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STATUS OF PROOXIDANT AND ANTIOXIDANT SYSTEMS IN THE SPERM AND SEMINAL PLASMA OF BREEDING BOARS OF LARGE WHITE BREED AND SS23 SYNTHETIC LINE

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The indicators of pro- and antioxidant systems in sperm and sperm plasma of breeding boars of Large White breed and SS23 synthetic line were studied. Measurements of antioxidant enzyme activity, determination of lipid peroxidation (LPO) product content, and antioxidant factor were performed. Lipid peroxidation in the semen of healthy breeding boars was characterized by a stable level of activity, which is necessary to ensure normal reproductive functions. Additionally, there was a high content of low molecular weight thiols and proteins. The concentration of SH-groups in spermatozoa was higher ($P \leq 0.05$) compared to sperm plasma. The number of total, protein, and free SH-groups in the semen of boars of the synthetic line was higher ($P < 0.05$) in relation to animals of the Large White breed. Low catalase (CAT) activity in the sperm was compensated by glutathione peroxidase (GPX). The content of ceruloplasmin (CP) in the sperm of boars was almost twice as high as that of sperm plasma. In spermatozoa, high content of reduced glutathione (GTH) was recorded, which was more than 3 times higher than in the seminal fluid. The main antioxidants of spermatozoa were superoxide dismutase (SOD), CP, SH-groups of proteins, and reduced content of GTH. We revealed that CAT is a key enzyme that neutralizes excess hydrogen peroxide in boar semen. In contrast, in sperm, hydrogen peroxide was inactivated mainly by GPX. Further research on the mechanisms of action of reactive oxygen species on boar semen will help to develop effective methods for sperm storage and successful fertilization of oocytes.

Key words: *boars, sperm, antioxidant enzymes, glutathione system, lipid peroxidation, superoxide dismutase, ceruloplasmin, spermatozoa, thiols, seminal plasma*

INTRODUCTION

Redox reactions by oxygen influence are an integral part of the existence of any aerobic organism. Among them, special attention should be paid to free radical reactions, in the process of which reactive oxygen species (ROS) are formed. About 98% of all oxygen entering the cell participates in the oxidation of substrates with the formation of adenosine triphosphate (ATP), and only 2% is used in reactions with the formation of ROS: superoxide anion radical, hydrogen peroxide, and hydroxyl radical (1-4). Reactive oxygen species may be of exogenous or endogenous origin. They are produced mainly by spermatozoa including immature cells, leukocytes, and epithelial cells. There are a number of environmental factors that have a direct or indirect impact on the intensity of ROS formation in the reproductive organs (5, 6).

The level of free radicals in the cell physiological state is always balanced. Depending on ROS concentration their activity can be regulatory or toxic. Usually, the intracellular level of ROS is quite low. The cell inactivates these compounds through antioxidant systems or repairs the damage caused by them through reparative processes. Mitochondria are the main source of ROS in male spermatozoa (7). Rich mitochondria in spermatozoa produce energy constantly used for cell motility. Reactive oxygen species exert inhibitory effects on motility that indicate a mitochondrial-independent mechanism (8). The reduction in motility may have been due to a ROS-induced lesion in ATP utilization or in the contractile apparatus of the flagellum. Mitochondrial dysfunction can increase electron loss and then increase the production of ROS to toxic levels disrupting homeostasis.

Sperm plasma membranes covering the acrosome and tail are the most sensitive to oxidative damage due to a significant amount of polyunsaturated fatty acids: 25% docosapentaenoic and 30% docosahexaenoic acid which they contain. Boar spermatozoa, in comparison with other animal species, have insufficient protective enzymes which probably indicates that the preventive function against ROS is fulfilled by low-or high-molecular-weight antioxidants of the seminal plasma (9-12). Supplements containing polyunsaturated fatty acids and antioxidants (PROSPERM) had a beneficial effect on the

biological characteristics of boar spermatozoa (13). In the LPO process, a number of mutagenic and genotoxic molecules of aldehydes (acrolein, malonic dialdehyde, and 4-hydroxynonenal) are formed causing protein and nucleic acid damage. Disruption of sperm membrane integrity coincidences with a decrease in their motility (5, 14, 15).

Thus, sperm can be considered a complex redox system that combines the antioxidant potential of seminal plasma and sperm with a prooxidant effect due to the production of ROS (16). The interaction of antioxidant and prooxidant mechanisms in semen determines the overall rate of LPO in spermatozoa. The main antioxidant enzymes of sperm are superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT) (17). However, extremely small cytoplasmic space for enzyme actions in spermatozoa does not allow for effective counteract oxidative damage. This function is performed by antioxidants of seminal plasma (17, 18). It contains SOD, CAT, GPX, and glutathione reductase (GR), which are synthesized by the prostate and other accessory glands, as well as non-enzymatic antioxidants, thiol compounds, ascorbic acid, tocopherol, carnitine, urate, albumin, taurine and hypotaurine (18).

