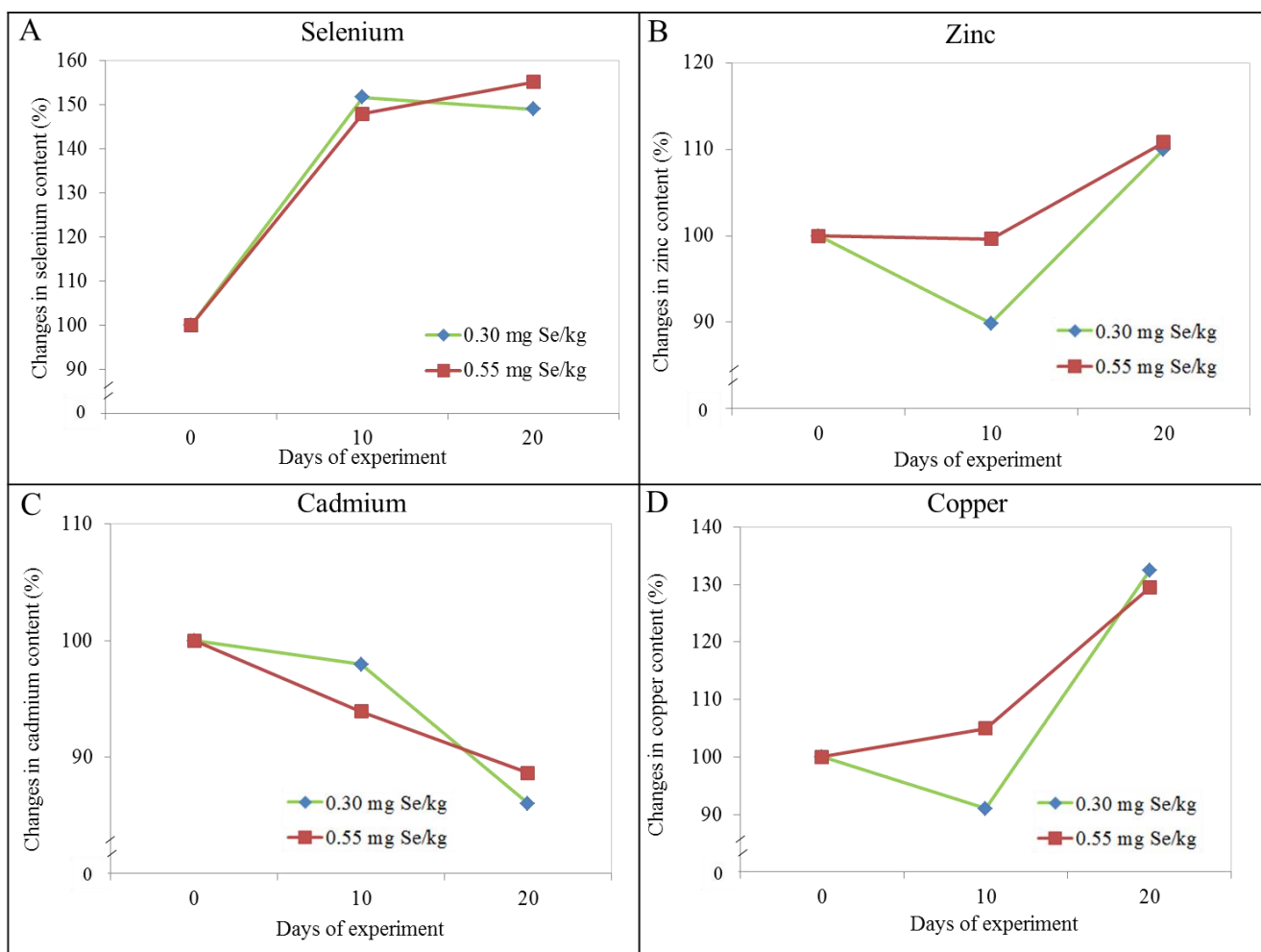


Figure 6. Effect of dosed selenium on levels of (A) selenium, (B) zinc, (C) cadmium and (D) copper in samples of erythrocytes.

