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Original article

EFFECT OF ZINC SUPPLEMENTATION TO DILUENT BOAR SEMEN ON SPERM CHARACTERISTICS AND ACTIVITY OF ANTIOXIDANT ENZYMES

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Background. Zinc is essential for male reproductive function as it is necessary for spermatozoa maturation, capacitation, acrosome reaction, and fertilization. It has been established that the use of chelates compounds of metals with amino acids, nucleotides, peptides, and carbohydrates is not only more effective but also economically justified.

Objective. The aim of this study was to evaluate and compare the effect of different concentrations of Zn glutamic amino acid chelate (ZnGlu) on boar spermatozoa motility, viability parameters, and prooxidant-antioxidant homeostasis during *in vitro* incubation.

Methods. Freshly ejaculated boar semen, after the addition of "Ecosperm" diluent, was divided into groups: a control group and three experimental groups. Zinc glutamate was supplemented to the experimental samples at concentrations of 1.0, 2.0, and 5.0 μg/ml, respectively. Semen samples were stored at 18 °C for 4 days. Every 24 hours, sperm motility and viability and the antioxidant status were assessed by the level of diene conjugates (DC), concentration of malondialdehydere (MDA), and the activities of catalase and superoxide dismutase.

Results. It was observed that boar sperm motility decreased with the extension of storage time, while the addition of 2.0 and 5.0 μg ZnGlu significantly improved sperm total motility and the percentage of vitality spermatozoa during 48–96 hours of incubation. Our studies also demonstrate that ZnGlu possess a protective effect in alleviating oxidative stress in boar sperm *in vitro*. The addition of ZnGlu significantly reduced the content of MDA and DC in ejaculate samples in all experimental groups during incubation, compared to the control group. Moreover, the activity of superoxide dismutase and catalase increased after adding ZnGlu to the boar semen, especially at concentrations of 2.0 and 5.0 μg/ml ($P < 0.05$).

Conclusions. These data demonstrate that the supplementation of zinc glutamate enhances the antioxidant defence system of sperm and improves quality of boar semen *in vitro*.

Keywords: sperm quality; sperm motility; zinc; boar semen; antioxidants.

Introduction

Zinc is an essential element with antioxidant properties and is a vital component of semen. Zinc ions play a crucial role in spermatozoa's fertilization ability, affecting aspects like motility, capacitation, membrane stability, and acrosome reaction [1–3]. A deficiency in zinc during the meiosis period can result in germ cell death without the formation of mature sperm cells and leads to the change can lead to changes in the structure, proliferation, and differentiation of Leydig cells, ultimately impairing spermatogenesis [4]. Notably, zinc is associated with protecting sperm cells from oxidative stress as it acts as a co-factor for superoxide dismutase (Cu/Zn-SOD) [5]. The absence or moderate deficiency of zinc in seminal plasma increases the risk of oxidative damage, resulting in reduced sperm quality and idiopathic male infertility [6, 7]. Adding Zn^{2+} to semen extenders before freezing has been shown to reduce the levels of reactive oxygen species (ROS) [8]. However, excessive Zn^{2+} can have a pro-oxidant effect leading to mitochondrial oxidative stress [9].

One of the causes of male infertility is oxidative stress, which results from elevated levels of reactive oxygen species (ROS) that can have harmful effect on spermatogenesis and sperm quality. The excessive production of ROS overwhelms the limited antioxidant defense system in sperm [10, 11]. Sperm cells are particularly vulnerable to oxidative stress as they contain very low levels of enzymatic antioxidants, while plasma membrane contains significant amounts of polyunsaturated fatty acids [12], which are insufficient for protecting sperm from high levels of ROS. Moreover, excessive ROS can lead to reduced spermatozoa vitality and fertilisation through the initiation of DNA damage, lipid peroxidation, loss of membrane integrity, increased permeability, inactivation of cellular enzymes, and cell apoptosis [13, 14]. To maintain the optimal functioning of sperm cells, it is essential to balance

the redox potential, which can be achieved, in part, through the use of zinc compounds.

In recent years, significant attention has been given to the study of organic trace elements due to their potentially higher bioavailability, although there are some conflicting findings [15, 16]. Furthermore, organic zinc compounds have a slower release within the cell, allowing cells ample time to regulate the free zinc ions produced after degradation of organic compounds within the cytoplasm [17]. It is well-established that the absorption of inorganic metal salts differs from that of metal-amino acid chelates [18, 19]. Even among metal-amino acid chelates, absorption rates can vary depending on factors such as the nature of ligand and the strength of chelation, solubility, stability constants, molecular weights, and so on.

