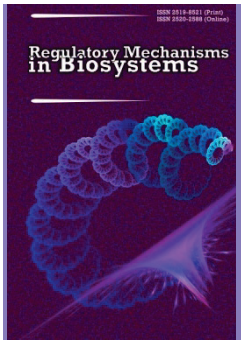

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Effects of copper citrate on physiological-biochemical parameters of ejaculate of sire boars

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Revealing peculiarities of the course of physiological and biochemical processes in the formation of parameters of ejaculate allows effective correlation of biological completeness of spermatozoids based on the use of chelate compounds of limiting microelements, particularly copper. The study was focused on determining changes in processes of spermatogenesis during correction of mineral nutrition. In the experiment, we used adult boars of the same age, live weight and quality of ejaculates. They consumed copper citrate during the 45 day experiment in doses higher than the norm by 10% (1.5 mg per 1 kg of combined feed) and 20% (3 mg per 1 kg of combined feed) compared with the control. We determined difference in the effects of different doses of copper citrate on the formation of parameters of ejaculates in sire boars. Addition of the mineral additive to the feed in the doses 10% exceeding the norm promoted increase in the parameters of functional activity of spermatozoids – mobility and survival – on day 45 of the intake, which occurred against the background of acceleration of peroxidation processes (increase in the concentration of thiobarbituric acid of active compounds) and activation of the system of antioxidant protection – increase in the activity of superoxide dismutase and decrease in the level of catalase in the sperm. At the same time, we observed increase in the morphometric parameters of spermatozoids – general length, width, length and volume of the head. On day 30, sire boars that had been consuming copper citrate in the amounts 20% above the norm were observed to have increase in concentration of spermatozoids, their mobility and survivability, though a decrease was seen in the morphometric parameters of the gametes. Further intake of this element caused increase in the amount of abnormal forms of spermatozoids. Under the action of this additive, we determined increase in the amount of metabolites of lipid peroxidation – conjugated dienes and thiobarbituric acids of active compounds, and also weakening of the system of antioxidative protection – decrease in the content of reduced glutathione and accumulation of dehydroascorbic acid. Thus, the intake of copper citrate in the amounts of 10% above the norm facilitated morpho-functional parameters of spermatozoids by activating the system of antioxidant protection. It would be promising to conduct further research to determine the effects of copper citrate on the processes of activation and capacitation of spermatozoids and fertilization of oocytes in in vivo and in vitro conditions.

Keywords: microelements; sperm; spermatozoids; peroxidation of lipids; antioxidants; homeostasis.

Introduction

Determining and removing factors that negatively affect the quality of obtained ejaculates helps increase the fertilizing ability of spermatozoids. Intensification of reproductive biotechnology requires obtaining biologically complete genetic material from sires, which depends greatly on breed (Knecht et al., 2014), age (Wang et al., 2017), seasonality (Rungruangsak et al., 2021), completeness of diet (Rodriguez et al., 2017) and intensity of sexual load (Shostya et al., 2021). Those factors need to be precisely controlled for the best manifestation of their reproductive potential. The volume of ejaculate, concentration, mobility and normal morphology of gametes are the main parameters for determining the quality of sperm production and are considered markers of fertility. Fertilizing ability of spermatozoids is closely related to their morphometric parameters. Shape, sizes and ratios between them determine the ability of gametes to direct progressive movement, capacitation and acrosome reaction, and the structure of spermatozoids influences the ability to penetrate the membranes of oocytes and the process of fusion (Garcia et al., 2016). Presence of spermaoztoids with abnormalities of different types indicates the extent of physiological abnormalities in the spermatogenesis process.

During maturation (process of moving through the epididymis), spermatozoids are characterized by a number of structural transformations: change in the composition and potential of the membranes, condensation of chromatin and acquisition of the ability to move (Shostia, 2009). Those changes occur with participation of active oxygen species (AOS) and under dynamic control of the prooxidant-antioxidant system (PAS). Increased generation of AOS leads to oxidative stress, accompanied by damage to the integrity of the membranes of spermatozoids and DNA fragmentation both at nuclear and mitochondrial levels. Oxidative damage to DNA at early stages of spermatogenesis (period of spermatogonia and spermatocytes) causes heightened level of apoptosis and increase in abnormal forms, and mature spermatozoids lose the ability to fertilize, decreasing their biological completeness (Kurkowska et al., 2020; Pintus & Ros-Santaella, 2021; Silvestre et al., 2021). Effects of negative factors on physiological-biochemical parameters of sperm production can be mainly leveled out using strategies of mineral nutrition, aimed at increasing tolerance of spermatozoids to oxidative stress, improvement of their functional activity and prolonging the period of spermodosis. A large number of studies indicates that micronutrients are valuable in metabolism of spermatozoids and in support of their functioning through antioxidant properties (Nenkova et al., 2017; Pipan et al., 2017; Sutovsky et al., 2019). By playing an important role in metabolism of carbohydrates, proteins and lipids, essential microelements provide realization of the genetic potential of males (Wu et al., 2019).

Formation of PAS in sperm has been confirmed, and therefore the quality of sperm-production is closely associated with the level of intake of microelements. Additional consumption of selenium, copper, iron, and zinc improves mobility and survivability of spermatozoids by decreasing

intensity of peroxidation processes, occurring against the background of strengthening the system of antioxidant protection (Horky et al., 2016; Usenko et al., 2020). Introduction of nanochelates of microelements directly into the semen extender increases the intensity of oxidative processes, thereby producing optimum level of active oxygen species, needed for the normal course of the processes of capacitation and hyperactivation (Rokotyanska, 2018; Kornyat et al., 2019). The leading role among the microelements determining the quality of sperm production is played by copper, which transports electrons to oxygen molecules in oxidation-reduction reactions. This is related to the fact that copper ions easily switch from one valent condition to other $Cu^{+} \leftrightarrow Cu^{2+}$, i.e. can be donors, as well as acceptors of electrons. Because of its oxidative-reducing potential, copper is present in active centers of proteins and is a cofactor of more than ten enzymes (Ogorek et al., 2017). Cu^{2+}/Zn^{2+}-superoxide dismutase (SOD) is the main enzyme of antioxidant protection, which dismutates the superoxide anions ($O_2^{\bullet-}$) into hydrogen (H_2O_2) and oxygen (O_2), protecting spermatozoids from negative effects of free radicals (Celino et al., 2011; Kodama et al., 2012). Other than protecting gametes from oxidative stress, SOD prevents early hyperactivation of spermatozoids before ejaculation (Esakky et al., 2013). Copper-containing ferroxidase – ceruloplasmin, a large amount of which is concentrated in Sertoli cells, participates in transport of iron ions, which are the basis for such fundamental cellular processes as oxidative phosphorylation and oxygen transport (Vashchenko & MacGillivray, 2013; Tvrda et al., 2015). Other than enzyme cofactor, copper takes part in realization of signal pathways of apoptosis development by inhibiting activation of caspases 3, 7 and 9 (Mufti et al., 2006; Devi et al., 2021). This confirms the leading role of copper in seminal plasma in the control of the number of live spermatozoids.

