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Effects of commercial selenium products on glutathione peroxidase activity and semen quality in stud boars

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ABSTRACT

The aim of this study was to determine how dietary supplementation of inorganic and organic selenium affects the selenium concentration and glutathione peroxidase activity in blood and sperm of sexually mature stud boars. Twenty-four boars of the Large White, Landrace, Pietrain and Duroc breeds of optimal breeding age (on average 2.5 years old) were used. The study lasted 90 days. The boars were randomly assigned to one of three dietary treatment groups: T1 = control; no added selenium ($n = 8$ boars), T2 = added 0.3 ppm inorganic selenium (sodium selenite, Microgran® Se 1% BMP) ($n = 8$ boars), and T3 = added 0.3 ppm organic selenium (Se-yeast, Sel-Plex 2000®) ($n = 8$ boars). The concentration of selenium was determined in whole blood and semen, while the activity of glutathione peroxidase (GPx) was measured in blood plasma and semen. In order to measure GPx activity in semen, reactivation of the enzymatic GPx activity was performed. The determined selenium concentration in blood was lowest in the non-supplemented group of boars. Blood plasma GPx activity was higher in boars fed organic selenium than in boars fed a diet without supplemented selenium. While the supplementation of sodium selenite significantly increased GPx activity in boar semen. The highest-concentration of selenium in semen at the end of the trial was determined in the group of boars supplemented with organic selenium, somewhat lower in boars fed supplemented inorganic selenium, and the lowest in the non-supplemented group of boars. The only significant difference between the selenite and Se-yeast diet supplementation was observed in the Se concentration of the semen. The supplementation of selenium affected semen quality, and organic selenium improved the progressive motility of the spermatozoa and increased their resistance in hypo-osmotic and thermal tests. The storage ability of short term preserved semen was improved by organic selenium supplementation, as well as also increasing the fertility rate in gilts.

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Abbreviations: AI, artificial insemination; β-NADPH, β-nicotinamide adenine dinucleotide 2′-phosphate; GPx, glutathione peroxidase; LMNS, live morphologically normal spermatozoa; NRC, National Research Council; PHGPx, phospholipid hydroperoxide glutathione peroxidase; PUFA, polyunsaturated fatty acids; TBH, *t*-butyl hydroxide; TGR, thioredoxin/glutathione reductase; $^{P}H_2O$, type 2 pure water; XFM, X-ray fluorescence microscopy.

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1. Introduction

Selenium is an essential trace element necessary for reproduction and embryonic growth. Selenium is known to be required for testosterone biosynthesis as well as for formation and development of spermatozoa (Behne et al., 1996). It has been shown that the mammalian selenium protein thioredoxin/glutathione reductase (TGR) has a role in disulfide bridge formation during the sperm maturation process (Su et al., 2005).

Phospholipid hydroperoxide glutathione peroxidase (PHGPx) or GPx4 is the fourth described selenoprotein from the family of glutathione peroxidases (GPxs) (Ursini et al., 1982). GPx4 is specific due to the fact that beside glutathione it can use a wide array of reducing substrates (Aumann et al., 1997) and is mostly present in testicles (Imai et al., 1995). The primary structural difference between GPxs is that GPx4 is a protein monomer while all other GPxs are tetramers. Since GPx4 can exist close to the membrane, it has important consequences for membrane protection (*in situ*) (Ursini et al., 1982). Recently, three different isoforms of GPx4: mitochondrial, non-mitochondrial and sperm nuclei specific forms have been characterized (Schneider et al., 2009).

Alvarez and Storey (1989) were the first to describe the role played by GPx in protecting mammalian spermatozoa from loss of motility caused by spontaneous lipid peroxidation. Many years later, it was reported that failure of GPx expression in spermatozoa was correlated with infertility in humans (Imai et al., 2001; Foresta et al., 2002). Within the last 6 years, the development of mouse GPx knockout models (Schneider et al., 2009; Imai et al., 2009; Liang et al., 2009) resulting in infertility or subfertility have demonstrated that GPx does indeed play an important role in mammalian sperm physiology.

Since the role that GPx plays in the removal of free radicals is well known, it is then not suprising that GPx is important in the protection of spermatozoa during spermatogenesis. The explaination for this is clear since lipid peroxidation was significantly increased either by GPx inhibition with mercaptosuccinate, a specific Se dependant GPx inhibitor, or by a decrease in the level of available reduced glutathione—GSH (Alvarez and Storey, 1989). The role of GPx in the protection of spermatozoa and male germinative tissue from peroxidative damage is even more important since catalase in mammal spermatozoa or sperm plasma is not present in significant amounts (Alvarez et al., 1987; Bilodeau et al., 2000). Therefore, in the absence of catalase, GPx plays a crucial role in the protection of spermatozoa during development and of testicular tissue from the effects of hydrogen peroxide. The GPx in spermatids (the developmental stage of spermatozoa) is in the form of the active peroxidase, while in mature spermatozoa it is transformed into structural protein (inactivated enzyme) which is incorporated in the mitochondrial capsule of spermatozoa. The mitochondrial capsule and the kinetic apparatus are located in the middle section of spermatozoa (Ursini et al., 1999). Alternatively to catalase, which reacts only with H_2O_2 as substrate and in relatively high-concentrations ($>10^{-6}$ M), GPx allows the subtle regulation of the H_2O_2 concentration. GPx also enables the repair of complex molecules (an example is cell membrane lipids) which are damaged by H_2O_2.

The spermatozoal plasma membrane is rich in PUFA (polyunsaturated fatty acids), the target of free radicals, which additionally emphasizes the significance of the protection afforded by GPx, as well as the fact that selenium is a necessary component of GPx, which makes the trace element selenium of utmost importance in reproduction. Also, widespread selenium deficiency can lead to lower egg production and decreased reproduction efficiency (Surai, 2002).

Mihailović et al. (1996) and Jovanović et al. (1998) have analyzed the selenium content in a total of 276 feed samples collected from 55 localities throughout Serbia and found the average selenium concentration to be 30.4 ± 25.3 μg/kg, which ranges from marginal deficiency (49.5 ± 25.3 μg/kg) in the northern part of the country to severe deficiency (8.8 ± 5.7 μg/kg) in the Sjenica-Pešter plateau in the Southwest region. Dimitrov et al. (2007) reported the beneficial effects of selenium supplementation on turkey semen. It is generally considered that organic forms of supplemented selenium have higher-biological availability when compared with inorganic forms in most domestic animals (Daniels, 1996), including swine (Mahan and Peters, 2004; Fortier and Matte, 2006).