However, there has been limited research on Zn(II) complexes with glutamine. We hypothesized that using glutamic acid as an organic complexing agent may enhance the effect of Zinc amino acid chelate on male reproductive function, as glutamate is involved as a neurotransmitter in the activation of the hypothalamo-pituitary axis. Therefore, the primary objective of the current study was to evaluate and compare the effect of different concentrations of zinc glutamic amino acid chelate (ZnGlu) on boars semen motility and viability parameters, as well as prooxidant-antioxidant homeostasis during *in vitro* incubation.

Materials and Methods

Experimental design

The study was performed on seven sexually mature and healthy Landrace boars, aged 24 months and of known fertility. All boars were individually housed in well-ventilated pens in appropriate welfare conditions. Semen was collected manually during routine exploitation of the boars, conducted twice a week to obtain semen for artificial insemination. For each ejaculate, ejaculate volume and sperm motility and concentration were determined. A 5 μL sample of sperm suspension was placed on a pre-warmed slide and sealed with a coverslip at 37 °C. Using 200× magnification, the percentage of normally motile spermatozoa was determined based on the number of sperm present in the microscope's field of vision. Spermatozoa concentration was determined using the colorimetric method with a Photometer SDM 5 (MiniTüb, Germany).

Ejaculates in which sperm motility was at least 70% were mixed with medium "Ecosperm" (Ukraine, Patent U200708849) for dilution and storage of boar sperm. For *in vitro* analyzes, the semen doses were divided into four aliquots: three treatments groups with ZnGlu concentrations of 1.0, 2.0, and 5.0, and a control group (CO) without any additives. These samples were preserved at 18 °C for 4 days. They were stored in a thermally controlled box set to 18 °C. Samples were collected every 24 hours for the analysis of sperm motility, viability, and biochemical parameters. Eight individual doses from seven individual boars were used for *in vitro* analysis.

Estimation of sperm motility

Sperm motility was assessed utilizing a phase-contrast system microscope (Olympus, Japan), which included a heated stage. After each incubation period, an aliquot of sperm suspension was placed on a pre-warmed slide and sealed with a coverslip at 37 °C. At each time point, the percentage of normally motile spermatozoa was determined using 200× magnification, based on the number of sperm visible within the microscope's field of view.

Sperm viability

To assess sperm viability, supravital staining was performed using a 1% aqueous Eosin Y and 10% aqueous Nigrosin solution (Sigma, USA) [20]. Briefly, 5 μl of sperm suspension was mixed with 5 μl of Eosin solution. After 15 s, 10 μl of the 10% Nigrosin solution was added and thoroughly mixed. A drop of this mixture was smeared onto a glass slide left to air-dry. The smears were examined under a light microscope at 100× magnification. Viable sperm cells were transparent, while dead sperm appeared pink.

Antioxidant activity analysis

The levels of superoxide dismutase (SOD), catalase activity (CAT), diene conjugates (DC), and malondialdehyde (MDA) were monitored at 24, 48, 72, and 96 hours after storage. Semen samples were centrifuged at 7500×g for 10 min to remove the supernatants. The obtained cell pellets were washed three times with PBS and then incubated with 0.2% Triton X-100 on ice to lyse cells at 4 °C for 20 min. The supernatant was collected by centrifugation (4000×g, 30 min, 4 °C) and kept on ice for further assays. The concentration of total protein in the tissue homogenates was measured using the Lowry [21] with kits "Simko LTD" (Ukraine, Lviv). All absorbance values were measured using a spectrophotometer.

Determination of diene conjugates content

DC, which form due to the rearrangement of double bonds in polyunsaturated fatty acids, were determined by Vlizlo [21]. After extraction in a mixture of heptane-isopropanol (2:1) and subsequent layering with HCI (pH 2.0), DC were detected in the heptane phase at $\lambda = 233$ nm. The content of DC was expressed in relative micromoles (μmol) per 1 mL of sperm.

Concentration of malondialdehyde

The assessment of MDA concentration relies on the reaction between MDA and thiobarbituric acid under conditions of acidity and elevated temperature [22]. The result of interaction is the color reaction. The colored product absorbance recording was performed spectrophotometrically ($\lambda = 535$ nm and $\lambda = 580$ nm). The concentration of thiobarbituric acid reactive substances (TBARS) was expressed in micromoles per milliliter (μmol/ml).

Activity of superoxide dismutase

SOD enzymatic activity was measured in the presence of NADH and phenazine methosulfate [21]. SOD activity was determined by a method based on its ability to reduce nitroblue tetrazolium. The degree of inhibition of the nitroblue tetrazolium reduction reflects the intensity of enzyme activity. The absorbance recording was performed spectrophotometrically ($\lambda = 540$ nm). SOD activity was expressed as units per milligram of protein (unit/mg protein).