Therefore, norming the mineral nutrition by giving copper allows biologically complete ejaculates to be obtained by increasing the functional activity of spermatozoids and decreasing the level of their abnormal forms. The objective of the study was determining the influence of copper on physiological-biochemical parameters of ejaculates of sire boars. The research was conducted in the following directions: determining the effects of copper citrate on qualitative and quantitative parameters of sperm production; determining the effect of copper citrate on morphological peculiarities of spermatozoids; studying the influence of copper citrate on the intensity of processes of peroxidation in sperm of sire boars.

Materials and methods

The studies of the effects of copper citrate on physiological-biochemical parameters of ejaculates of sire boars were performed in the conditions of Plemservis Ltd and the Laboratory of Physiology of Reproduction of the Institute of Pig Breeding and Agroindustrial Production of the National Academy of Sciences of Ukraine. The studies' protocols were approved by the local ethics commission of the Poltava State Agrarian University. Feeding, maintenance and withdrawal of animals from the experiment were carried out according to the Law of Ukraine "On Protection of Animals from Cruel Treatment" (Ukraine, 28.03.2006) and the rules of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Strasbourg, 13.03.1986).

For the experiment, we selected 9 Large White adult sire boars aged 18–24 months with live weight 250–300 kg, of which three groups were formed according to analogue principle, three individuals in each (the control and two experimental). The animals were kept in individual animal holding devices of 7 m^2 area. The animals were fed twice a day, according to the fodder norms (3.5 kg/day of mixed feed of our own production). The diet of sire boars of the control group remained without changes (15.4 mg of copper per 1 kg of mixed feed), whereas the diets of I and II experimental groups were supplemented with copper citrate in doses respectively higher by 10% (1.5 mg per 1 kg of mixed food) and 20% (3 mg per 1 kg of mixed feed) compared with the control. The experiment lasted for 105 days, in particular: preparation period – 30 days, the main – 45 days (feeding copper citrate), and the final – 30 days.

Sperm of sire boars was obtained twice a week manually (without artificial vagina) (Melnik, 2003). In the preparation period, 24 ejaculates were collected, in the main period 36 (24 ejaculates – day 30, 12 ejaculates – day 45) and 24 ejaculates in the final period from each group of the animals, providing optimum functioning of the reproductive system and quality of sperm production. Freshly collected ejaculates were analyzed according to weight (g), concentration of spermatozoids (M of cells/cm^3), total number of spermatozoids in the ejaculate (B), number of live spermatozoids in the ejaculate (B), mobility of spermatozoids (%) and survivability of spermatozoids (%). Mass of ejaculate after filtration was determined in a disposable polyethylene sperm collector by weighing on TBE-0.6 laboratory scales (Techno Wagy, Ukraine, 2018) with the accuracy of 10 mg. Concentration of spermatozoids was determined using Photometer SpermaCue (Minitüb, Germany, 2013). Total number of spermatozoids in the ejaculate was determined by multiplying the volume of ejaculate by concentration of spermatozoids (Melnik & Kravchenko, 2016). The number of live spermatozoids in the ejaculate was determined by multiplying the total number spermatozoids in the ejaculate by percentage of mobile spermatozoids (Melnik & Kravchenko, 2016). Mobility of spermatozoids was determined visually under a microscope. For this purpose, a drop of sperm was placed on a dry microscope slide using a pipette and covered by another microscope slide. The obtained preparation was placed on the object stage of the microscope, connected to heated stage (38–40 ºC). The activity of gametes was determined at 180–300x magnification of the microscope. Mobility of spermatozoids was evaluated in points, according to a ten-point scale. The highest score – 10 points – was given to sperm where practically all 100% of spermatozoids moved in direct-progressing manner. A total of 90% of spermatozoids moving with direct progression were given 9 points, 80% – 8, and so on (Melnik, 2003). Survivability of spermatozoids was determined by accelerated method (thermoresistant probe). For this purpose, the examined samples of sperm were held in a thermostat at a temperature of 38 ºC for 3 h. The parameter of mobility of spermatozoids after the incubation was determined using the abovementioned methods (Melnik, 2003).

On a defattened, dry microscope slide, we put a drop of extended sperm (using 1% solution of sodium chloride by two times) and made a thin smear, which was dried in air, and further fixed in 96% alcohol rectifier for 1–2 minutes. The obtained smear was rinsed in distilled water and stained by azur-eosin solution (Yablonskij, 2002). On the obtained preparations, we determined morphological sizes of spermatozoids employing the method of cytomorphometry with a PZO Eclipse E-200F micromicroscope followed by video-microscopic cytometry using a digital camera Micromed MDC-500 in the software environment Vividia Able Scope. From each sire boar, we measured 100 spermatozoids, which had directly-progressing spatial positions. Morphological analysis of sizes of spermatozoids was performed according to the following parameters: general length, width and volume of the heads, body length. Volume of the head of spermatozoids was determined using the formula of Melnyk & Kravchenko (2016). Along with the determining sizes of spermatozoids, we carried out their visual evaluation for the purpose of determining abnormalities in the morphology according to the Blom's classification.