The aim of this study was to determine how the supplementation of dietary inorganic and organic selenium affects the selenium concentration, GPx activity in blood and sperm as well as boar semen quality and fertility in sexually mature stud boars.

2. Materials and methods

2.1. Materials

Inorganic selenium (sodium selenite) Microgran® Se 1% BMP, was obtained from DSM Nutritional Products Ltd., Basel, Switzerland. Organic selenium (Se-yeast) Sel-Plex 2000® was obtained from Alltech Inc., Dublin, Ireland. HCl, 320331; HNO_3, 02650 (Fluka); H_2O_2, H0904; TraceCERT®, 1000 mg/L Pd in hydrochloric acid, 78437; glutathione reduced, G4251; glutathione reductase from baker's yeast, G3664; β-NADPH, N7505; β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate, 82059 (Fluka); *t*-butyl hydroxide, 58448; and 0.1 M $NaHCO_3$ in H_2O, 36486 were obtained from Sigma–Aldrich, St. Louis, MO, USA. Vacutainers, LH102 I.U., were purchased from BD, Plymouth, Great Britain; semen extender Androstar Plus® was purchased from Minitüb, Tiefenbach, Germany; and the disposable catheters, Golden Gilt® were purchased from Kruuse, Denmark.

2.2. Animals

Twenty-four boars of the Large White, Landrace, Pietrain and Duroc breeds of optimal breeding age (on average 2.5 years old), all previously selected for artificial insemination on the basis of their ability to produce high-quality ejaculates and on being trained to mount an artificial sow, were used for semen collection. To account for the potential difference in genetic lines, each treatment group of boars contained the same number of breed individuals (two boars each of Large White, Landrace, Pietrain and Duroc breeds). Furthermore, we have analyzed the results of breed-specificity and have found no difference between the breeds in terms of selenium status, semen quality, semen resistance or in fertility. Before the start of the experiment all boars were fed a diet supplemented with 0.3 mg/kg of inorganic selenium (sodium selenite). The boars were housed in individual pens and fed a diet based on corn–soybean meal up to 3 kg a day. The diet met or exceeded the National Research Council (NRC) nutrient recommendations for breeder boars (NRC, 1998). The control and treatment diets were identical, except for the additional selenium supplements. The boars were randomly assigned to one of three dietary treatments: T1 = control, no added selenium ($n = 8$ boars), T2 = added 0.3 ppm inorganic selenium ($n = 8$ boars), and T3 = added 0.3 ppm organic selenium ($n = 8$ boars). The composition of the diets is provided in Table 1.

Seventy-two crossbred gilts (Landrace sows × Yorkshire boars), averaging 8 months old and weighing between 100 and 110 kg, were used for artificial insemination (AI). The gilts were housed in individual pens and fed a diet based on corn–soybean meal that met or exceeded the NRC nutrient recommendations for gilts (NRC, 1998). During gestation all gilts were fed 1.8–2.2 kg/d of a 16.8% crude protein diet having 13.7 MJ DE/kg.

Experimental procedures used in this study were in accordance with guidelines of the European Community (Directive 86/609/EEC) and guidelines of the Ethics Committee of the Faculty of Veterinary Medicine, University of Belgrade, Serbia.

2.3. Sample collection

2.3.1. Semen

Prior to and during the experiment, semen was routinely collected weekly using the gloved-hand technique (King and Macpherson, 1973). Semen quality evaluation (ejaculate volume, spermatozoa concentration, progressive motility and semen morphology in raw semen) was done on day 0 of the study, and again on the 45th, 60th and 90th day of the study. The spermatozoa progressive motility in short term preserved semen was evaluated at the moment of production and during the storage period. Evaluation started at the 45th, 60th and 90th day of the study and lasted for 7 days on a daily basis.

The sperm-rich fraction of semen was used for determination of the selenium content and GPx activity. After determination of the spermatozoa concentration of semen by the SpermaCue™ spectrophotometer (Minitüb GmbH, Tiefenbach, Germany), the semen samples were standardized at 300×10^6 spermatozoa/mL and then frozen at −18 °C until needed. The activity of GPx was determined on the 60th and 90th day of the study, and the selenium concentration of the semen was determined on the 90th day of the study.

2.3.2. Blood

Samples of blood were obtained by venipuncture of the jugular vein at the end of the study (90th day). Blood was collected into vacutainers containing lithium heparin as anticoagulant. The blood samples for detection of GPx activity were centrifuged (2500 × g for 20 min at 20 °C) immediately after collection and processed for further analyses. The heparinized whole blood samples used for determination of selenium were frozen at −18 °C and kept for later processing.

Table 1
Composition of diet fed to each group of boars.

Diet component[a]	(% diet component)		
	T1 Control natural	T2 Inorganic selenium	T3 Organic selenium
Corn	32.0	32.0	32.0
Barley	28.3	28.3	28.3
Dehydrated Lucerne meal, 18% protein	10.0	10.0	10.0
Soybean meal	8.0	8.0	8.0
Wheat bran	10.0	10.0	10.0
Livestock yeast	0.7	0.7	0.7
Dicalcium phosphate	0.5	0.5	0.5
Salt	0.5	0.5	0.5
Fish meal, 62% protein	8	8	8
Premix 1	2	–	–
Premix 2	–	2	–
Premix 3	–	–	2

[a] Crude protein 18% CP; ME 2915 Mcal; Premix 1–3: Vitamin A, 2.7×10^5 I.U.; vitamin D_3, 3.4×10^4 I.U.; vitamin E, 580 mg; vitamin K_3, 33 mg; vitamin B1, 25 mg; vitamin B2, 130 mg; vitamin B3, 470 mg; vitamin B6, 30 mg; vitamin B12, 1 mg; niacin, 580 mg; choline, 8500 mg; Co, 8.5 mg; Cu, 2100 mg; Fe, 1800 mg, Mn 1330 mg; Zn 1800 mg. Selenium: Premix 1 = 0, Premix 2 = 15 mg (sodium selenite), Premix 3 = 15 mg (selenized yeast).