Wang et al. [43] supplemented diet of chickens with selenium in organic form in a dose of 0.15 mg/kg FM. Activity of GPx was significantly increased ($P < 0.05$); similarly as in our experiment SOD activity increased ($P < 0.05$) under the reduction of MDA production ($P < 0.05$). These parameters were measured in blood serum. On the contrary, another study describes that an absence of selenium leads to the significant reduction of GPx activity ($P < 0.05$) and significant increase in SOD activity (P

<0.05) in the kidneys and blood serum of guinea pigs [44]. Sochor et al. [45] in the experiments on Norway rats determined significantly increased activity of GPx and increased levels of reduced glutathione in animals supplemented with selenium in organic form at a dose of 1.5 mg Se/kg FM in comparison with animals supplemented with the same dose of selenium in inorganic form. In the experiment, which lasted for 5 months, were pigs supplemented with selenium in organic form at a dose of 0.3 mg Se/kg FM. Pigs supplemented by this selenium form demonstrated significantly higher activity of GPx ($P < 0.05$) and significantly higher levels of hemoglobin and total proteins in blood serum ($P < 0.05$) [46]. Our study correlates with these results. Some other studies document increase in GPx activity in erythrocytes of different animal species supplemented with selenium in diet [47-50]. In our experiment the GPx activity was significantly increased already after relative short time of supplementation with selenium (10th day of the experiment). Experiment that was carried out by Oda et al. [51] proved that decrease in selenium content in organism leads to a reduction of reduced glutathione in testes of experimental animals. Experiment on Norway rats confirmed fact that selenium at a dose of 1 mg Se/kg FM (sodium selenite) reduces production of MDA [52]. Reduction in MDA content is being associated with increasing selenium supplementation (and thus with the its increasing concentration in the tissues) and with increased activity of GPx in mice [53], Norway rats [54], poultry [55], and cattle [56]. In our experiment selenium supplementation had no effect on the production of MDA in any group of sows that was included in the experiment. As we can conclude from this fact, duration of the experiment, which was set to 20 days, was too short to cause reduction in the MDA content in investigated samples. On the other hand, it should be noted that organisms after birth are exposed to higher oxidative stress [57].

In the experiment, where growing chickens were supplemented with selenium at a dose of 0.5 mg Se/kg FM in the diet, supplemented and unsupplemented groups of animals had no significant differences in the level of zinc in the blood serum and concentration of copper was similarly to our experiment increased but without statistical significance in comparison with the group of animals without supplemented selenium. Activity of GPx was significantly increased ($P < 0.05$) in the group with an income of 0.5 mg Se/kg FM in comparison with the group without selenium supplementation [55]. Pechova et al. [58] compared two groups of goats. Experimental group was supplemented with selenium in the diet at a dose of 0.3 mg/kg FM (organic form of selenium – yeasts enriched by selenium), second experimental group of goats served as a control group. Concentration of selenium in the blood of animals of experimental group was 3.5 times higher ($P < 0.001$) after six weeks compared with the control group. We observed the increase of selenium in erythrocytes by 52 % in the Se2 group of sows in our experiment. Pechova et al. [58] found no significant differences in the concentration of copper and zinc in both groups of goats. Also Cortinhas et al. [59] who in similar experiment supplemented cows with organic form of selenium for 60 days found no significant differences in levels of zinc and copper in blood of control and experimental animals. At supplementation of selenium and zinc in dairy cattle at a dose of 0.3 mg Se/FM + 40 mg Zn/FM Mudgal et al. [60] stated that this dose did not affect (in contrast to our study) level of copper in blood. On the other hand, they determined significant increase in content of thyroidal hormones. This finding is supported by the study of Qin et al. [61]. In the study, which was performed in Poland, a research team of Tomza-Marciniak et al. [62] monitored relations between concentrations of selenium and heavy metal ions in