To evaluate the intensity of the course of peroxidation in sperm, we determined: 1. Concentration of conjugated dienes (CD) – spectrophotometry. The method is based on the property of conjugated dienes to absorb light emission in the ultraviolet area of the spectrum at the wavelength of 233 nm. At the same time, optical density of the solution is proportionate to the concentration of conjugated dienes in the examined sample (Vlizlo, 2004). Concentration of TBA-active compounds (aldehydes and ketones) was determined photoelectrocolometrically. The method is based on the reaction between malondialdehyde (MDA) and thiobarbituric acid (TBA), which at high temperature and in acid environment takes place with formation of a coloured complex, containing one molecule of MDA and two molecules of TBA (Vlizlo, 2004). To evaluate the level of antioxidant protection in the samples of sperm, we determined:

1) activity of superoxide dismutase (SOD) – photometrically, according to the speed of inhibition of autoxidation of adrenaline. The method is based on the ability of superoxide dismutase to inhibit autoxidation of adrenaline, which is initiated by superoxide radicals that are produced by interaction of adrenaline and metals in an alkaline environment (Shabunin, 2010);

2) catalase activity (CA) – using the method based on ability of hydrogen peroxide to form a stable coloured complex with ammonia molybdenum salts (Korolyuk et al., 1988);

3) concentration of reduced form of glutathione – photoelectrocolometrically with Elman's reagent. The method is based on the fact that the sulfhydryl group of reduced glutathione enters the reaction with 5 5′-dithiobis(2-nitrobenzoic) acid (Elman's reagent), which results in formation of thio-nitrophenyl anion, stained yellow, which has maximum of 412 nm, in equimolar quantities (Shabunin, 2010);

4) concentrations of ascorbic (AA) and dehydroascorbic (DHA) acids were determined according to their qualitative reaction with 2,4-dinitrophenylhydrazine with further determining the number of formed osazones using a modified method (Rybalko, 2005).

The results are presented as mean ± standard error (x ± SE). To compare the difference of mean parameters between the control and experimental groups, we used the Tukey test, where the differences were considered statistically significant at $P < 0.05$ for all the data.

Results

The data of the experiment indicate the difference between the effects of the level of mineral additive on biological completeness of ejaculates. On day 45 of the intake, the mass of ejaculate of the animals that had received copper citrate in the amount 10% higher than the norm was 230 ± 9 g, which – compared with the initial period and on day 30 of the main period was higher by 12.5% and 16.8% respectively. At the end of the experiment, ejaculate of males that had consumed the mineral additive in the amount exceeding the norm by 20% equaled 260 ± 8 g, which was 9.1% higher than the preparation period and 20.5% ($P < 0.01$) and 8.4% compared with the 30th and 45th days of the main period respectively. In the same period, weight of the ejaculate from sire boars of experimental group II was higher compared with the control and experimental group I respectively by 12.9% ($P < 0.05$) and 18.1% ($P < 0.01$) (Fig. 1a).

Intake of copper citrate in the amount 10% higher than the norm for a month promoted increase in the concentration of spermatozoids by 5.7%, which was higher by 17.6% ($P < 0.01$) compared with the control, though on day 45 of the main period and at the end of the experiment, this parameter was 208 ± 5 and 213 ± 8 M cells/cm^3, which was lower by 14.1% ($P < 0.05$) and 12.2% ($P < 0.01$) compared with the 30th day of the main period respectively. Consumption of copper citrate in the amount that exceeded the norm by 20% was accompanied by 13.2% ($P < 0.05$) and 9.4% increases in the concentration of spermatozoids on the 30th day (228 ± 7 M cells/cm^3) and the 45th (220 ± 6 M cells/cm^3) day of the main period compared with the beginning (201 ± 5 M cells/cm^3), which – compared with the control – were higher by 10.5% and 17.1% ($P < 0.01$) respectively (Fig. 1b).

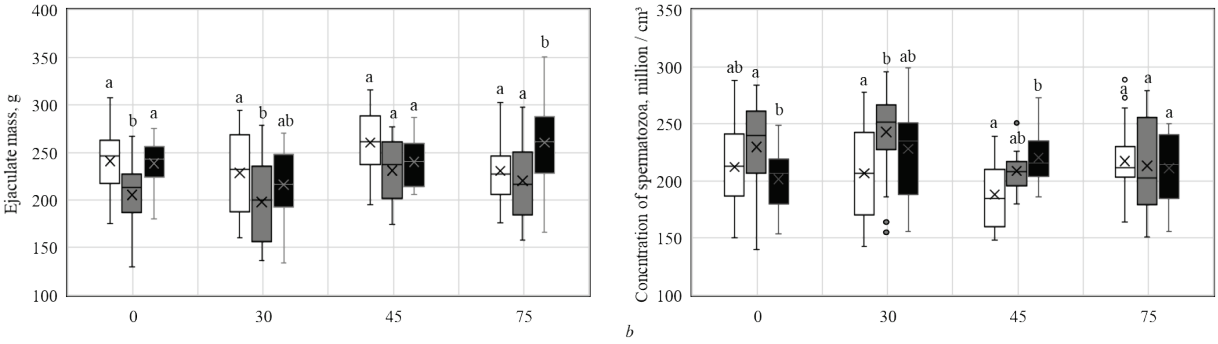

Fig. 1. Changes in mass of ejaculate (*a*) and concentration of spermatozoids (*b*) under the action of copper citrate: on abscissa axis – periods of the experiment (0 – preparation period (n = 24); 30 – 30th day of the main period (n = 24), 45 – 45th day of the main period (n = 12), 75 – final period (n = 24)); white rectangle – control group, grey rectangle – experimental group I (10% copper citrate), black rectangle – experimental group II (20% of copper citrate); upper and lower edges of rectangle – 25% and 75% quartiles, vertical lie – minimal and maximal values, circles – emissions, x (inside rectangle) – mean value, horizontal line on rectangle – median; letters on each figure indicate significant differences between groups in each period at ($P < 0.05$) according to the results of Tukey test

Intake of the microelement increased by 20% had significant effect on the total number of spermatozoids and number of their live forms in ejaculate. At the end of the experiment, the number of spermatozoids in animals of this group equaled 55 ± 2 B in ejaculate, which – compared with animals of the control and experimental group I – was higher by 9.9% and 18.3% ($P < 0.05$) respectively. On day 45 of the experiment, the number of live spermatozoids in ejaculates of sire boars of experimental group II equaled 47 ± 2 B in ejaculate, which was 20.7% higher compared with the beginning. At the end of the experiment, the greatest share of live spermatozoids in ejaculate was seen in animals that had been consuming the highest level of copper citrate, which – compared with the control (41 ± 1 B) and experimental I (38 ± 2 B) groups – was higher by 13.1% and 22.1% ($P < 0.05$).