2.4. Determination of the selenium content

The selenium content of the feed samples was determined at the beginning of the study. The content of selenium in heparinized blood and raw semen was determined at the end of the study.

The selenium content was determined in homogenized feed samples (3 g), heparinized blood (1 mL) and raw semen samples (1 mL). The determined spermatozoa concentration in semen was adjusted to 300×10^6 spermatozoa mL^{-1} of semen in order to exclude the differences due to variable semen concentration.

Samples were treated with 10 mL of a digestion mixture (concentrated HNO_3/H_2O_2, 4:1), and digested in a microwave oven (MILESTONE TC, Sorisole, Italy) until the organic matter was fully oxidized. Samples were then treated with a solution of HCl (6 M) and palladium diluted with hydrochloric acid to reduce selenium to SeH_2. The generated hydride gas was injected into the electric heater (900 °C) and the result was recorded on the spectrophotometer. Analyses of each sample were performed in duplicate to increase the analysis accuracy.

The selenium content of samples was obtained by Atomic Absorption Spectrometry—HGAAS (THERMO SOLAAR S4 VP90 system, Thermo Fisher Scientific, Waltham, MA, USA) employing continuous hydride generation with a deuterium connector and selenium lamp (electricity current of 7 mA, wave length at 196 nm and 1 nm slit width).

2.5. Determination of glutathione peroxidase activity (EC 1.11.1.9.)

The GPx activity in blood plasma was determined at the end of the study and in semen on the 60th day and at the end of the study.

The GPx activity was determined using the method described by Günzler et al. (1974) with modifications by Sankari (1985). The principle of this method is based on measuring the NADPH consumption in enzyme systems where GPx is in surplus, and is used to reduce *t*-butyl hydroxide (TBH) with the participation of reduced glutathione (GSH) as the immediate hydrogen donor. The GSH concentration was maintained at a constant level by addition of the glutathione reductase (GR) enzyme, which utilizes NADPH for glutathione regeneration. Two minutes after addition of TBH (start of the reaction) the consumption of NADPH was measured for 3 consecutive minutes (in 60 s intervals) on a Cecil CE 2021 spectrophotometer (Cecil Instruments Ltd., Cambridge, UK) at 37 °C, $\lambda = 366$ nm. Type 2 pure water ($^{P}H_2O$) produced by the Millipore Elix Essential 10 UV water purification system (EMD Millipore Corp., Billerica, MA, USA) was used to prepare stock solutions of GR and GSH, and 0.1% $NaHCO_3$ in $^{P}H_2O$ was used for the preparation of NADPH. A low TBH concentration (<2.32 mM) allows the exclusive measurement of selenium dependent GPx activity (Burk et al., 1978). The difference in the rate of NADPH consumption between the test and blank samples represents the GPx activity. The hemoglobin (Hb) concentration was determined using the cyanmethemoglobin method described by Crosby et al. (1954). The GPx activity in heparinized blood plasma was calculated for 110 g Hb/L of blood.

It was necessary to reactivate the enzyme in mature spermatozoa prior to analysis, because the raw semen in our study had GPx activity below the detection limit. In order to reactivate the GPx in semen, low temperature lyses treatment was applied (freezing at −18 °C) over a 30 day period. The frozen semen samples were then thawed in a water bath at 38 °C for 2 min, and the GPx activity was determined as previously described for blood plasma samples. GPx activity of lysed semen was calculated on the basis of 300×10^6 spermatozoa/mL of semen.

The results were expressed in microkatals per liter (μkat/L), where one katal is the SI unit equivalent to the amount of enzyme required to convert 1 mole of substrate per second.

2.6. Semen quality analyses

The ejaculate volume (sperm rich fraction) was measured in a graduated glass cylinder. The determination of spermatozoa concentration (number of spermatozoa $\times 10^6$/mL of semen) was obtained on a SpermaCue™ spectrophotometer.

The progressive motility of spermatozoa in both raw and short term preserved semen was determined by direct microscopy with 100× magnification at 38 °C (Krüss MBL 2100, Hamburg, Germany) of a pressed droplet (15 μL) in 3 different visible fields by the same person.

Short term semen preservation was carried out according to the extender producer manual by using Androstar Plus® semen extender (Minitüb; Tiefenbach, Germany). The insemination dose contained 3×10^9 spermatozoa in the 100 mL volume. The short preserved semen was stored in a temperature controlled semen storage box at 17 ± 1 °C, and rotated daily in order to prevent spermatozoa precipitation. The spermatozoa progressive motility in short term preserved semen was expressed on a four grade scale system as described by Mortimer (1994).

The percentage of live morphologically normal spermatozoa (LMNS) was evaluated by the staining method of Blom (1950), and commercially prepared eosine/nigrosine stain (Hankok 1® Alfapanon, Novi Sad, Serbia) was used. The gross spermatozoa morphology using the staining procedure to determine the live/dead sperm ratio was examined at 400× magnification (Krüss MBL 2100, Hamburg, Germany) on a total number of 200 spermatozoa per sample (Bellwood and Andrasik-Catton, 2014).

2.7. The semen resistance tests

The semen thermo resistance test was performed over the duration of 45 min at the temperature of 42 °C according to the method of Fiser et al. (1991). The evaluation of spermatozoa progressive motility in thermal resistance tests was carried out as previously described above for raw and short term preserved semen.

The hypo-osmotic swelling test (HOS test) was performed using the modified method of Vazquez et al. (1997). A 150 mOsm/L fructose hypo-osmotic solution was used in the tests. Sterile Biopur Safe-lock Tubes® (1.5 mL, Eppendorf, Germany) were filled with 900 μL of the hypo-osmotic solution and 100 μL of fresh undiluted semen. The tubes were capped and incubated in a water bath at 37 °C for 60 min. After incubation, 15 μL of the incubated fluid was placed on a microscope slide, covered with a cover glass and examined with a phaze contrast microscope (Krüss MBL 2100, Hamburg, Germany) at 400× magnification. The spermatozoa with swollen and coiled tails, from the center onward, were deemed intact and functionally active. A total of 200 spermatozoa were examined in each preparation in order to achieve 0.5% accuracy.