milk of dairy cattle. They found negative correlation between concentrations of selenium and cadmium ($P < 0.05$). Increasing concentration of selenium in blood led to the decrease in cadmium level in blood. This fact was observed also in our experiment, where the highest level of cadmium was determined in erythrocytes of the control unsupplemented group and the lowest in the Se1 and Se2 groups supplemented with selenium in organic form ($P < 0.05$). Supplementation of diet with selenium may reduce impact of cadmium that has strong pro-oxidative properties [11,63]. It has been established in the study focused on the human population that increased levels of cadmium, lead and arsenic are responsible for an increase of MDA and GSH levels in blood [64,65]. Increased level of MDA is an important marker of oxidation stress that may lead to the increased risk of cancer. Patients with higher levels of selenium in blood showed reduced content of MDA and increased activity of GPx [66-68]. This fact was confirmed also in our experiment, where sows supplemented by selenium showed increased level of GSH; however, reduced content of MDA in the experimental groups of animals supplemented by 0.30 a 0.55 mg Se/kg FM was not confirmed. Increased level of zinc that was not affected by selenium supplementation has, according to many authors, a direct effect on metallothionein, which serves as a protection against free oxygen radicals (generally reactive oxygen species) [69-74]. This effect was observed also in plants, where increased level of selenium was accompanied by reduced level of cadmium in leaves [75]; this fact has been confirmed also by work of Krystofova et al. [76]. High amounts of heavy metals can also affect the antioxidant capacity in fodder resulting in an increased amount of oxidized glutathione in different species of grasses [65]. Similar results were observed also in the case of flax [71], maize [77], and tobacco [78]. High intake of heavy metals in the organism can cause oxidative stress [65,78] and reduce levels of antioxidant enzymes. Hill et al [79] at the measurement of content of zinc and copper in the blood of pigs observed increased activity of SOD. Our experiments confirmed this fact. We observed increased activity of SOD in the Se1 and Se2 experimental groups of sows, where increased concentrations of copper in erythrocytes were determined.

5. CONCLUSIONS

In our experiment, which was based on supplementation of selenium in organic form, we monitored its effect on the antioxidant status of postpartum sows. According to our results, the doses of selenium 0.30 and 0.55 mg/kg FM significantly increased the activity of the antioxidant enzymes glutathione peroxidase, superoxide dismutase and glutathione-S-transferase. We determined also a significant increase in antioxidant activity measured by FRAP, FR and ABTS methods. The amount of reduced glutathione was also statistically significantly higher in both experimental groups. The levels of MDA and SH- groups were not significantly affected by the supplementation with selenium in any of the experimental groups. Selenium and copper were significantly increased in erythrocytes of the both groups of sows supplemented with selenium (Se1 and Se2). On the contrary, the level of cadmium in erythrocytes of supplemented animals was significantly reduced. Selenium had no significant effect on content of zinc in erythrocytes of any group of sows included in the experiment. We can conclude that supplementation with selenium in organic form may reduce oxidation stress in postpartum sows and overcome this stressful period for organism.

ACKNOWLEDGEMENTS

This study was supported by the Research centrum SIX CZ.1.05/2.1.00/03.0072 and IGA TP3/2013. The authors would like to express their thanks to Martina Stankova for technical assistance.