Any changes in equilibrium lead to an increase in the content of ROS, which directly affects the reduction of the biological value of sperm: disruption of their formation, ability to fertilize, DNA integrity being one of the main causes of death of zygotes, embryos, and abnormalities in the offspring. The content of ROS in seminal plasma can be used to predict fertility in animals. Their and their level is an independent marker of infertility (6). In boar semen, hydrogen peroxide is the major free radical mediating the damage (19). The extracellular signal-regulated kinase (ERK) pathway regulates tyrosine phosphorylation in boar spermatozoa through its ERK1/2 component and ROS mediates cyclic adenosine monophosphate-dependent protein kinase A and ERK pathway signaling during sperm capacitation, through phosphorylation/dephosphorylation of specific proteins. Thus, the disclosure of the patterns of free radical oxidation and the functioning of the antioxidant defense system in semen will allow for the development of effective methods and techniques for improving the quality of sperm products with subsequent production of healthy offspring. Therefore, the aim of the study was to compare the antioxidant and LPO systems in sperm and seminal plasma of the Large White boars with those of the SS23 synthetic line.

MATERIALS AND METHODS

Animals

The experiments were carried out in the conditions of the selection center, pig breeding farm LLC ‘Elita’ Terezyne of Bila Tserkva district of Kyiv region, and the research laboratory of Bila Tserkva National Agrarian University, Ukraine. Purebred boars (2-years old) of Large White breeds and a specialized synthetic line SS23 were used for research. To clarify the breed characteristics of the free radical oxidation processes in the semen of boars on the principle of analogy, two groups of animals (n=8/group) were used. Boars were kept in the standard housing conditions and fed with the complete feed (PK-57-2), and free access to feed and water. The conditions of controlled animal keeping met the general biological requirements for semen collection.

The animals were involved in experiments in compliance with the requirements of the European Convention for the Protection of Vertebrate Animals; Strasbourg, 1986. The use of boar semen (according to European Union Council Directive 2010-63-EU) was approved by the Local Ethics Committee in Krakow, Poland (permission number: 144b/2015).

Semen collection and treatment of ejaculates for biochemical analysis

Ejaculates were obtained once a week using the glove-hand method from each animal (from May to June; n=12 ejaculates/animal). Semen (150–380 ml of volume, 177–580×10^6 sperm/ml of concentration and 60–80% of motility) was centrifuged (3000 rpm for 10 min), supernatant (semen plasma) was separated, and sperm pellet was washed twice in phosphate-buffered saline. The cytoplasm of spermatozoa was obtained by destroying sperm cell membranes through sonication in RIPA buffer and next by differential centrifugation (14000 g/min at 4ºC for 10 min). Total protein concentrations in sperm and seminal plasma were quantified using the Bio-Rad DC Protein Assay Kit with BSA as a standard (Bio-Rad Labs, GmbH, Munchen, Germany) (for details please see (17)). Samples were assayed immediately upon collection. An aliquot of appropriate volume was used for further analyses to determine the activity of antioxidant enzymes, LPO products, CP, SH-groups of proteins, and GTH content.

Determination of antioxidant enzyme activity and content of non-enzymatic antioxidants

1. Determination of antioxidant enzyme activity

Superoxide dismutase (SOD) activity was determined by a method based on the SOD ability to compete against nitroblue tetrazolium (TheroFisher, Waltham, MA, USA) for superoxide radicals coming from the photooxidation of riboflavin at λ=560 nm (20). SOD activity was expressed as relative activity units per 1 mL plasma and sperm.

Catalase activity (CAT, EC 1.11.1.6) was determined by the reaction of hydrogen peroxide with ammonium molybdate (Sigma-Aldrich, Saint Louis, MO, USA) (21). The reaction mixture contained 2.8 mL of 0.1 M solution of hydrogen peroxide and 0.1 mL of a diluted plasma sperm. The reaction was carried out for 5 min and stopped the reaction by the addition of 1.0 mL of a 0.3 M diammonium molybdate solution. The level of the colored product was measured at λ=410 nm against water and the values were expressed in μcat/mL in plasma and sperm, respectively.

GPX (glutathione hydrogen-peroxide oxidoreductase, EC 1.11.1.9) activity was determined by measuring the rate of GSH oxidation before and after incubation with tert butyl hydroperoxide (Sigma-Aldrich, Saint Louis, MO, USA) as described earlier (22). The color reaction is based on the interaction of SH-groups with the 5,5-dytiobis2-nitrobenzoic acid (DTNBA), resulting in the formation of colored product - dinitrophenyl anion. The quantity of the latter is directly proportional to the number of SH-groups that have reacted with DTNBA. Enzyme activity was expressed as μmol GSH/min×g of protein.

GRD (glutathione NADP+ oxidoreductase, EC 1.6.4.2) activity was measured as described earlier (22). This method is based on the catalytic reduction, dependent on nicotinamide adenine dinucleotide phosphate (NADPH, Merck, Frankfurt, Germany), of the oxidized form of GTH. The reaction intensity can be assessed by the rate of decrease of the extinction on the wavelength of NADPH maximum absorption (340 nm). GR activity was calculated using the molar absorption ratio for NADPH at a wavelength of λ=340 nm. The enzyme activity was expressed in μmol NADPH NADP•H_2/min×g of protein.