2.8. The fertility evaluation

Semen fertility was evaluated in a biological test using the AI of gilts at the end of the study (90th day). Short term preserved boar semen was used for AI up to five days from the production date. The insemination dose per service contained 3×10^9 spermatozoa in the 100 mL volume. Prior to insemination, microscopic examination of preserved semen was performed, and all semen samples had spermatozoa with over 45% progressive motility. Three gilts were inseminated with the semen from each boar, making it 24 gilts per group or 72 gilts in total. In order to inseminate the gilts in a short period of time and exclude the possible influence of inadequate detection of estrus, the estrus cycle in all gilts was synchronized. The gilts received i.m. injections of 750 I.U. PMSG (Folligon®, Intervet, Boxmeer, The Netherlands) and 72 h later received 500 I.U. hCG (Chorulon®, Intervet, Boxmeer, The Netherlands). The gonadotropin-stimulated gilts with visible estrus signs ($n = 72$, 24 gilts per each group) were inseminated twice, 24 and 48 h after hCG injection. The day following the second insemination was designated as the first day of pregnancy in all gilts. The insemination was carried out using a disposable catheter (Golden Gilt®, Kruuse, Denmark), while the pregnancy check was made using a 3.5 MHz transabdominal B-mode ultrasound (Mindray DP 6600, Shenzhen Upnmed Equipment Co. Ltd., Guangdong, China).

2.9. Statistical analyses

Statistical analyses of intergroup differences were performed by ANOVA, and then the Tukey multiple comparison test was used to separate the means, chi square test of proportions was used in the evaluation of the fertility success. The software package Prism Pad v. 5.0 (Graph Pad Software Inc., San Diego, CA, USA) was used for statistical calculations. Data were expressed as means ± standard deviation. Differences with $P < 0.05$ and $P < 0.01$ were considered statistically significant or highly significant, respectively.

3. Results

3.1. Selenium content

The selenium content of the control diet (group T1) was 0.098 mg/kg, the selenium content of the diet with supplemented inorganic selenium (group T2) was 0.389 mg/kg, and the selenium content of the diet with supplemented organic selenium (group T3) was 0.387 mg/kg.

At the end of the study (90th day), the determined selenium content in boars blood was higher when they were fed the T2 (2.33 ± 0.33 μg/mL) and T3 (2.69 ± 0.32 μg/mL) diets ($P < 0.01$) compared to the control group (T1) (1.49 ± 0.29 μg/mL), which are shown in Table 2.

A similar trend was observed with the selenium content in semen of stud boars. The selenium content in semen of the supplemented organic selenium group T3 (113.0 ± 7.09 ng/mL) was higher than that of group T2 ($P < 0.01$) (100.3 ± 8.69 ng/mL) and of the control group T1 ($P < 0.05$) (91.6 ± 10.6 ng/mL). The concentration of selenium in semen is shown in Table 2.

3.2. Glutathione peroxidase activity

The lowest activity of the selenoenzyme GPx at the end of the study (90th day) was observed in blood plasma from boars in the control group T1 (25.1 ± 6.88 μkat/L), and was the highest in blood plasma from boars supplemented with organic selenium in group T3 (32.0 ± 3.72 μkat/L). The difference was statistically significant ($P < 0.05$) and is shown in Table 2. The group of boars fed a diet supplemented with inorganic selenium (group T2) had GPx activity in blood plasma of 25.6 ± 0.76 μkat/L.

On the 60th day of the study, reactivated semen GPx activity was not significantly different between treatment groups. It was 1.75 ± 0.35 μkat/L in group T1, 2.29 ± 0.60 μkat/L in group T2 and 1.93 ± 0.71 μkat/L in group T3. On the 90th day of the study, the highest reactivated GPx activity was measured in group T2 (2.63 ± 0.56 μkat/L) and the lowest reactivated GPx activity in semen was observed in boars from the control group T1 (1.77 ± 0.31 μkat/L) ($P < 0.05$). Interestingly, the measured

Table 2
The selenium status of stud boars.

		T1, Control no supplemented selenium	T2, Supplemented inorganic selenium 0.3 ppm	T3, Supplemented organic selenium 0.3 ppm
The selenium content in whole blood of stud boars (μg/mL)	$\bar{x} \pm SD$	1.49 ± 0.29^{A}	2.33 ± 0.33^{B}	2.69 ± 0.32^{B}
	Std. Error	0.12	0.14	0.13
	CV	19.4%	14.2%	12.0%
The selenium content (ng/g calculated at 300×10^6 spermatozoa/mL) in semen of stud boars at the 90th day of the study	$\bar{x} \pm SD$	91.6 ± 10.6^{A}	100.3 ± 8.69^{a}	$113.0 \pm 7.03^{B,b}$
	Std. Error	3.76	3.07	2.49
	CV	11.6%	8.7%	6.2%
Blood plasma GPx activity (μkat/L) in boars (calculated at 110 g Hb/L blood) at the 90th day of the study	$\bar{x} \pm SD$	25.1 ± 6.88^{a}	25.6 ± 0.77	32.1 ± 3.72^{b}
	Std. Error	3.08	0.34	1.66
	CV	27.4%	3.0%	11.6%
Semen GPx activity (μkat/L) (calculated at 300×10^6 spermatozoa/mL) at the 90th day of the study	$\bar{x} \pm SD$	1.77 ± 0.31^{a}	2.63 ± 0.56^{b}	2.26 ± 0.62
	Std. Error	0.13	0.23	0.25
	CV	17.6%	21.1%	27.5%

The values with unlike superscripts differ at $P < 0.05$ (small letters) or $P < 0.01$ (capital letters).

reactivated GPx activity in group T3 (2.26 ± 0.62 μkat/L) was not significantly different ($P > 0.05$) from that in group T2 or in the control group T1, shown in Table 2, this finding needs further investigation.

3.3. Semen quality

The ejaculate volume (mL) was not significantly different between all groups of boars at the end of the study (206.3 ± 43.1 mL in T1, 240. 0 ± 99.4 mL in T2 and 212.5 ± 49.2 mL in the T3 group).