Intake of copper citrate was accompanied by changes in the activity of spermatozoids. Sire boars that had been consuming copper citrate in 10%-above-the-norm amounts were seen to have 5.5% ($P < 0.05$) and 5.3% ($P < 0.05$) increases in mobility of gametes on days 30 and 45, which was higher than the control by 4.4% ($P < 0.05$) and 7.0% ($P < 0.01$) respectively (Fig. 2a). In the final period, after the intake of the mineral additive had been stopped, the mobility of spermatozoids of males of experimental group I was 82 ± 1%, which was lower by 6.1% ($P < 0.01$) and 7.0% ($P < 0.01$) respectively compared with days 30 and 45 of the main period. Increase in the level of copper citrate in diet by 20% promoted 7.8% ($P < 0.01$), 9.7% ($P < 0.01$) and 5.6% ($P < 0.05$) increases in mobile spermatozoids throughout the experiment. The highest parameters of survivability of spermatozoids after three-hour incubation at the temperature of 38 ºC were observed in sire boars that had been consuming copper citrate in the amount higher than the norm by 10%. Therefore, at the ends of the main and final periods, the survivability of spermatozoids was at the levels of 70 ± 1% and 71 ± 1%, higher by 13.5% ($P < 0.01$) and 15.5% ($P < 0.01$) compared with the beginning and by 12.0% ($P < 0.01$) and 12.5% ($P < 0.01$) compared with the control group (Fig. 2b). On the 30th day of consumption of the mineral additive, the survivability of spermatozoids in animals of experimental group II was 13.8% ($P < 0.01$) higher than at the beginning of the experiment and 10.9% ($P < 0.01$) and 4.2% ($P < 0.05$) higher compared with the control and experimental group I respectively. Further intake of high doses of copper citrate caused 7.5% ($P < 0.01$) and 6.9% ($P < 0.05$) decreases in survivability of spermatozoids at the ends of the main and final periods compared with the 30th day of the main period.

We determined changes in morphometric parameters of spermatozoids depending on the level of intake of copper citrate (Table 1). General length of sex cells in animals of experimental groups depended on the consumed dose. Therefore, sire boars that had been consuming copper citrate in the amount of 10% above the norm for a month were observed to have 1.9% ($P < 0.01$) decrease in general length of spermatozoids, though at the end of the main and final periods, this parameter increased by 3.4% ($P < 0.01$) and 3.2% ($P < 0.01$) compared with day 30 of the main period, which – compared with the control – was higher by 2.3% ($P < 0.01$) and 0.9%. The animals whose diet was supplemented with copper citrate in 20%-above-the-norm doses were observed to have decrease in the general length of spermatozoids throughout the experiment, particularly by 4.7% ($P < 0.01$) (day 30), 3.7% ($P < 0.01$) (day 45) and 1.9% ($P < 0.01$) (final period) compared with the beginning. After the

intake of the mineral additive had been stopped (final period), the animals of experimental group II were observed to have 2.9% ($P < 0.01$) and 1.9% ($P < 0.01$) increases in spermatozoids compared with the 30th and 45th days of the main period, which were higher by 2.2% ($P < 0.01$) and 1.0% ($P < 0.01$) compared with the control and experimental group I.

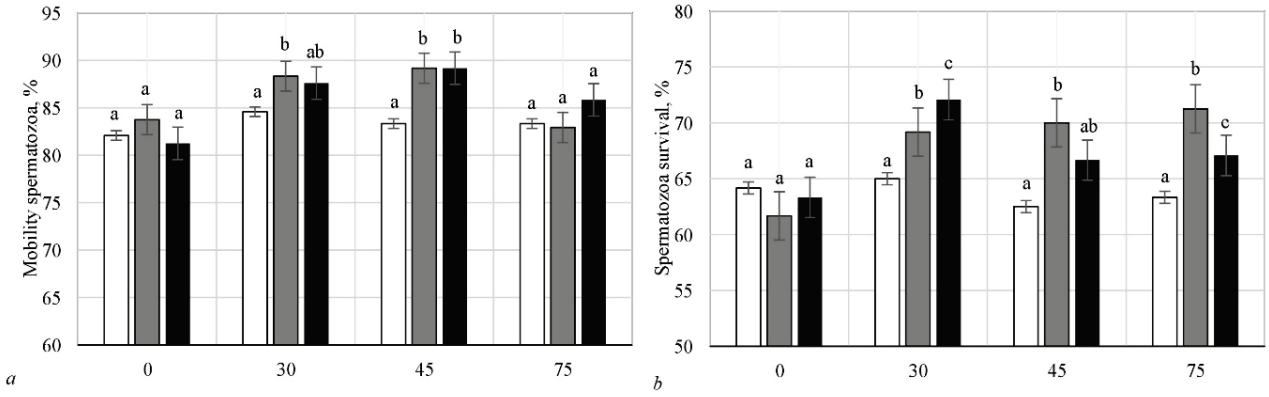

Fig. 2. Changes in mobility (*a*) and survivability (*b*) of spermatozoids under the action of copper citrate (x ± SE): abscissa axis – periods of the experiment (0 – preparation period (n = 24); 30 – 30th day of the main period (n = 24), 45 – 45th day of the main period (n = 12), 75 – final period (n = 24)); white rectangle – control group, grey rectangle – experimental group I (10% copper citrate), black rectangle – experimental group II (20% copper citrate); letters on each figure indicate significant differences between the groups in each period at ($P < 0.05$) according to the results of the Tukey test

Table 1
Changes in morphometric parameters of spermatozoids under the influence of copper citrate (x ± SE, n = 300)

Parameter	Groups	Period of the experiment			
		preparation	main		final
			30th day	45th day	
General length of spermatozoids, μm	C	54.12 ± 0.14[a]	53.52 ± 0.16[a]	54.02 ± 0.15[a]	54.61 ± 0.17[a]
	I	54.51 ± 0.19[a]	53.43 ± 0.15[a]	55.28 ± 0.18[b]	55.13 ± 0.18[a]
	II	56.92 ± 0.16[b]	54.22 ± 0.18[b]	54.79 ± 0.15[b]	55.82 ± 0.16[b]
Length of the head, μm	C	8.19 ± 0.03[a]	8.35 ± 0.03[a]	8.63 ± 0.03[a]	8.95 ± 0.04[a]
	I	8.11 ± 0.02[a]	8.41 ± 0.02[a]	8.68 ± 0.03[a]	8.92 ± 0.04[ab]
	II	8.60 ± 0.03[b]	8.39 ± 0.03[a]	8.65 ± 0.03[a]	8.79 ± 0.03[b]
Width of the head, μm	C	4.04 ± 0.11[a]	4.05 ± 0.02[a]	4.18 ± 0.02[a]	4.21 ± 0.02[a]
	I	3.98 ± 0.02[a]	4.13 ± 0.02[b]	4.20 ± 0.01[a]	4.20 ± 0.02[a]
	II	4.17 ± 0.02[b]	4.11 ± 0.02[b]	4.17 ± 0.02[a]	4.18 ± 0.01[a]
Volume of the head, μm^3	C	67.21 ± 0.85[a]	72.21 ± 0.92[a]	79.55 ± 0.76[a]	83.91 ± 0.88[a]
	I	68.23 ± 0.92[a]	75.69 ± 0.70[b]	80.80 ± 0.75[a]	83.24 ± 0.86[a]
	II	79.64 ± 1.06[b]	75.28 ± 0.94[b]	79.25 ± 0.73[a]	81.29 ± 0.79[a]
Body length, μm	C	10.34 ± 0.07[a]	10.48 ± 0.05[a]	10.26 ± 0.04[a]	10.62 ± 0.05[ab]
	I	10.46 ± 0.05[a]	10.74 ± 0.05[b]	10.52 ± 0.05[b]	10.78 ± 0.05[a]
	II	11.16 ± 0.08[b]	10.87 ± 0.06[b]	10.55 ± 0.05[b]	10.61 ± 0.05[b]