Glutathione-S-transferase (GST, EC 2.5.1.18) activity was measured in both the seminal plasma and sperm according to Vlasova (23) using 1-chloro-2,4-dinitrobenzene (Sigma-Aldrich, Saint Louis, MO, USA) as a substrate. Enzyme activity was

determined at 25ºC by monitoring changes in absorbance at λ=340 nm for 2 min at a constant temperature. The GST activity was expressed as nmol of conjugate/min×g of protein.

2. Determination of content of non-enzymatic antioxidants

Glutathione (GSH) content was determined by the level of thionitrophenyl anion formation as described earlier (22). The color reaction is based on the interaction between SH-groups of GSH and 5,5'-dithiobis-(2-nitrobenzoic acid) (Sigma-Aldrich, Saint Louis, MO, USA). GSH content was measured using the calibration graph and expressed in mmol GSH per 1 mL of plasma and sperm, respectively.

For CP concentration measurement samples included 0.05 mL of plasma, 4 mL 0.4 M of acetic buffer solution (pH 5.5) and 0.5 mL 0.5% aqueous solution of 1,4-phenylenediamine dihydrochloride (Sigma-Aldrich, Saint Louis, MO, USA). The control sample had the same quantity of reagent and biological materials with the additional 1 mL of 3% solution of sodium fluoride. All samples were incubated for 1 hour at 37ºC next to the experiment sample was added 1 mL of 3% solution of sodium fluoride. The absorbance was measured at λ=530 nm and CP concentration expressed in μg/cm^3 (24).

For analysis of the concentration of SH-groups 0.05 mL of serum was dissolved to 0.5 mL with distilled water and next 0.5 mL of 6 M potassium iodide solution, 2 drops of 5% starch solution, and 1.8 mL of 0.1 M phosphate buffer (pH 7.6) were added. The absorbance was measured at λ=500 nm before and after the application of 0.3 mL 0.001 N of iodine solution (Sigma-Aldrich, St. Louis, MO, USA). The SH-group concentration was expressed as mmol/l (25).

All measurements were performed using a UV/Vis spectrophotometer SF2000 (OKB SPECTR LLC, Saint Petersburg, Russia).

Determination of lipids peroxidation products content and antioxidant factors

Diene conjugates (DC), formed as a result of the transfer of double bonds in polyunsaturated fatty acids, were determined by Vlizlo (25). After extraction in a mixture of heptane-isopropanol (2:1) and subsequent layering with HCI (pH 2.0), diene conjugates were detected in the heptane phase at λ=233 nm. The content of diene conjugates was expressed in relative units per 1 mL of plasma and sperm, respectively.

The content of lipid hydroperoxides (LHP) in sperm was determined by Vlizlo (25). This method is based on spectrophotometrical optical density measurement of the products of ammonium thiocyanate, hydrochloric acid, and Mohr salt (Sigma-Aldrich, Saint Louis, MO, USA) reaction. Lipids from the samples were preliminarily extracted with ethanol. The selection of tissue samples and preparation for extraction was performed at 4ºC. Ethanol (2.8 mL) and 0.05 mL of 50% trichloroacetic acid were added to 0.2 mL of hemolisate (dissolved in buffer solution with pH 7.4), and shaken for 5–6 min. Obtained protein precipitate was separated by centrifugation at 700 g. Ethanol (1.2 cm^3), 0.02 mL of concentrated HCl, and 0.03 mL of 1% Mohr salt solution in 3% HCl were added to 1.5 mL of supernatant. The mixture was stirred. After 30 s, 0.2 mL of 20% ammonium thiocyanate (Sigma-Aldrich, Saint Louis, MO, USA) was added, and then the absorbance of the solution was determined at λ=480 nm. In a control sample, the appropriate amount of water was added instead of supernatant. The content of LHP was calculated by the difference between experimental and control values and expressed in arbitrary units of optical density for 1 mL of plasma and sperm, respectively.

The concentration of thiobarbituric acid reactive substances (TBA-RS), characterizing the LPO rate, was assessed based on the reaction between malondialdehyde (MDA) (Sigma-Aldrich, Saint Louis, MO, USA) and thiobarbituric acid (TBA) (Sigma-Aldrich, Saint Louis, MO, USA). The 0.1 mL plasma was added to 1.5 mL 0.025 M Tris-buffer with potassium chloride (pH 7.4). These samples were incubated for 30 min at 37ºC; 1 mL 20% solution of trichloroacetic acid was added and centrifuged for 15 min at 3000 g. Supernatants (2 mL) were added to 1 mL of 0.8% solution of TBA and heated for 10 min at 100ºC. The absorption was measured at λ=532 nm and TBA-RS concentration was expressed as nmol MDA/mL plasma and sperm, respectively (25).

The value of the antioxidant protection index (API) gives an idea of the general trends of the changes of redox balance in a living organism towards recovery, and was calculated by the formula (26):

$$API=A_{SOD}\times A_{CAT}/C_{TBA\text{-}RS},$$

where API - antioxidant index; A_{SOD} - superoxide dismutase activity; A_{CAT} - catalase activity; $C_{TBA\text{-}RS}$ - the content of thiobarbituric acid reactive substances.