Semen concentration at the end of the study (number of spermatozoa/mL) was highest ($P > 0.05$) in the group of boars supplemented with organic selenium ($411.9 \pm 79.0 \times 10^6$ spermatozoa/mL in group T3), lower in the group fed inorganic selenium (T2) $369.1 \pm 127.6 \times 10^6$ spermatozoa/mL, and lowest in the control group of boars (T1) $367.3 \pm 96.8 \times 10^6$ spermatozoa/mL.

The total number of spermatozoa per ejaculate was not statistically different during the study period, and at the end of the study (90th day) the totals ranged between $73.2 \pm 14.1 \times 10^9$ spermatozoa in the control (T1), $81.4 \pm 27.6 \times 10^9$ spermatozoa in T2, and $85.9 \pm 19.4 \times 10^9$ spermatozoa in the T3 group of boars.

The percentage of progressively motile spermatozoa in raw semen ranged from 83.1 ± 2.58% to 88.1 ± 2.59% during the study and is shown in Fig. 1. The percentage of live morphologically normal spermatozoa is shown in Fig. 2.

3.4. Semen resistance tests

The percentage of metabolically inactive spermatozoa (dead spermatozoa), determined by the HOS test at the end of the study (90th day), was higher in the group of boars fed no supplemented selenium (24.4 ± 13.58%) or inorganic selenium

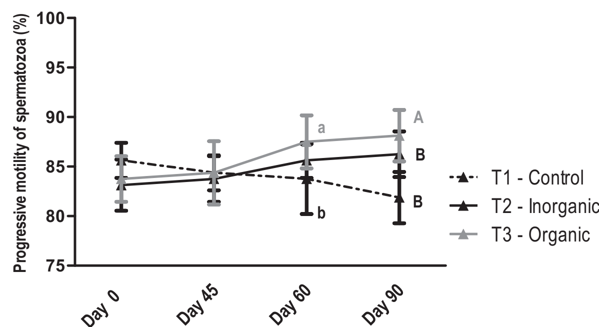

Fig. 1. The mean percentage (±SD) of progressive motile spermatozoa in raw semen. The values with unlike superscripts differ at $P < 0.05$ (small letters) or $P < 0.01$ (capital letters). SD, standard deviation.

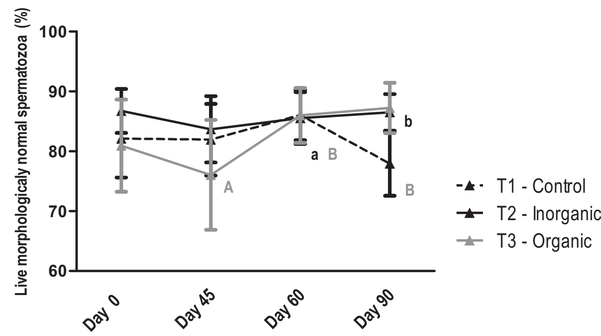

Fig. 2. The mean percentage (±SD) of live morphologically normal spermatozoa in boar ejaculate. The values with unlike superscripts differ at $P < 0.05$ (small letters) or $P < 0.01$ (capital letters). SD, standard deviation.

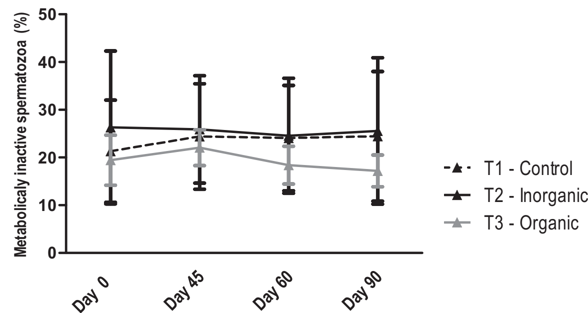

Fig. 3. The mean percentage (±SD) of metabolically inactive spermatozoa in boar ejaculate. The percentage of metabolically inactive spermatozoa (dead spermatozoa), determined by the HOS test during the study ($P > 0.05$ at all times). SD, standard deviation.

(25.6 ± 15.3%) than for the group of boars fed a diet supplemented with organic selenium (17.2 ± 3.32%) ($P > 0.05$), shown in Fig. 3.

A significant decrease ($P < 0.05$) of spermatozoa progressive motility upon exposure to the thermo resistance test in the control group of boars (T1), between the beginning of the study (60.5 ± 14.6%, day 0) and the end of the study (44.5 ± 6.85%, 90th day), was recorded. At the end of the study (90th day), significantly higher ($P < 0.05$) progressive motility was observed in the group of boars fed organic selenium (T3) (63.0 ± 4.83%) than in the group of boars fed a diet with no supplemented selenium (T1) (44.5 ± 6.85%). The progressive motility of spermatozoa after exposure to the thermal test is shown in Fig. 4.

The decrease in spermatozoa progressive motility of short term preserved semen (stored at 17 ± 1 °C), over a 7 day period is shown in Fig. 5. The decrease of spermatozoa progressive motility in short term preserved semen during storage was highly significant between all groups of boars ($P < 0.01$) at the end of the study (90th day). The spermatozoa progressive motility, after 5 days of storage (120 h), was higher ($P < 0.01$) in the T3 group (3.75 ± 0.26) than in the T2 (3.20 ± 0.26) and T1 (2.90 ± 0.32) groups of boars. After storage for 7 days, the spermatozoa progressive motility decreased even more ($P < 0.01$) in the control T1 group (2.15 ± 0.41) compared to the T2 (2.80 ± 0.23) and T3 (3.20 ± 0.26) groups. Interestingly, the motility of spermatozoa in boars fed organic selenium (T3, 3.20 ± 0.26) decreased at a slower rate ($P < 0.01$) than that of the boars fed inorganic selenium (T2, 2.80 ± 0.23).