Note: C – control group, I – experimental I (10% of copper citrate), II – experimental II (20% of copper citrate); letters indicate significant differences between the groups in each period within column at ($P < 0.05$) according to the results of the Tukey test.

The length of the head of spermatozoids in sire boars of experimental group I on days 35 and 45 of the main period increased by 3.7% ($P < 0.01$) and 7.0% ($P < 0.01$) compared with the beginning. At the end of the experiment, the length of the head in animals of this group was higher by 9.9% ($P < 0.01$) compared with the preparation period and by 6.6% ($P < 0.01$) and 2.8% ($P < 0.01$) compared with the 30th and 45th days of intake of the microelement respectively. On the 30th day of the main period, intake of copper citrate in 20%-above-the-norm doses led to 2.4% ($P < 0.01$) decrease in the length of the head. However, further intake of the mineral additive promoted 3.9% ($P < 0.01$) (day 45) and 4.7% ($P < 0.01$) (final period) increases in this parameter. At the end of the experiment, the shortest length of the head was determined for animals that consumed copper in 20%-above-the-norm amounts, which – compared with the control and experimental group I – was lower by 1.8% ($P < 0.05$) and 1.5% respectively.

Significant changes in sizes of the width of the head were determined in the experimental group of animals that had been additionally consuming copper citrate in the doses higher than the daily norm by 10%. Therefore, the width of the spermatozoid head of animals of this group increased as early as one month after the intake, by 3.8% compared with the beginning and by 1.9% ($P < 0.01$) compared with the control group. At the ends of the main and final periods, we observed further increase in this parameter by 1.7% ($P < 0.05$) compared with the 30th day of the main period. Sire boars that had been consuming the highest dose of copper citrate (20%) for 30 days were observed to have decrease in the width of the head by 1.4%, though after the end of the intake of mineral additive, and in the final period, this parameter increased by 1.7% ($P < 0.05$) compared with the 30th day of the main period.

With increase in length and width of the head, sire boars of experimental group I were observed to have simultaneous increase in the volume of the head throughout the period of the experiment, particularly by 10.9% ($P < 0.01$) (the 30th day), 18.4% ($P < 0.01$) (the 45th day), and 21.9% ($P < 0.01$) (the final period). On day 30 of the main period, the volume of the head of sperm of animals of experimental group II decreased by 5.5% ($P < 0.01$), though at the ends of the main and final periods, there was increase in the volume of the head respectively by 10.2% ($P < 0.01$) and 7.9% ($P < 0.01$) compared with the 30th day of the main period.

Intake of copper citrate in 10%-above-the-norm doses promoted 2.7% ($P < 0.01$) increase in length of the body on the 30th day of the main period, which was higher by 2.5% ($P < 0.01$) compared with the control group. On the 45th day of intake of the mineral additive, sire boars were observed to have 2.5% ($P < 0.01$) decrease in the body length of spermatozoids compared with the 30th day. On days 30 and 45 of the intake of the mineral additive, the body length of spermatozoids of animals of experimental group II was 2.6% ($P < 0.01$) and 5.5% ($P < 0.01$) shorter compared with the beginning, though 3.7% ($P < 0.01$) and 2.8% ($P < 0.01$) longer compared with the control group. At the end of the experiment, body sizes of spermatozoids of males of experimental group I were 1.5% ($P < 0.01$) greater compared with the control and experimental group II.

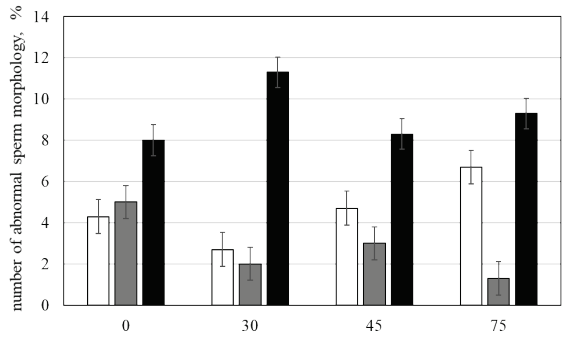

Fig. 3. The number of spermatozoids with abnormal morphology in the conditions of consuming various doses of copper citrate (n = 300): on abscissa axis – periods of the experiment (0 – preparation period; 30 – 30th day of the main period, 45 – 45th day of the main period, 75 – final period); white rectangle – control group, grey rectangle – experimental group I (10% of copper citrate), black rectangle – experimental group II (20% of copper citrate)

The number of spermatozoids with abnormal morphology varied depending on the level of intake of copper citrate (Fig. 3). In samples from males that had been additionally consuming mineral additive in the optimum amount (10% above the norm), the number of spermatozoids with abnormal morphology on the 30th and 45th days of the main period of the experiment accounted for 2% and 3%, which was lower by 2.5 and 1.6 times respectively compared with the beginning. In the same periods, the highest number of abnormal forms of spermatozoids was determined in animals that had been consuming the highest level of the additive, equaling 11.3% and 8.3%, which was 2.8–5.6 times higher compared with the other groups. A similar tendency was also seen at the end of the experiment.

During the experiment, we determined difference in the intensity of the peroxidation processes in sperm of sire boars. The intake of copper citrate in 10%-above-the-norm doses caused 11.5% and 21.9% decreases in the concentration of conjugated dienes at the end of the main and final periods respectively. At the end of the main and final periods, the concentrations of conjugated dienes in the studied samples of the animals of experimental group II equaled 2.29 ± 0.45 and 2.19 ± 0.21 μmol/L, which was higher than the control group by 63.6% and 34.4% (1.40 ± 0.35 and 1.63 ± 0.29 μmol/L) and by 49.7% and 62.2% (1.53 ± 0.26 and 1.35 ± 0.24 μmol/L, Fig. 4a) compared with experimental group I. On day 45 of the intake, the concentrations of TBA-active compounds in animals of experimental groups I and II were at the levels of 29.80 ± 4.04 and 30.64 ± 4.12 μmol/L, which were higher by 22.0% and 42.7% compared with the preparation period (24.42 ± 2.24 and 21.47 ± 3.14 μmol/L). After the end of the intake of the mineral additive, in sire boars of the experimental groups (I and II), we found 9.6% and 23.9% decreases in the content of TBA-active compounds compared with the 45th day of the main period, which were 25.7% and 9.0% lower than the control group respectively (Fig. 4b).