All measurements were performed using a UV/Vis spectrophotometer SF2000 (OKB SPECTR LLC, Saint Petersburg, Russia).

Statistical analysis

Statistical evaluation of the results was conducted using the arithmetic mean and standard error (M±SE) and the adequate interval for assessing the degree of probability (*P*) using Student's criterion (t). Differences were statistically significant at $P<0.05$. The results of the study are processed using the statistical package Statistica 6.0 (StatSoft Inc, USA). The normality of the distribution of actual data was checked by using the criterion of Shapiro-Wilk (27).

Table 1. The content of lipid peroxidation products (M±m; *n*=8).

Indexes	Large white breed		Synthetic line SS23	
	plasma	**sperm**	**plasma**	**sperm**
Lipids hydroperoxides unit. act/cm^3	3.29±0.11	7.57±0.29	2.94±0.14	6.92±0.30
Diene conjugates unit. act/cm^3	0.19±0.01	0.31±0.02	0.22±0.01	0.80±0.04***
TBA-RS nmol MDA/cm^3	3.54±0.20	3.44±0.23	3.79±0.22	2.80 ± 0.13
Antioxidant protection index	111.31±4.66	64.94±4.23	61.15±5.13***	65.66±6.37

The difference is probably relative to purebred boars of the large white breed: *$P<0.05$; **$P<0.01$; ***$P<0.001$. TBA-RS, thiobarbituric acid reactive substances.

RESULTS

Lipids peroxidation products content and antioxidant factor

The average values of the content of the primary (DC, LHP) and secondary (TBA-RS) products of LPO in sperm and seminal plasma of boars of Large White breed and synthetic line SS23 are presented in *Table 1*.

In the sperm plasma of the Large White boars level of LHP ranged from 2.75 to 3.71 units/mL and in sperm it ranged from 6.72 to 8.77 units/mL. It was noted that the range of fluctuations in the content of LPO products in spermatozoa was higher compared to sperm plasma (*Table 2*). The concentration of LHP in spermatozoa of purebred animals was inversely proportional to the activity of SOD (r= –0.83) and proportional to the content of CP (r=0.54). In the sperm of boars of synthetic lineage, a high negative correlation between LHP content and SOD activity (r= –0.71) was found.

The content of TBA-RS in the plasma of sperm of animals of the synthetic line was slightly higher than in boars of the Large White breed, while in sperm the opposite trend was observed. It was noted that in sperm of both studied groups of animals the range of fluctuations in the content of LPO products was higher than in sperm plasma. Purebred boar seminal plasma was characterized by positive correlations between TBA-RS content with SOD activity (r=0.60) and CAT (r=0.75). In the sperm of Large White boars, there was a high correlation between the concentration of TBA-RS and the DC content (r=0.60). In spermatozoa of boars of synthetic lineage, the DC content was significantly higher ($P<0.01$), compared with the group of purebred animals. Moreover, the concentration of this LPO product in seminal plasma of both studied groups of breeding boars was almost the same.

Antioxidant enzymes activity

In the seminal plasma of boars of the Large White breed, the low activity of SOD (*Table 3*) and the highest level of SOD in sperm (exceeding 16.1% ($P<0.05$)) when compared to animals of the synthetic line were found. The purebred sperm was

Table 2. Statistical indicators of the content of lipid peroxidation products (n=8).

Indexes	LHP				DC				TBA-RS			
	plasma		sperm		plasma		sperm		plasma		sperm	
	Large White breed	Synthetic line SS23	Large White breed	Synthetic line SS23	Large White breed	Synthetic line SS23	Large White breed	Synthetic line SS23	Large White breed	Synthetic line SS23	Large White breed	Synthetic line SS23
Min	2.75	2.26	6.67	5.76	0.10	0.18	0.22	0.66	2.82	3.08	2.73	2.05
Max	3.71	3.63	8.77	8.52	0.24	0.26	0.42	0.98	4.61	4.53	4.62	3.25
Range	0.96	1.37	2.05	2.76	0.14	0.08	0.20	0.32	1.79	1.45	1.88	1.19
Dispersion	0.10	0.17	0.66	0.73	0.002	0.001	0.004	0.02	0.33	3.78	0.44	0.14
Mean of deviation	0.32	0.41	0.81	0.85	0.04	0.03	0.06	0.14	0.57	1.94	0.66	0.37
Coefficient of variation	0.09	0.14	0.11	0.12	0.24	0.14	0.19	0.15	0.16	0.16	0.19	0.13
Asymmetry	–0.23	0.11	0.44	0.64	–0.58	–0.13	0.09	0.23	0.75	0.02	0.86	–0.85
Kurtosis	2.19	2.58	1.56	2.72	2.71	1.55	2.63	1.62	2.63	1.31	2.33	3.01
Median	3.31	2.84	7.31	6.79	0.19	0.23	0.32	0.78	3.33	3.72	3.25	2.82
Shapiro-Wilk criterion	0.97	0.96	0.88	0.96	0.96	0.90	0.98	0.93	0.93	0.86	0.86	0.92

The difference is probably relative to purebred boars of the Large White breed: *$P<0.05$; **$P<0.01$; ***$P<0.001$. DC, djene conjugates; LHP, lipid hydroperoxides; TBA-RS, thiobarbituric acid reactive substances.