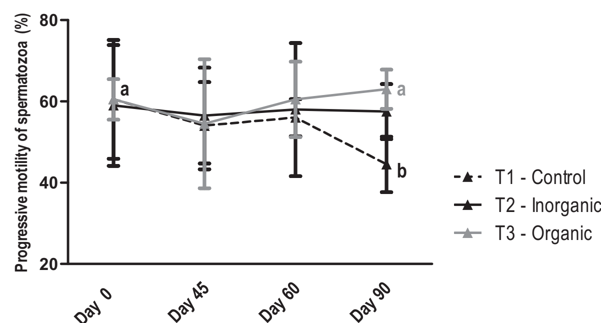

Fig. 4. The mean percentage (±SD) of progressive motility of spermatozoa after exposure to thermal resistance. Values with unlike superscripts differ at $P < 0.05$. SD, standard deviation.

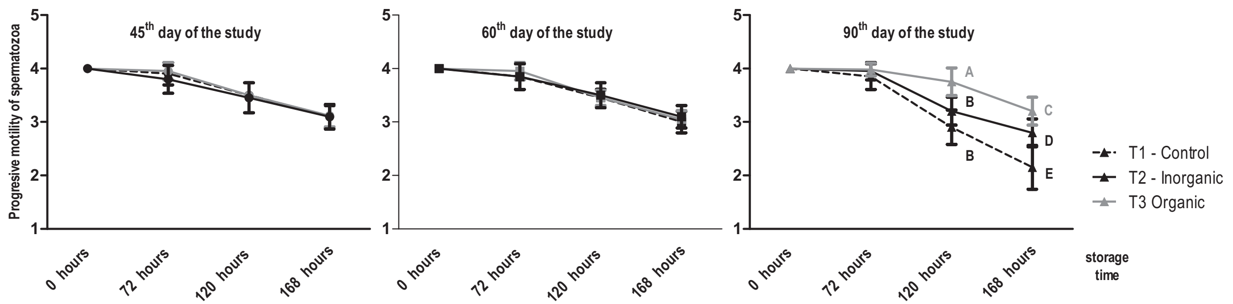

Fig. 5. The decrease in spermatozoa progressive motility (mean ± SD) of short term preserved semen (stored at 17 ± 1 °C), over a 7 day period. The values with unlike superscripts differ at $P < 0.01$. SD, standard deviation.

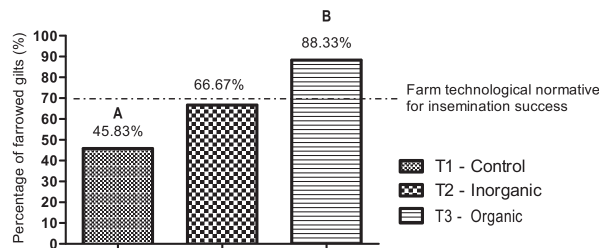

Fig. 6. Fertility success (conception rate). The values with unlike superscripts differ at $P < 0.01$.

3.5. Fertility success rate

A total of 72 gilts were inseminated (24 gilts for each group of boars). The conception rate was expressed as the percent of farrowed gilts, and is shown in Fig. 6.

In the group of gilts inseminated with semen from the control group of boars (T1), 11 gilts farrowed yielding a total of 114 piglets; in the group of gilts inseminated with semen from the T2 group of boars, 16 gilts farrowed yielding a total of 175 piglets; and in gilts inseminated with semen from the T3 group of boars, 20 gilts farrowed yielding a total of 252 piglets. The higher number of piglets per farrowed gilt obtained in the T3 group (12.60 ± 1.85) was highly significant ($P < 0.01$) compared with the T2 group (10.94 ± 1.65) or the T1 group (10.36 ± 1.63) ($P < 0.01$).

4. Discussion

The selenium content of control feed was 0.098 mg/kg, and was somewhat higher than expected. Serbian Feed Law (2010) provides for a minimal level of 0.1 mg/kg of selenium in feed for boars. Therefore, it is clear that the control group of boars (T1) was fed a diet that nearly met the accepted standard for selenium. According to Mihailović et al. (1996) and Jovanović et al. (1998) cereals originating from Serbian fields contain on average 27.0 ± 23.8 μg/kg selenium and, therefore, may be considered selenium deficient. The selenium content in our control feed contained about three-times the average amount of selenium in Serbian cereals. Since no selenium was added to the control feed, the observed high-selenium content may be attributed to a relatively high-percentage of fish meal in the diet (8%). Although the selenium content in fish meal can be high, ranging from 62.7 to 507.0 μg/kg, it is often considered to be an “unreliable” source of selenium, since the presence of mercury and other heavy metals can bind selenium and form insoluble complexes rendering it unavailable for absorption in the animal (Pappa et al., 2006).

The selenium content of boars blood at the end of the study was higher in group T2 (2.33 ± 0.33 μg/mL) and group T3 (2.69 ± 0.32 μg/mL) ($P < 0.01$) compared to the control group T1 (1.49 ± 0.29 μg/mL). The potential role of selenium in male fertility was studied in Croatia from 1988 to 1990 (Kršnjavi et al., 1992). Eighteen blood plasma samples originating from patients with low spermatozoa counts and 23 blood plasma samples from patients with normal spermatozoa counts were tested. Kršnjavi et al. (1992) determined a significantly lower selenium concentration in blood plasma from men with oligospermia and astenospermia than in blood plasma from males having normal sperm counts.

Lasota et al. (2004) measured the selenium content and GPx activity in blood plasma ($n = 58$) and semen ($n = 155$) of sexually mature boars, of variable ages, and found no correlation between the selenium content and GPx activity in blood plasma and semen. The authors then hypothesized that the mechanisms regulating the concentration of selenium and GPx activity in blood plasma and semen were independent. They also suggested that the age of the boars may affect the selenium concentration and GPx activity in blood plasma and semen.

In our study, the highest-blood plasma GPx activity was observed in boars (group T3) supplemented with organic selenium (32.0 ± 3.72 μkat/L), and the lowest GPx activity was found in boars in the control group T1 without supplemental selenium (25.1 ± 6.88 μkat/L) ($P < 0.05$). The group of boars fed inorganic selenium (T2) had blood plasma GPx activity of 25.6 ± 0.76 μkat/L. Our findings differ from those of Mahan and Peters (2004) who determined about 6% lower blood plasma GPx activity in sows supplemented with organic selenium than in sows fed a diet supplemented with inorganic selenium. Our results also differs from Fortier and Matte (2006), who found an 8.5% lower GPx activity in blood plasma from gilts fed supplemental organic selenium compared with gilts supplemented at the same level with inorganic selenium.