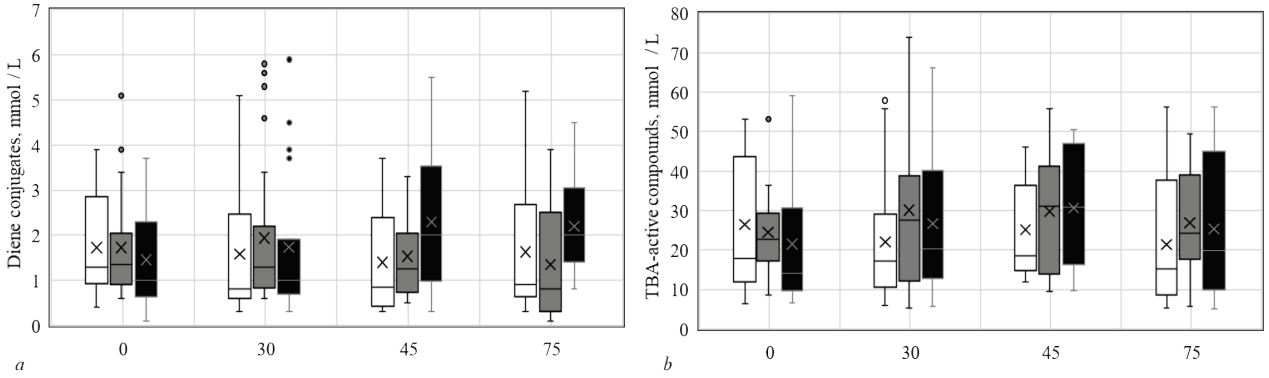

Fig. 4. Intensity of peroxidation processes in sperm of sire boars: *a* – concentration of TBA-active compounds, *b* – concentration of conjugated dienes, on abscissa axis – periods of the experiment (0 – preparation period (n = 24); 30 – 30th day of the main period (n = 24), 45 – 45th day of the main period (n = 12), 75 – final period (n = 24)); white rectangle – control group, grey rectangle – experimental group I (10% of copper citrate), black rectangle – experimental II (20% of copper citrate); upper and lower edges of rectangle – 25% and 75% quartiles, vertical line – minimal and maximal values, circles – emissions, x (inside rectangle) – mean value, horizontal line on rectangle – median; no significant differences were found according to the Tukey test

Table 2
Condition of antioxidant system in sperm of sire boars (x ± SE)

Parameters	Groups	Period of the experiment			
		preparation (n = 24)	main		final (n = 24)
			30th day (n = 24)	45th day (n = 12)	
Superoxide dismutase, conventional units/mL	C	0.371 ± 0.044[a]	0.313 ± 0.027[a]	0.410 ± 0.046[a]	0.354 ± 0.038[a]
	I	0.284 ± 0.039[a]	0.264 ± 0.029[a]	0.513 ± 0.083[a]	0.418 ± 0.080[a]
	II	0.444 ± 0.046[a]	0.526 ± 0.053[b]	0.529 ± 0.114[a]	0.503 ± 0.089[a]
Catalase, H_2O_2/min/L	C	27.85 ± 3.40[a]	23.99 ± 4.07[a]	19.60 ± 4.26[a]	19.20 ± 2.66[a]
	I	25.11 ± 3.83[a]	23.23 ± 3.54[a]	14.18 ± 2.16[a]	21.69 ± 2.69[a]
	II	22.33 ± 3.62[a]	24.05 ± 3.86[a]	15.68 ± 2.12[a]	21.87 ± 2.68[a]
Reduced glutathione, μmol/L	C	0.327 ± 0.056[a]	0.305 ± 0.043[a]	0.358 ± 0.061[a]	0.295 ± 0.050[a]
	I	0.337 ± 0.074[a]	0.266 ± 0.029[a]	0.281 ± 0.042[a]	0.268 ± 0.041[a]
	II	0.332 ± 0.057[a]	0.218 ± 0.026[a]	0.245 ± 0.033[a]	0.229 ± 0.028[a]
Ascorbic acid, μmol/L	C	8.69 ± 1.25[a]	10.36 ± 1.23[a]	8.73 ± 0.99[a]	10.74 ± 0.89[a]
	I	10.52 ± 0.85[a]	9.25 ± 0.88[a]	10.27 ± 0.90[a]	12.97 ± 0.79[a]
	II	8.25 ± 0.65[a]	9.69 ± 0.99[a]	10.32 ± 1.04[a]	10.95 ± 0.93[a]
Dehydroascorbic acid, μmol/L	C	8.86 ± 1.12[a]	10.28 ± 1.00[a]	7.20 ± 1.03[a]	10.26 ± 1.11[ab]
	I	10.84 ± 0.84[a]	12.45 ± 1.08[ab]	8.76 ± 1.28[a]	7.54 ± 0.79[a]
	II	8.43 ± 0.71[a]	14.75 ± 1.27[b]	14.17 ± 1.04[b]	13.16 ± 0.89[b]

Note: C – control group, I – experimental I (10% of copper citrate), II – experimental II (20% of copper citrate); letters indicate significant differences between groups in each period within one column at ($P < 0.05$) according to results of the Tukey test; n – number of examined samples (24 ejaculates were collected in the preparation period, 36 in the main (24 ejaculates – day 30, 12 ejaculates – day 45) and 24 in the final, from each group of animals).

The level of antioxidants in the samples of sperm of sire boars during the experimental period ranged depending on the consumed dose of copper citrate (Table 2). At the end of the main and final periods, in sperm of sire boars that had been consuming mineral additive in 10%-above-the-norm doses, the activity of SOD was higher by 80.6% and 47.2% compared with the beginning of the experiment respectively. The highest level of SOD was determined in animals of experimental group II on day 30 of the consumption, which was higher respectively by 68.1% ($P < 0.01$) and 99.2% ($P < 0.01$) compared with the control and experimental group I. A similar tendency was also seen at the end of the experiment. Intake of various doses of copper citrate (10% and 20%-above-the-norm) during 45 days promoted 43.5% and 29.7% decreases in the level of CA in sperm, which was 27.7% and 20.0% lower respectively compared with the control group. After the intake of the mineral additive, activity of this enzyme in males of experimental groups I and II increased by 52.9% and 39.6% compared with the main period, which was higher than the control group by 12.9% and 13.9% respectively.