Table 3. The activity of enzymes of the antioxidant system and the content of CP (M±SE; n=8).

Indexes	Large White breed		Synthetic line SS23	
	seminal plasma	sperm	seminal plasma	sperm
Superoxide dismutase unit. act/mL	0.99±0.04	1.18±0.06	1.06±0.06	0.99±0.05*
Catalase µcat/mL	395.60±16.31	189.14±15.10	217.78±15.77***	182.48±7.91
Ceruloplasmin µg/mL	72.63±2.45	185.06±9.71	73.50±2.68	170.28±11.89

The difference is probably relative to purebred boars of the Large White breed: *$P<0.05$; **$P<0.01$; ***$P<0.001$.

characterized by a high negative correlation between SOD and CAT activity (r= –0.71). A positive correlation was found between the activity of SOD and GPX (r=0.74), SOD and GST (r=0.52).

The activity of CAT in seminal plasma and sperm markedly varied (*Table 4*). In seminal plasma of animals of the synthetic line, it was lower by 45% ($p<0.001$) compared with purebred boars. The concentration of CP in seminal plasma and sperm of boars of both breeds was approximately at the same level. A negative correlation was found between CP content and CAT activity (r= –0.64). In the plasma of sperm of Large White boars, the content of CP was positively correlated with the activity of SOD (r=0.54).

To assess the overall antioxidant properties of the animal body, such an integrated indicator as antioxidant protection was calculated. In the seminal plasma of purebred boars, the studied

Table 4. Statistical indicators of the antioxidant system enzymes activity and the content of ceruloplasmin (n=8).

Indexes	Superoxide dismutase				Catalase				Ceruloplasmin			
	seminal plasma		sperm		seminal plasma		sperm		seminal plasma		sperm	
	Large White breed	Synthetic line SS23	Large White breed	Synthetic line SS23	Large White breed	Synthetic line SS23	Large White breed	Synthetic line SS23	Large White breed	Synthetic line SS23	Large White breed	Synthetic line SS23
Min	0.85	0.86	0.98	0.86	322.3	151.8	143.9	138.5	60.3	63.8	150.5	143.5
Max	1.24	1.28	1.42	1.28	463.5	274.3	245.1	213.1	84.0	85.7	236.2	201.2
Range	0.39	0.42	0.45	0.42	141.1	122.5	101.2	74.5	23.6	21.8	85.7	57.7
Dispersion	0.02	0.03	0.03	0.03	2127	1990	1824	500.8	48.1	57.3	754.0	392.3
Mean of deviation	0.14	0.17	0.17	0.17	46.12	44.6	42.7	22.3	6.9	7.5	27.4	19.8
Coefficient of variation	0.12	0.16	0.14	0.16	0.11	0.21	0.23	0.12	0.09	0.10	0.15	0.12
Asymmetry	0.75	0.20	0.51	0.20	–0.08	–0.24	0.22	–0.78	–0.16	0.39	0.83	1.25
Kurtosis	3.11	1.44	1.95	1.44	2.08	1.74	1.36	3.09	2.81	1.87	2.65	3.15
Median	0.99	1.02	1.16	1.02	399.6	222.4	178.4	186.4	72.6	71.7	177.6	153.1
Shapiro-Wilk criterion	0.91	0.89	0.89	0.89	0.98	0.95	0.86	0.93	0.95	0.95	0.89	0.79

The difference is probably relative to purebred boars of the Large White breed: **P*<0.05; ***P*<0.01; ****P*<0.001.