The group of boars supplemented with organic selenium (T3) had higher-semen selenium levels (113.0 ± 7.09 ng/mL) compared to the control group of boars (T1) (91.6 ± 10.6 ng/mL) ($P < 0.01$) and the group of boars fed inorganic selenium (T2) (100.3 ± 8.69 ng/mL) ($P < 0.05$). Our findings are in accordance with the findings of Marin-Guzman et al. (1997) who found that boar semen selenium levels ranged from 31.0 to 134.0 ng/g. Hurst et al. (1999) investigated the selenium levels in semen of infertile men and found the levels to be lower compared to levels in men with normal fertility. According to the findings of Boitani and Puglisi (2008) selenium was acknowledged to be essential in normal spermatogenesis of mammals, due to its important role in GPx activity and in selenoprotein P. On the contrary, Takasaki et al. (1987) found no significant difference between semen selenium levels in men with normal fertility ($n = 32$) and in infertile men ($n = 73$). Moreover, Takasaki et al. (1987) found the level of selenium higher and the spermatozoa less motile in infertile men, which suggest that high-levels of selenium might have a negative effect on sperm motility, and therefore may affect fertility. In our study, supplementation of both organic and inorganic selenium at approximately 0.3 mg/kg above the levels recommended by Serbian Feed Law, did not show any adverse effect on boars' health, semen production nor semen quality. This finding indicates that the real requirements of stud boars during the maximal exploitation phase may be higher than the current requirements prescribed by law.

The reactivated semen GPx activity, on the 90th day of the study, was higher in boars fed inorganic selenium (group T2) (2.63 ± 0.56 μkat/L) ($P < 0.05$) and organic selenium (group T3) (2.26 ± 0.62 μkat/L) ($P > 0.05$) compared to boars fed no supplemental selenium (group T1) (1.77 ± 0.31 μkat/L). It was necessary to reactivate the enzyme in mature spermatozoa prior to its analysis, because raw semen in our study had GPx activity below the detection limit, suggesting that most of the GPx was embedded in the spermatozoa mitochondrial capsule. Infertility in male domesticated mammals fed suboptimal amounts of selenium is characterized by spermatozoa of lower progressive motility and the morphological changes that are most notable are in the central section of the spermatozoa. As confirmation to our findings, Foresta et al. (2002), who investigated semen GPx activity in 37 men of normal and 75 men of reduced fertility, found the GPx activity to be lower ($P < 0.001$) in infertile men.

No significant difference in the ejaculate volume nor in the spermatozoa concentration was recorded between treatment groups in our study ($P > 0.05$). This finding is similar to the findings of Jacyno et al. (2002) who observed no effect of selenium (inorganic or organic) on ejaculate volume and progressive motility but found higher spermatozoa concentrations in boars fed a diet supplemented with organic selenium. In our study, on the 60th day, the spermatozoa progressive motility in raw semen was significantly higher ($P < 0.05$) in the group of boars supplemented with organic selenium (88.1 ± 2.59%) than in the group of boars fed a diet without supplemented selenium (83.7 ± 3.54%). Similarly at the end of the study (90th day), spermatozoa progressive motility was higher ($P < 0.01$) in boars fed diets supplemented with inorganic (86.2 ± 2.31%) and organic selenium (87.5 ± 2.67%) compared to boars fed a diet with no supplemental selenium (81.9 ± 2.59%). Our findings are in agreement with the findings of Marin-Guzman et al. (1997), who found semen quality to be more consistent in boars fed supplemental selenium than in boars fed no supplemental selenium. Our findings support the fact that selenium is essential for normal kinetics of spermatozoa. The GPx in spermatids is in a form of active peroxidase, while in spermatozoa it is in a form of an inactive enzyme (structural protein) mainly built into the mitochondrial capsule (located in the middle piece of the spermatozoa). Kehr et al. (2009) have visualized selenium by X-ray fluorescence microscopy (XFM) and identified a dramatic Se enrichment specifically in late spermatids. They claim that this enrichment was due to elevated levels of the mitochondrial form of glutathione peroxidase 4 and was fully dependent on the supplies of Se provided by selenoprotein P. In sperm, Se was primarily found in the middle piece and co-localized with Cu and Fe.

Martins et al. (2014) reported that organic Se feeding increased PHGPx in raw boar semen, although it did not significantly influence the Se concentration in the seminal and blood content. The remaining analyzed seminal characteristics in boars' raw semen were not influenced. Stradaioli et al. (2009) measured PHGPx in spermatozoa from 92 yearling bulls of three different Italian breeds (Chianina, Romagnola, and Marchigiana), and revealed the presence of two quite well separated populations. A PHGPx activity of 130 mU/mg separated the high-PHGPx group (H-PHGPx, $n = 73$) from the low-PHGPx group (L-PHGPx, $n = 19$). Forward motility was markedly higher in the H-PHGPx group, which also contained a lower percentage of detached heads, abnormal midpieces, and proximal droplets. On the other hand, different from human studies, no correlation was observed between PHGPx activity and the number of spermatozoa in the ejaculate. Apart from the sperm count, which typically differed among breeds, and the number of detached heads in the L-PHGPx group, which correlated with a higher sperm count, no other significant difference in seminal parameters among breeds was apparent. They have concluded that the assay for sperm PHGPx activity to be a unique tool to evaluate semen quality for sire selection. In our study, a slower rate of decrease in spermatozoa progressive motility during the 7 day storage ($P < 0.01$) was observed in the Se-yeast group than in the sodium selenite or no selenium supplemented boars. Also, the highest GPx4 activity was observed in the Se-yeast group, which supports the claim that GPx is mainly incorporated into the spermatozoal mitochondrial capsule (middle piece of the spermatozoa). The kinetic apparatus of spermatozoa is located in the middle piece; hence, decreased motility can be

attributed to lower GPx activity. A well-known effect of selenium deficiency includes instability of the middle piece leading to defective sperm motility (Hansen and Deguchi, 1996).