The highest level of reduced glutathione throughout the experiment was seen in animals of the control group, whereas the lowest level was in animals of experimental group II. As early as on the 30th day of the intake of copper citrate in the maximal amount (20% above the norm), reduced glutathione in sperm of sire boars decreased by 26.2%, which was 28.5% and 18.0% lower compared with the control and experimental group I respectively. At the ends of the main and final periods, the levels of reduced glutathione in animals of experimental group II were 31.6%, 12.8%, 22.4% and 14.6% lower than in the control and experimental group I respectively.

During the first month of the main period, the intake of copper citrate in 10%-above-the-norm doses led to 12.1% decrease in CA compared with the beginning, though at the end of the main and final periods, we determined 11.0% and 40.2% ($P < 0.05$) increases in the level of this antioxidant. Concentration of CA in sperm of sire boars of experimental group II increased throughout the experiment and at the end of the main and final periods, which was higher by 25.1% and 32.7% compared with the beginning respectively. The level of DHA in sire boars of experimental group I increased on the 30th day of the main period and was higher by 14.9% than the beginning. At the end of the main and final periods, we observed 29.6% and 39.4% ($P < 0.05$) decreases in DHA in the animals of this group, compared with the 30th day of the intake of mineral additive. The intake of copper citrate in the doses that were 20% higher than the norm promoted increase in the concentration of DHA compared with the beginning of the experiment ($P < 0.01$), which – compared with the control group – was lower by 43.5% ($P < 0.05$) (day 30), 96.8% ($P < 0.01$) (day 45). At the end of the experiment, the highest level of DHA was determined in animals of experimental group II, which was 28.3% and 74.5% ($P < 0.01$) higher compared with the control and experimental group I respectively.

Discussion

Determining peculiarities of formation of reproductive ability of males in the conditions of action of various factors is the main condition of obtaining complete genetic material, and therefore offspring. Micronutrients are significant for reproductive ability, and therefore their level in sperm is one of the main biomarkers of quality of sperm production (Ogorek et al., 2017; Fallah et al., 2018; Erohina & Zernaeva, 2020). At the same time, a special role in the normal formation of reproductive function is played by copper. This is confirmed by the obtained results of the studies on influence of intake of copper citrate in doses higher than the norm by 20% on the quantitative parameters of sperm production in sire boars – increase in mass of ejaculates and concentration of spermatozoids. A positive effect of content of this microelement in blood and sperm on quality of sperm production was observed by Eidi et al. (2010). At the same time, insufficient nutrition of animals with copper was accompanied by decrease in this microelement in sperm and saturation with spermatozoids, conditioned by disruption of functioning of Sertoli cells, and therefore processes of spermatogenesis (Tvrda et al., 2015).

Increase in total amount of spermatozoids in studied samples from sire boars that had been receiving maximal level of the microelement is explained by positive correlation between the mass of ejaculate and quantity of gametes, as observed earlier (Smital et al., 2005). Increase in those parameters allows a larger amount of spermatozoids to be obtained from one male, providing more efficient use of genetic potential (Gorski et al., 2017). Positive changes in the parameters of ejaculate under the influence of feeding factor, in particular mineral nutrition, were observed in the study by Semenov et al. (2015).

Sire boars that had been consuming various doses of copper citrate had the highest parameters of the functional activity of spermatozoids. This is conditioned by the fact that the level of copper in seminal plasma is considered an important factor in formation of cyclic adenosine monophosphate (c-AMP) in the internal environment of spermatozoids, activating their mobility (Roy et al., 2013). However, excessive concentration of copper in the blood decreased pH of seminal plasma, deteriorating mobility and survivability of gametes (Eidi et al., 2010).

The pattern we observed in the improvement of survivability of spermatozoids after three-hour incubation in sire boars that had been consuming copper citrate in the optimal amount (10% above the norm) was obviously conditioned by the fact that copper is cofactor of SOD, which protects spermatozoids from oxidative stress and cytochrome *c* oxidase, which participates in energy metabolism and gamete development and support of humoral immunity (Pipan et al., 2017). In males that had been consuming copper citrate in the amounts higher than the norm by 20%, survivability of sex cells decreased. According to the data of Pesch et al. (2006), increase in the copper concentration leads to impairment of glycolysis in seminal plasma, which inhibits mitochondrial potential of spermatozoids, decreases their mobility and survivability. A negative effect of high level of microelements in seminal plasma on mobility and survivability of spermatozoids was also observed in the studies by Shostia et al. (2018).

Morphometric parameters of spermatozoids are believed to determine their mobility and survivability. General length of spermatozoids has positive correlation relationship with speed and type of their movement. Spermatozoids with longer flagella are considered to move faster through sexual pathways of female and be the first to reach oviducts (Gorski et al., 2018; Barquero et al., 2021). During our studies, we determined that the general length of spermatozoids was the highest in sire boars which had been consuming the mineral additive for 45 days in optimum amount, which likely increased their fertilizing ability. Positive influence of correction of nutrition of males on morphology of spermatozoids (general length and length of the head) was reported by Pavlova (2020).

We also determined dependence of mobility (hydrodynamics) of gametes on the form of their head. Spermatozoids with elongated heads, compared with rounded, move more intensively, and the ratio of the length of the head to its width characterizes the ability of sex cells to move in a directly progressing manner (Gil et al., 2009; Gillies et al., 2009). Intake of copper citrate in 10%-above-the-norm doses promoted significant increase in the length, width and the area of the head ($P < 0.01$) compared with the beginning of the experiment. The importance of those changes was confirmed by the study of Montoto et al. (2011), who determined that a larger area of the head increased the likelihood of attachment of spermatozoid to epithelial cells of the lower layer of the oviduct, where spermatozoids remain in the condition of rest, which is necessary for their further functioning.

According to Gorski et al. (2017), there is a relationship between the parameters of ejaculate and the sizes and form of spermatozoids. It was determined that with increase in the weight of ejaculate, there was observed decrease in the length and width of the head, and therefore its volume, though such spermatozoids had longer flagella (Gorski et al., 2017). In sire boars with lower fertilizing ability, spermatozoids had a larger head with more rounded shape (Hirai et al., 2001). A similar tendency was confirmed by our research, in which sire boars that had been additionally receiving copper were observed to have maximum mass of ejaculate, and spermatozoids had longer flagella and more rounded heads.