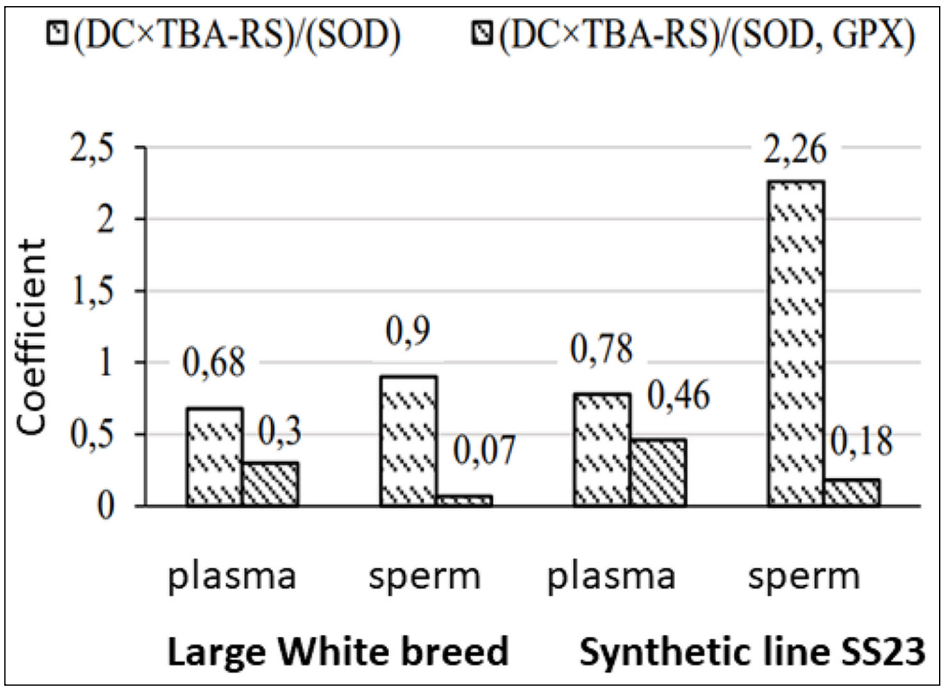

Fig. 1. Redox balance coefficient in the semen of boars of Large White breed and synthetic line SS23 (n=8) determined by the products of lipid peroxidation (thiobarbituric acid reactive substances (TBA-RS), diene conjugates (DC)) and the activity of antioxidant enzymes (superoxide dismutase (SOD), glutathione peroxidase (GPX)).

index was 1.8 times (P<0.01) higher compared to the group of animals of the synthetic line.

To calculate this coefficient, the following parameters: the content of TBA-RS, DC, SOD, and GPX activity in relative units were used. Two redox balance variants were calculated. In the first case, the ratio between TBA-RS, DK, and SOD activity was calculated. The results of the calculations indicated an increased level of prooxidants in the sperm of boars of both experimental groups. In the sperm of synthetic lineage, this level was 2.5 times higher than in purebred boars. According to another calculation option, the redox balance coefficient was determined by taking into account the products of LPO (TBA-RS, DC) and the activity of antioxidant enzymes (SOD, GPX) (*Fig. 1*).

Thiol compounds content

The concentration of SH groups in spermatozoa of boars of both breeds was much higher than in seminal plasma. Most thiol groups were bound to proteins. Seminal plasma and sperm of purebred boars contained on average 140.40 and 330.53 mmol/l of SH-groups. In boars of the synthetic line - 179.85 and 357.01 mmol/l SN-groups, respectively (*Table 5*).

The variability in the ejaculates of purebred animals was 124.8–164.4 mmol/dm^3 in seminal plasma and 297.6–374.4 mmol/dm^3 in spermatozoa. The content of total thiol groups in seminal plasma of breeding boars of the synthetic line was in the range of 147.3–216.0 mmol/dm^3, in the sperm - 304.8–410.4 mmol/dm^3. Protein thiol groups predominated in sperm and seminal plasma of the studied animals. The ratio of protein SH-groups to free (non-protein) in seminal plasma and spermatozoa of purebred breeders was 4.03 and 4.79 units, in animals of synthetic line - 4.41 and 3.37, respectively (*Table 6*).

The variability of the indicator within the studied ejaculates of purebred animals was 11.05–16.15 μmol/mL in seminal plasma and 35.70–59.50 μmol/mL in spermatozoa. The content of GSH in sperm of Large White boars was higher by 7.3% compared with similar indicators in the group of animals of the synthetic line SS23. The relationship between GR activity and reduced GTH was confirmed by a moderate negative correlation. In sperm of Large White boars, there was a moderate positive correlation between the content of reduced GTH and SOD activity (r=0.51), and a moderate negative correlation with CAT activity (r= –0.69).

The activity of GPX in the plasma of sperm of boars of the synthetic line was lower (P<0.001) compared to purebred animals. The range of fluctuations in GPX activity in the semen of boars of the synthetic line SS23 was lower compared to purebred animals. Seminal plasma of purebred boars was characterized by a positive correlation between GPX activity with CP content (r=0.63) and SOD activity (r=0.78). Sperm showed a negative correlation between GPX activity and LHP (r= –0.60). Glutathione peroxidase in sperm of animals of synthetic line SS23 correlated with the content of LHP (r= –0.64) and SOD activity (r=0.66).

The activity of GST in the seminal plasma of Large White boars was inversely proportional to the content of reduced GTX (r= –0.52) and directly proportional to the number of diene conjugates (r=0.74). Sperm was characterized by a positive correlation of GST activity with the amount of GSH (r=0.86), negative with the content of CP (r=0.59), TBA-RS (r=0.76) and CAT activity (r=0.62). The correlative dependence of GST activity was observed in the seminal plasma of boars of the synthetic line SS23 with the amount of CP (r= –0.70), and CAT activity (r=0.55). In sperm, GST correlated with the amount of LHP (r= –0.52), TBA-RS (r=0.56), and CAT activity (r= –0.62).

It was found that the activity of GR in the semen of boars of the synthetic line was lower (P<0.001) compared to purebred animals. The range of oscillations in the groups of boars of the synthetic line was lower compared to purebred animals. In sperm of boars of Large White breeds, there was a moderate positive correlation between GR activity and CP content (r=0.69) and a moderate negative correlation with LHP content

Table 5. The content of SH-groups (mmol/l) (M±SE; n=8).