References

1. C. H. Hu, Y. L. Li, L. Xiong, H. M. Zhang, J. Song and M. S. Xia, *Anim. Feed Sci. Technol.*, 177 (2012) 204.
2. X. A. Zhan, Y. Z. Qie, M. Wang, X. Li and R. Q. Zhao, *Biol. Trace Elem. Res.*, 142 (2011) 481.
3. A. G. Bertechini, E. J. Fassani, J. A. G. de Brito and P. R. Barrios, *Rev. Bras. Zootecn.*, 41 (2012) 624.
4. B. Lelyon, V. Chatellier and K. Daniel, *Inra Prod. Anim.*, 25 (2012) 67.
5. T. L. Xue, H. Hartikainen and V. Piironen, *Plant Soil*, 237 (2001) 55.
6. H. J. Hu, M. Wang, X. A. Zhan, X. Li and R. Q. Zhao, *Biol. Trace Elem. Res.*, 142 (2011) 471.
7. M. Pohanka, J. Sochor, B. Ruttkay-Nedecky, N. Cernei, V. Adam, J. Hubalek, M. Stiborova, T. Eckschlager and R. Kizek, *J. Appl. Biomed.*, 10 (2012) 155.
8. P. Sobrova, A. Vasatkova, J. Skladanka, M. Beklova, L. Zeman, R. Kizek and V. Adam, *Chem. Pap.*, 66 (2012) 1092.
9. O. Zitka, M. Kominkova, S. Skalickova, H. Skutkova, I. Provaznik, T. Eckschlager, M. Stiborova, V. Adam, L. Trnkova and R. Kizek, *Int. J. Electrochem. Sci.*, 7 (2012) 10544.
10. A. Kleckerova, P. Sobrova, O. Krystofova, J. Sochor, O. Zitka, P. Babula, V. Adam, H. Docekalova and R. Kizek, *Int. J. Electrochem. Sci.*, 6 (2011) 6011.
11. D. Hynek, J. Prasek, J. Pikula, V. Adam, P. Hajkova, L. Krejcova, L. Trnkova, J. Sochor, M. Pohanka, J. Hubalek, M. Beklova, R. Vrba and R. Kizek, *Int. J. Electrochem. Sci.*, 6 (2011) 5980.
12. O. Krystofova, P. Majzlik, J. Zehnalek, L. Havel, V. Adam and R. Kizek, *Lis. Cukrov. Repar.*, 126 (2010) 409.
13. M. Pohanka, D. Hynek, A. Kracmarova, J. Kruseova, B. Ruttkay-Nedecky, J. Sochor, V. Adam, J. Hubalek, M. Masarik, T. Eckschlager and R. Kizek, *Int. J. Electrochem. Sci.*, 7 (2012) 11978.
14. O. Rop, T. Jurikova, J. Sochor, J. Mlcek and D. Kramarova, *J. Food Qual.*, 34 (2011) 187.
15. O. Rop, J. Mlcek, T. Jurikova, M. Valsikova, J. Sochor, V. Reznicek and D. Kramarova, *J. Med. Plants Res.*, 4 (2010) 2431.
16. O. Rop, V. Reznicek, J. Mlcek, T. Jurikova, J. Balik, J. Sochor and D. Kramarova, *Hortic. Sci.*, 38 (2011) 63.
17. O. Rop, J. Sochor, T. Jurikova, O. Zitka, H. Skutkova, J. Mlcek, P. Salas, B. Krska, P. Babula, V. Adam, D. Kramarova, M. Beklova, I. Provaznik and R. Kizek, *Molecules*, 16 (2011) 74.
18. J. Sochor, P. Babula, B. Krska, A. Horna, I. Provaznik, J. Hubalek and R. Kizek, in J. Jan, R. Jirik, R. Kolar, J. Kolarova, J. Kozumplik, I. Provaznik (Editors), *Analysis of Biomedical Signals and Images*, Brno Univ Technology Vut Press, Brno, 2010, p. 209.
19. J. Sochor, P. Majzlik, P. Salas, V. Adam, L. Trnkova, J. Hubalek and R. Kizek, *Lis. Cukrov. Repar.*, 126 (2010) 414.
20. J. Sochor, H. Skutkova, P. Babula, O. Zitka, N. Cernei, O. Rop, B. Krska, V. Adam, I. Provaznik and R. Kizek, *Molecules*, 16 (2011) 7428.
21. J. Sochor, O. Zitka, H. Skutkova, D. Pavlik, P. Babula, B. Krska, A. Horna, V. Adam, I. Provaznik and R. Kizek, *Molecules*, 15 (2010) 6285.
22. B. Prieto-Simon, M. Cortina, M. Campas and C. Calas-Blanchard, *Sens. Actuator B-Chem.*, 129 (2008) 459.
23. L. Campanella, E. Martini and M. Tomassetti, *Talanta*, 66 (2005) 902.
24. M. F. Barroso, C. Delerue-Matos and M. Oliveira, *Food Chem.*, 132 (2012) 1055.
25. W. Andlauer and J. Heritier, *Food Chem.*, 125 (2011) 1517.