Finding abnormal morphology of spermatozoids and its localization (head, body, flagellum) allows impairments in spermatogenesis to be determined and therefore removed. Ejaculates of normal quality are known to contain abnormal forms of gametes within 10–15% (Said et al., 2005; Saravia et al., 2007). During our studies, deviations in the structure

of spermatozoids, manifested as curved flagellum and presence of cytoplasmatic drop, were mainly found in males that had been consuming copper citrate in 20%-above-the-norm amounts. Defects of morphology of spermatozoids' flagella are associated with change in its structural components – damage to the structure of the central and peripheral pairs of microtubes of axonemes, uneven arrangement of mitochondria and dysplasia of the fibrous layer of the main part of the flagellum (Hayat et al., 2012; Rudneva & Chernyh, 2018). We determined that the beginning of bending of the flagellum was observed in the period of passage of spermatozoids through the epididymis, which is characteristic of detachment of the cytoplasmic drop. Such type of abnormalities as residual cytoplasmatic drop in the distal section of spermatozoids is considered to cause no decrease in fertility, though it may be a reason for the so-called "Dag defect" (Saravia et al., 2007; Banaszewska & Andraszek, 2021).

Increase in copper accumulation in the testicles leads to histological changes, manifesting in atrophy of the ductus deferens, decrease in the amount of spermatogonial cells and Sertoli cells, and also decrease in meiosis index. Toxic effect of high levels of copper causes various levels of damage to pachytene spermatocytes and early spermatids (vacuolization, karyorrhexis, pyknosis), leading to heightened level of apoptosis and a high proportion of abnormal forms of mature spermatozoids (Javed & Michael, 2014). Roychoudhury et al. (2010) conducted studies on influence of different doses of copper (*in vitro*) on morpho-functional condition of spermatozoids and confirmed the negative effect of high concentration of $CuSO_4$ on the morphology of gametes. Most abnormalities are presented as impairments in acrosome and curved flagellum (Roychoudhury et al., 2010). In our study, the lowest level of abnormal spermatozoids was determined in sire boars that had been consuming the mineral additive in optimum amount, which – compared with the groups – was 2.8–5.6 times lower. Decrease in the amount of abnormal morphology of gametes in the conditions of influence of factor of mineral nutrition was reported by Rybalko et al. (2020).

Biochemical composition of sperm plays quite an important role in maintenance of functional activity of spermatozoids. During our studies, we determined the effect the level of influx of copper to the organism has on biochemical transformation in sperm, mainly on formation of the PAS. The effect of intake of copper citrate in 10%-above-the-norm amounts was seen in sire boars on the 45th day of the main period of the experiment. The positive influence of this microelement manifested in increase in the system of antioxidant protection, namely increase in SOD activity ($P < 0.01$), which stabilizes the formation of free radicals in the period of spermatogenesis and during maintenance of spermodoses (Abdul-Rasheed, 2010).

Superoxide dismutation is accompanied by formation of hydrogen peroxide, which has a more reactive effect on spermatozoids. Residual of H_2O_2 activates catalase, which mainly functions in the endoplasmic reticulum, peroxisomes, mitochondria, cytosol and seminal plasma (Nandi et al., 2019; Rubio-Riquelme et al., 2020). Intake of optimum amount of copper citrate decreased the CAT level ($P < 0.05$), and therefore controlled the concentration of H_2O_2 in seminal plasma, thereby increasing the survivability of spermatozoids on day 45 of the intake (Peruma et al., 2013). The animals of this group were observed to have maximum concentration of ascorbic acid and intense oxidation of glutathione as a result of reduction of dehydroascorbic acid by thiol proteins. Against the background of increase in antioxidant protection in sperm of males, there was seen insignificant decrease in the number of conjugated dienes, indicating slowing of peroxidation processes.

Reduced form of copper (Fenton reaction) is able to catalyze hydroxyl radical, which has high reacting ability to spermatozoids (Mario, 2014). According to our studies of sperm of animals which had been consuming the maximum amount of the mineral additive, we observed increases in the levels of conjugated dienes and TBA-active compounds, indicating that copper ions inhibit antioxidant reactions (Liu et al., 2016). Increased concentration of metabolites indicates peroxidation of lipids of membrane of spermatozoids with further inhibition of their mobility after three-hour incubation compared with the animals that had been consuming the optimum level of copper, and was confirmed by the study by Abdul-Rasheed (2010). At the same time, we determined low concentration of reduced glutathione and high level of DHA in the studied secretions of animals that had been consuming copper citrate in 20%-above-the-norm doses, indicating synergic interaction of those biologically active substances. Decrease in the level of reduced glutathione is likely to be associated with combination with glutathione peroxidase, which participates not only in inactivation of peroxide hydrogen, but is also used in metabolism of cells and detoxification of Cu^+ (Remita et al., 2020).

Conclusions

During the influence of various doses of copper citrate, we observed distinct effects on quality of ejaculates of sire boars. On the 45th day, the intake of this element in 10%-above-the-norm amounts promoted increase in morphometric parameters of spermatozoids (overall length, width and volume of the head), and also improvement of functional activity of spermatozoids (mobility and survivability). The indicated changes in quality of sperm production occurred against the background of acceleration of the peroxidation processes and activation of the system of antioxidant protection in sperm.

Increase in the dose of copper citrate in fodder equaling 20% above the norm caused changes in the quality of ejaculate as early as the 30th day of the intake. Sire boars of this group were characterized by higher concentration, mobility and survivability of spermatozoids, with simultaneous decrease in the general length of spermatozoids ($P < 0.01$), length ($P < 0.01$), volume of the head ($P < 0.01$) and body length ($P < 0.01$). At the same time, on day 45 of the intake of this element, we observed further decrease in the general length ($P < 0.01$), body length ($P < 0.01$) and increase in the number of abnormalities of spermatozoids. Influence of this additive shifts proxidant-antioxidant homeostasis in the direction of acceleration of peroxidation processes – increase in the number of conjugated dienes and TBA-active compounds, and also decrease in the level of the system of antioxidant protection – intense use of reduced glutathione and accumulation of dehydroascorbic acid ($P < 0.01$).

The presented results of the study indicate that in order to improve the quality of ejaculates, it is practical to feed animals with copper citrate in the amount that exceeds the norm by 10%, which improves the quality of sperm production and optimizes the condition of prooxidant-antioxidant homeostasis.

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