Indexes	Large White breed		Synthetic line SS23	
	seminal plasma	**sperm**	**seminal plasma**	**sperm**
Total SH-group	140.40±5.22	330.53±9.63	179.85±8.09**	357.01±13.40
Free SH- group	27.90±1.42	57.03±3.01	33.23±1.59*	81.60±3.14***
Protein SH- groups	112.50±3.92	273.50±14.14	146.62±9.42**	275.41±14.52

The difference is probably relative to purebred boars of the Large White breed: *P<0.05; **P<0.01; ***P<0.001.

Table 6. The content of reduced glutathione (GTH) and the activity of GTH-dependent enzymes (M±SE; n=8).

Indexes	Synthetic line SS23		Large White breed	
	seminal plasma	**sperm**	**seminal plasma**	**sperm**
Glutathione μmol/mL	13.39±0.73	49.73±2.92	13.49±0.49	46.11±2.70
GPO μmol/min×g protein	2.28±0.12	12.77±0.69	1.69±0.07***	12.06±0.35
Glutathione-S-transferase nmol of conjugate/min×g protein	0.61±0.03	2.99±0.20	0.25±0.02***	3.97±0.24**
Glutathione NADP+oxidoreductase, EC1.6.4.2 μmol NADP•H_2/min×g protein	1.01±0.06	6.42±0.15	0.65±0.03***	5.51±0.14***

The difference is probably relative to purebred boars of the Large White breed: *P<0.05; **P<0.01; ***P<0.001.

(r= –0.69) and GPX activity (r= –0.70). A correlation between GRD activity and CP activity (r=0.52) and GSH (r= –0.68) was observed in the seminal plasma of animals of the SS23 synthetic line. In sperm GR correlated with CAT (r= –0.58).

DISCUSSION

The imbalance in redox homeostasis is one of the causes of infertility in males of various species (14-16, 28, 29). It needs to be noted that ROS production is also important in ensuring the physiological functions of sperm (30-32). Breeding boars are an excellent model for studying the functioning of the redox system in sperm and seminal plasma therefore the results can be used to understand mechanisms that take place during fertility disturbances. The intensity of ROS processes is determined primarily by the specific features of the tissue (33, 34). This study is a continuation of the previous one that showed that the level of endogenous intoxication in sperm and seminal plasma of the Large White boars was higher than in animals of the synthetic line SS23 however the content of total proteins was lower in sperm and seminal plasma of the Large White boars when compared to those of animals of the synthetic line (17). In addition, the processes of the oxidative modification of proteins in boars of the synthetic line were more intensive as evidenced by the higher content of the aldehyde and ketodinitrophenyl hydrazones in sperm.

The intensity of LPO processes is controlled by the antioxidant system. Superoxide dismutase, CAT, CP, GTH, and enzymes dependent on it are the main line of antioxidant protection in semen (2, 3, 16). Superoxide dismutase (SOD) catalyzes the dissociation of the superoxide anion radicals to hydrogen peroxide and oxygen. However, there is evidence that hydrogen peroxide is much more toxic to sperm than the superoxide anion radical. In our study, various levels of SOD were revealed in sperm and seminal plasma of the White Large boars versus synthetic line animals. Such dynamics can be attributed to the high content of LPO products (lipid hydroperoxides and TBA-RS).

According to Nandi (35), there is a correlation between CAT and GPX activity. These enzymes reduce the content of hydrogen peroxide in cells, converting it into water and oxygen. Boar semen plasma of both experimental groups was characterized by a sufficiently high CAT activity. Increased CAT activity against the background of reduced SOD activity can be considered an adaptive response of the body because the toxicity of hydrogen peroxide is 10 times less than the superoxide anion radical (17). It should be noted that in excess of SOD-produced hydrogen peroxide is able to form highly reactive hydroxyl radicals, which lead to fragmentation of the protein part of the enzyme molecule and loss of its activity. The accumulation of hydrogen peroxide in semen adversely affects the functioning of spermatozoa and inhibits the acrosome response (36). For effective action, SOD requires the presence of low molecular weight antioxidants or coordinated work with peroxidases. The activity of CAT in the sperm of both breeds was almost at the same level. The low activity of the studied enzyme in spermatozoa is compensated by the high activity of GPX. With effective antioxidant protection in tissues, a certain proportionality between the activity of SOD and CAT must be maintained, because they are involved in the regulation of two successive stages of the same chain of transformations. In purebred boar sperm, this consistency is manifested in the form of a high negative correlation (r= –0.71). No significant changes in API values were detected in spermatozoa probably due to the high activity of CAT in the biomaterial. In some findings, the redox balance coefficient is used as an additional criterion for assessing the functioning of the antioxidant system. This indicator characterizes the ratio of the total amount of prooxidants to the activity of antioxidants (37). The negative correlation of the indicator with sperm motility and the *in vivo* fertility of liquid-stored boar semen (artificial insemination doses) was reported (38).