26. W. Ying, C. Calas-Blanchard, M. Cortina-Puig, B. H. Liu and J. L. Marty, *Electroanalysis*, 21 (2009) 1395.
27. M. Kremplova, L. Krejcova, D. Hynek, P. Barath, P. Majzlik, V. Horak, V. Adam, J. Sochor, N. Cernei, J. Hubalek, R. Vrba and R. Kizek, *Int. J. Electrochem. Sci.*, 7 (2012) 5893.
28. H. Park and Y. K. Choung, *Hum. Ecol. Risk Assess.*, 13 (2007) 1147.
29. Y. L. Hong, S. L. Yeh, C. Y. Chang and M. L. Hu, *Clin. Biochem.*, 33 (2000) 619.
30. G. L. Ellman, *Arch. Biochem. Biophys.*, 82 (1959) 70.
31. D. Potesil, J. Petrlova, V. Adam, J. Vacek, B. Klejdus, J. Zehnalek, L. Trnkova, L. Havel and R. Kizek, *J. Chromatogr. A*, 1084 (2005) 134.
32. J. Petrlova, R. Mikelova, K. Stejskal, A. Kleckerova, O. Zitka, J. Petrek, L. Havel, J. Zehnalek, V. Adam, L. Trnkova and R. Kizek, *J. Sep. Sci.*, 29 (2006) 1166.
33. V. Supalkova, D. Huska, V. Diopan, P. Hanustiak, O. Zitka, K. Stejskal, J. Baloun, J. Pikula, L. Havel, J. Zehnalek, V. Adam, L. Trnkova, M. Beklova and R. Kizek, *Sensors*, 7 (2007) 932.
34. O. Zitka, O. Krystofova, P. Sobrova, V. Adam, J. Zehnalek, M. Beklova and R. Kizek, *J. Hazard. Mater.*, 192 (2011) 794.
35. V. Diopan, K. Stejskal, M. Galiova, V. Adam, J. Kaiser, A. Horna, K. Novotny, M. Liska, L. Havel, J. Zehnalek and R. Kizek, *Electroanalysis*, 22 (2010) 1248.
36. O. Zitka, H. Skutkova, O. Krystofova, P. Sobrova, V. Adam, J. Zehnalek, L. Havel, M. Beklova, J. Hubalek, I. Provaznik and R. Kizek, *Int. J. Electrochem. Sci.*, 6 (2011) 1367.
37. O. Zitka, M. A. Merlos Rodrigo, V. Adam, N. Ferrol, M. Pohanka, J. Hubalek, J. Zehnalek, L. Trnkova and R. Kizek, *J. Hazard. Mater.*, 203-204 (2012) 257.
38. V. Adam, I. Fabrik, V. Kohoutkova, P. Babula, J. Hubalek, R. Vrba, L. Trnkova and R. Kizek, *Int. J. Electrochem. Sci.*, 5 (2010) 429.