The percentage of live morphologically normal spermatozoa in the boars fed no supplemental selenium was not statistically different from the beginning (82.1 ± 6.53%) of the study to the 90th day (77.9 ± 5.35%) ($P > 0.05$). In the group of boars fed a diet supplemented with inorganic selenium no significant variation in the percentage of live morphologically normal spermatozoa was observed from the beginning of the study (86.8 ± 3.67%) or at the end of the study (86.5 ± 3.05%). Interestingly, the group of boars fed a diet supplemented with organic selenium had a higher percentage of live morphologically normal spermatozoa on the 90th day (87.2 ± 4.21%) ($P < 0.01$) and the 60th day (86.0 ± 4.60%) ($P < 0.05$), than on the 45th day of the study (76.1 ± 9.18%). Our findings are in accordance with the findings of Marin-Guzman et al. (2000a) who found spermatozoa in boars fed a diet deficient in selenium to have lower ATP concentrations as well as structural abnormalities of the middle piece (an electron microscopy study). Our findings correlate with the findings of the above mentioned authors regarding selenium retention since the percentage of live morphologically normal spermatozoa significantly increased on the 60th and 90th day of the study.

A lower number of spermatozoa did not react upon exposure to the hypo-osmotic solution (lower number of metabolically inactive spermatozoa) in boars fed organic selenium (17.2 ± 3.32%) than in boars fed inorganic supplemented selenium (25.6 ± 15.3%) ($P > 0.05$). This is in agreement with the findings of Jacyno et al. (2002), who found that the spermatozoa of organic selenium fed boars had better results with the hypo-osmotic tests, and had a higher percentage of morphologically normal spermatozoa as well.

A decrease in spermatozoa progressive motility upon exposure to the thermal test was observed in boars fed no selenium supplemented diets on the 90th day (44.5 ± 6.85%) compared to the 60th day of the study (60.5 ± 14.6%) ($P < 0.05$). Also, at the end of the study (90th day), a higher spermatozoa progressive motility ($P < 0.05$) was observed in the boars fed organic selenium (63.0 ± 4.83%) compared to the boars fed no supplemental selenium (44.5 ± 6.85%). These findings confirm the importance of selenium in spermatozoa protection and point out the need for further investigations.

The spermatozoa progressive motility (expressed on the Mortimer four grade scale system) in freshly preserved semen at the end of the study, after storage for 5 days was higher ($P < 0.01$) in the group of boars fed organic selenium (3.75 ± 0.26) than in boars fed inorganic selenium (3.20 ± 0.26) or a no selenium supplemented diet (2.90 ± 0.32). A similar trend was observed after storage for 7 days, where a lower ($P < 0.01$) progressive motility was observed in the control (2.15 ± 0.41) than in the group of boars fed inorganic selenium (2.80 ± 0.26) or organic selenium (3.20 ± 0.26) supplemented diets. The better spermatozoa progressive motility in short term preserved (stored) semen observed from selenium supplemented boars clearly indicate that such semen could possibly have higher fertility as well. Marin-Guzman et al. (2000b) reported lower progressive motility of spermatozoa in short term preserved semen as well as defects in the spermatozoa middle piece of boars fed a selenium deficient diet. Short term preserved boar semen is typically used up to 8 days for artificial insemination; however, if used after the 5th day of production it usually results in lower fertility. Christensen et al. (2004) reported the decrease in litter size when semen older than 3 days was used for sow artificial insemination. Dilution of semen reduces the concentration of semen plasma and decreases the concentration of natural antioxidants which have a protective effect on spermatozoa (Maxwell and Johnson, 1999). Interestingly, we observed that spermatozoa progressive motility tended to decrease faster in selenium deficient boars (T1 group) than in boars fed a diet supplemented with selenium, which speaks in favor of an anti-oxidative role for selenium in boar spermatozoa.

The highest fertility rate in our study was observed in the group of gilts inseminated with semen from boars fed organic selenium (88.3%), lower fertility with semen from boars fed inorganic selenium (66.7%) and the lowest fertility with semen from boars fed no supplemented selenium (45.8%); this phenomenon needs further investigation. The fertility rate in the group of boars fed organic selenium was significantly higher than in the group of boars fed no supplemented selenium ($P = 0.024$, chi square test of proportions). No significant differences between the sodium selenite group and the control group of boars, or between the sodium selenite and Se-yeast groups in regard to fertility were recorded. The highest number of piglets was obtained in the group of gilts inseminated with semen obtained from the boars fed organic selenium, lower in the group of gilts inseminated with semen obtained from boars fed inorganic selenium, and lowest in the group of gilts inseminated with semen obtained from boars in the no selenium supplemented group ($n = 252$, 175, and 114 piglets, respectfully). The average number of piglets per farrowed gilt was higher in the group of boars fed organic selenium (12.6 ± 1.85) compared to the group fed inorganic selenium (10.9 ± 1.65) ($P < 0.05$) or the group fed no supplemental selenium (10.4 ± 1.63) ($P < 0.01$). Generally, the fertility rate and the number of piglets are not easily comparable between farms but rather to the farm normative. The fertility obtained in control (T1) and the inorganic selenium supplemented (T2) group were below the farm normative (see Fig. 6). Only the group of boars fed organic selenium (group T3) yielded fertility above the farm set normative.

5. Conclusions

The highest-level of selenium in blood was recorded in the group of boars supplemented with organic selenium and the lowest-level of selenium was observed in the control group of boars fed a diet without supplemented selenium. The highest-level of selenium in semen at the end of the trial was determined in the group of boars supplemented with organic selenium, somewhat lower in the boars supplemented with inorganic selenium, and the lowest in the non-supplemented group of boars. Blood plasma GPx activity was higher in boars fed organic selenium than in boars fed a diet without supplemented

selenium. The supplementation of inorganic selenium efficiently increased the GPx activity in semen. The supplementation of selenium affected semen quality, and improved semen quality was more notable in the group of boars supplemented with the organic form of selenium through increased progressive motility of the spermatozoa, resistance to hypo-osmotic shock and thermal tests. The storage ability of short term preserved semen was improved by selenium supplementation, most notably in the boars fed organic selenium. The selenium supplementation increased the fertility rate, yielding the highest rates in boars fed a diet supplemented with organic selenium.

Conflict of interest

We state that the authors or author's institution have no financial or other relationship with other people or organizations that may inappropriately influence the author's work.

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