Copper-containing protein, CP, plays an important role in the inactivation of ROS. It is the main extracellular antioxidant that inhibits LPO and neutralizes the superoxide anion radical. The content of CP in the sperm of boars was almost twice as high as that of seminal plasma. The significant content of this protein is probably associated with low SOD activity. Moreover, the relatively stable content of CP in the ejaculates of breeding boars was due to the high resistance of this protein to the toxic effects of ROS.

Thiol compounds play a significant role in the regulation of redox homeostasis in cells and tissues. They participate in the regulation of energy metabolism, play the role of natural antioxidants, and create optimal conditions for the fertilization of the egg. Currently, there are more than 100 enzymes whose activity is inhibited by blocking in their structure SH-groups (17). Mammalian semen is rich in sulfur, the main part of which is part of amino acids, peptides, and proteins (cysteine, GTH, ergothioneine). These compounds are the main line of protection of cells from hydroxyl radicals (39). SH-groups protect proteins from the destructive action of ROS, so it is likely that the physiological activity of sperm obtained from boars of different breeds depends on the quantitative content of sulfhydryl groups in spermatozoa.

The relatively high level of thiol groups in the semen of breeding boars is probably due to the intensification of synthetic processes and greater accumulation of protein than fat in boars. There are scarce literature data on the relationship between SH-groups and sperm production. The protection of cells from the destructive action of ROS is also due to the GTH system. The coordinated action of all its components (GSH, GR, GPX, GST) helps to establish the optimal level of peroxide compounds and maintain antioxidant homeostasis. In mammalian semen, the non-enzymatic link of antioxidant protection is represented mainly by GTH. It is a coenzyme of a number of enzymes required for the maintenance of intracellular homeostasis and detoxification of peroxides (occurs with the participation of GPX and GST), involved in maintaining redox homeostasis of the mitochondrial matrix, thereby protecting mitochondria DNA and metabolism. GSH is a 'trap' for free radicals, due to which the antioxidant potential of GTH is realized. Under physiological conditions, most of the GTH is in reduced form (oxidized - does not exceed 1%). Decreased GSH is an indicator of redox status and reduces the resistance of cells to oxidative damage.

The high activity of GST against the background of reduced GPX activity can be regarded as the competition of two GTH-dependent enzymes for reduced GTH. The obtained data are consistent with the existing ideas about the role of the GTH antioxidant system in the neutralization of LPO products (40). It was found that the redox balance coefficient was the highest in the plasma of sperm of animals of the synthetic line. It should also be noted that in seminal plasma the coefficient was significantly higher compared to sperm. The obtained data confirm the finding that seminal plasma has a higher antioxidant potential compared to spermatozoa (41, 42). Comparing the coefficients obtained by different calculations, the features of redox regulation in seminal plasma and sperm can be recognized. The imbalance of pro-and antioxidants in the semen of boars of the synthetic line was higher. This may indicate that the semen of Large White breeds has a greater supply of antioxidant status compared to animals of the synthetic line SS23. Interestingly, recent studies by O'Brien (43) add to our data important information that sperm from wild boars showed more resistance to ROS than sperm from domestic ones.

It should be highlighted here that the studied sperm and seminal plasma parameters of the Large White boars and synthetic

line SS23 boars besides genetic regulation are under hormonal impact. Both environmental hormonally active chemicals with antiandrogenic or estrogenic properties including not-well studied metalloestrogens (44) as well as endogenous factors are implicated in this regulation. Therefore, further studies counting their specific effects are urgently needed with the use of new markers of reproductive function development and aging *e.g.* aquaporins (45).

It should be emphasized that the presence of a multi-stage antioxidant system of protection against free radical oxidation causes the complexity of the causal relationship between pro- and antioxidants and aims to establish a balance between them and maintain the optimal metabolic balance of the cell. The intensity of LPO processes in spermatozoa is much higher than in the extracellular space. All components of the antioxidant defense system are normally in a mutual compensatory relationship. As a rule, a decrease in the concentration or activity of some antioxidants leads to corresponding changes in others. Superoxide dismutase, CAT, CP, GTH, and enzymes dependent on it form the main antioxidant system of boar sperm in both intracellular and extracellular space.

The semen of boars studied here contains a significant number of SH- groups of low molecular weight thiols and proteins, which are apparently involved in complex biochemical processes in cells, as well as in the preservation of structural elements of cells, gamete membranes, and sperm quality. Further research on the mechanisms of action of ROS on semen in breeding boars and the influence of various biologically active substances on semen as well as pro- and antioxidant components are needed. This will help to deepen ideas about the biological significance of these compounds and processes in the body, as well as find effective methods and drug storage outside the body together with the oocyte fertilization procedure.

Authors' contribution: S. Polishchuk - head of the project; S. Tsekhmistrenko - designed the experiment; V. Polishchuk - wrote the manuscript; O. Tsekhmistrenko - collected the samples; L. Zdorovtseva - analyzed the data; O. Yakoviichuk and M. Kotula-Balak - corrected the manuscript; K. Tarasiuk, Y. Ievstafiieva and T.Hustol - performed the laboratory analysis.

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