39. D. Huska, O. Zitka, O. Krystofova, V. Adam, P. Babula, J. Zehnalek, K. Bartusek, M. Beklova, L. Havel and R. Kizek, *Int. J. Electrochem. Sci.*, 5 (2010) 1535.
40. M. A. C. Danes, L. J. Chagas, A. M. Pedroso and F. A. P. Santos, *J. Dairy Sci.*, 96 (2013) 407.
41. Y. Y. Zhang, S. Z. Zhu, X. P. Wang, C. Y. Wang and F. C. Li, *Anim. Feed Sci. Technol.*, 169 (2011) 259.
42. P. Horoky, P. Jancikova, J. Sochor, D. Hynek, G. J. Chavis, B. Ruttkay-Nedecky, N. Cernei, O. Zitka, L. Zeman, V. Adam and R. Kizek, *Int. J. Electrochem. Sci.*, 7 (2012) 9643.
43. Y. X. Wang, X. A. Zhan, D. Yuan, X. W. Zhang and R. J. Wu, *Czech J. Anim. Sci.*, 56 (2011) 305.
44. T. V. Sirota, *Bull. Exp. Biol. Med.*, 149 (2010) 412.
45. J. Sochor, M. Pohanka, B. Ruttkay-Nedecky, O. Zitka, D. Hynek, P. Mares, L. Zeman, V. Adam and R. Kizek, *Cent. Eur. J. Chem.*, 10 (2012) 1442.
46. V. Sarkuniene, V. Oberauskas, D. Baranauskiene, D. Urbsiene, J. Kulpys, V. Andruleviciute and M. Sarkunas, *Vet. Zootec.*, 50 (2010) 88.
47. V. Petrovic, J. Novotny, V. Hisira, R. Link, L. Leng and G. Kovac, *Acta Vet. BRNO*, 78 (2009) 365.
48. J. C. Zhou, H. Zhao, J. G. Li, X. J. Xia, K. N. Wang, Y. J. Zhang, Y. Liu, Y. Zhao and X. G. Lei, *J. Nutr.*, 139 (2009) 1061.
49. D. T. Juniper, R. H. Phipps, E. Ramos-Morales and G. Bertin, *Anim. Feed Sci. Technol.*, 149 (2009) 228.
50. W. B. Yue, C. X. Zhang, L. G. Shi, Y. S. Ren, Y. S. Jiang and D. O. Kleemann, *Asian Australas. J. Anim. Sci.*, 22 (2009) 365.
51. S. S. Oda and Z. K. El-Maddawy, *Exp. Toxicol. Pathol.*, 64 (2012) 813.
52. M. Guney, *Biol. Trace Elem. Res.*, 149 (2012) 234.
53. X. M. Ren, G. G. Wang, D. Q. Xu, K. Luo, Y. X. Liu, Y. H. Zhong and Y. Q. Cai, *Food Chem. Toxicol.*, 50 (2012) 3521.
54. C. Sanchez-Gonzalez, C. Bermudez-Pena, F. Guerrero-Romero, C. E. Trenzado, M. Montes-Bayon, A. Sanz-Medel and J. Llopis, *Br. J. Nutr.*, 108 (2012) 893.

55. S. G. Harsini, M. Habibiyani, M. M. Moeini and A. R. Abdolmohammadi, *Biol. Trace Elem. Res.*, 148 (2012) 322.
56. S. S. Sahoo, R. C. Patra, P. C. Behera and D. Swarup, *Vet. Res. Commun.*, 33 (2009) 281.
57. M. Chai, G. Barker, R. Menon and M. Lappas, *Placenta*, 33 (2012) 604.
58. A. Pechova, L. Sevcikova, L. Pavlata and R. Dvorak, *Vet. Med.*, 57 (2012) 394.
59. C. S. Cortinhas, J. E. de Freitas, J. D. Naves, M. A. D. Porcionato, L. Silva, F. P. Renno and M. V. dos Santos, *Rev. Bras. Zootecn.*, 41 (2012) 1477.
60. V. Mudgal, A. K. Garg, R. S. Dass and V. P. Varshney, *Biol. Trace Elem. Res.*, 145 (2012) 304.
61. F. Qin, X. P. Zhu, W. Zhang, J. P. Zhou, S. W. Zhang and Z. H. Jia, *Biol. Trace Elem. Res.*, 143 (2011) 1480.
62. A. Tomza-Marciniak, B. Pilarczyk, M. Bakowska, R. Pilarczyk, J. Wojcik, A. Marciniak and D. Hendzel, *Biol. Trace Elem. Res.*, 144 (2011) 517.
63. I. Zwolak and H. Zaporowska, *Cell Biol. Toxicol.*, 28 (2012) 31.
64. J. Gumulec, M. Raudenska, M. Hlavna, T. Stracina, M. Sztalmachova, V. Tanhauserova, L. Pacal, B. Ruttkay-Nedecky, J. Sochor, O. Zitka, P. Babula, V. Adam, R. Kizek, M. Novakova and M. Masarik, *Exp. Ther. Med.*, 5 (2013) 479.
65. J. Skladanka, V. Adam, O. Zitka, O. Krystofova, M. Beklova, R. Kizek, Z. Havlicek, P. Slama and A. Nawrath, *Int. J. Environ. Res. Public Health*, 9 (2012) 3789.
66. S. Guzel, L. Kiziler, B. Aydemir, B. Alici, S. Ataus, A. Aksu and H. Durak, *Biol. Trace Elem. Res.*, 145 (2012) 23.
67. M. Salman, R. Rehman, T. Mahmud, Z. Waheed Uz, U. Shafique, J. Anwar and S. Z. Ali, *J. Chem. Soc. Pak.*, 33 (2011) 869.
68. Y. B. Cihan, S. Sozen and S. O. Yildirim, *Biol. Trace Elem. Res.*, 144 (2011) 360.
69. S. Krizkova, M. Ryvolova, J. Hrabeta, V. Adam, M. Stiborova, T. Eckschlager and R. Kizek, *Drug Metab. Rev.*, 44 (2012) 287.
70. M. Masarik, J. Gumulec, M. Sztalmachova, M. Hlavna, P. Babula, S. Krizkova, M. Ryvolova, M. Jurajda, J. Sochor, V. Adam and R. Kizek, *Electrophoresis*, 32 (2011) 3576.
71. J. Najmanova, E. Neumannova, T. Leonhardt, O. Zitka, R. Kizek, T. Macek, M. Mackova and P. Kotrba, *Ind. Crop. Prod.*, 36 (2012) 536.
72. L. Krejcová, I. Fabrik, D. Hynek, S. Krizkova, J. Gumulec, M. Ryvolova, V. Adam, P. Babula, L. Trnkova, M. Stiborova, J. Hubalek, M. Masarik, H. Binkova, T. Eckschlager and R. Kizek, *Int. J. Electrochem. Sci.*, 7 (2012) 1767.
73. M. Ryvolova, V. Adam and R. Kizek, *J. Chromatogr. A*, 1226 (2012) 31.
74. M. Ryvolova, D. Hynek, H. Skutkova, V. Adam, I. Provaznik and R. Kizek, *Electrophoresis*, 33 (2012) 270.
75. L. Lin, W. H. Zhou, H. X. Dai, F. B. Cao, G. P. Zhang and F. B. Wu, *J. Hazard. Mater.*, 235 (2012) 343.
76. O. Krystofova, V. Adam, P. Babula, J. Zehnalek, M. Beklova, L. Havel and R. Kizek, *Int. J. Environ. Res. Public Health*, 7 (2010) 3804.
77. O. Zitka, M. A. Merlos, V. Adam, N. Ferrol, M. Pohanka, J. Hubalek, J. Zehnalek, L. Trnkova and R. Kizek, *J. Hazard. Mater.*, 203 (2012) 257.
78. O. Krystofova, O. Zitka, S. Krizkova, D. Hynek, V. Shestivska, V. Adam, J. Hubalek, M. Mackova, T. Macek, J. Zehnalek, P. Babula, L. Havel and R. Kizek, *Int. J. Electrochem. Sci.*, 7 (2012) 886.
79. G. M. Hill, J. E. Link, L. Meyer and K. L. Fritsche, *J. Anim. Sci.*, 77 (1999) 1762.