Catalase activity

CAT (EC 1.11.1.6) was assessed using the reaction between hydrogen peroxide and ammonium molybdate [23]. The intensity of the resulting product color was measured spectrophotometrically at $\lambda = 410$ nm. Enzyme activity was quantified as millimoles per minute per milligram of protein (mmol/minute × 1 mg of protein).

Statistical analysis

All experimental data underwent statistical analysis using Microsoft Excel software with one-way analysis of variance (ANOVA). The normality of data was assessed using the Shapiro–Wilk test. Statistical comparisons with the control group were conducted using the paired *t*-test, as all variables exhibited a normal distribution in all groups. Results were presented as mean ± standard error of the mean (SEM) and considered as statistically significant at $P < 0.05$.

Bioethics

The study was conducted in accordance with the guidelines of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes.

Results

Total sperm motility in fresh semen did not change significantly in all groups after 24 hours of storage. After 48 hours of incubation, a significant increse was noted in the group containing 1 μg ZnGlu compared to the remaining gropus (Fig. 1a). In all samples, sperm motility continued to decrease in successive hours of storage. In turn, no significant differences were observed in sperm motility between groups after 72 hours of storage. Notably, the total motility was significantly higher

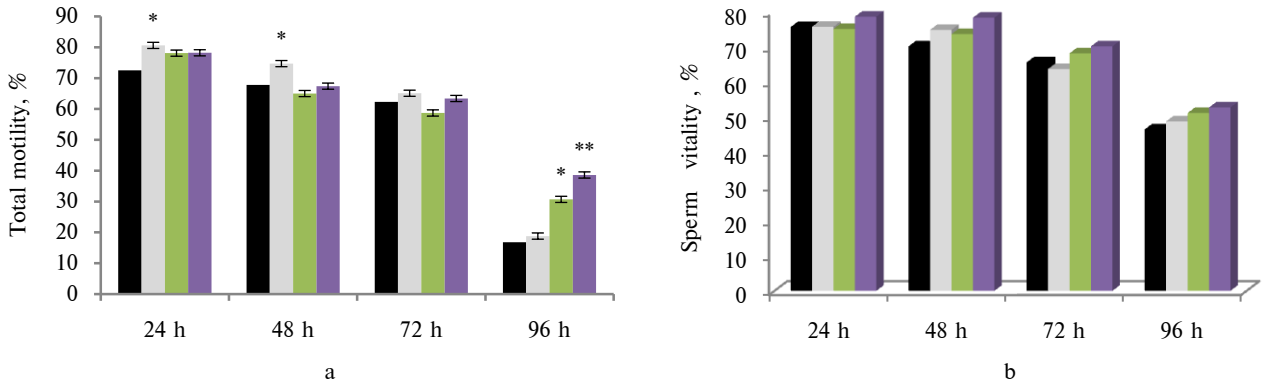

Figure 1: Effects of ZnGlu at different concentrations on sperm motility (a) and vitality (b). Boar spermatozoa were incubated in non-treatment media (control) or in the presence of different concentrations of ZnGlu (1.0, 2.0, and 5.0) at 18 °C for 96 hours: ■ – control, ■ – ZnGlu 1.0 μg/ml, ■ – ZnGlu 2.0 μg/ml, ■ – ZnGlu 5.0 μg/ml. This experiment was conducted 3 times ($n = 3$), and the results are presented as the mean percentage of total spermatozoa ± SEM. Experimental groups were compared pairwise with the control. Statistical differences are indicated with * – $P < 0.05$, ** – $P < 0.01$

($P < 0.05$) after 96 hours of storage in samples preserved with the addition of 2 and 5 μg ZnGlu compared to the control and incubation with the lowest concentration of ZnGlu.

The study revealed that the viability of boar spermatozoa was dependent on the concentration of the compound (Fig. 1b). There were no differences in the percentage of boar sperm vitality among all groups at the initial time of storage (24 hours). With the extension of preservation time, addition of ZnGlu improved the sperm vitality, and the treatment groups with ZnGlu supplementation had higher values at the 48 hours of storage compared to those in the control group. As observed in Fig. 1b, ZnGlu at concentrations of 2 and 5 μg led to an increase in the percentage of vital spermatozoa during 48–96 hours of storage, although these changes were not statistically significant. Additionally, ZnGlu increased the total motility and vitality of boar sperm.

To investigate the protective role of ZnGlu in modulating redox homeostasis in boar sperm, the levels of DC and MDA, as well as the activities of catalase (CAT) and superoxide dismutase (SOD), were evaluated after storage for up to four days under different conccentrations of ZnGlu. As observed in Fig. 2a, the spermatozoa DC level decreased starting from 48 hours after addition of ZnGlu at concentration of 2 and 5 μg ($P < 0.05$). DC values were significantly lower than those of the controls after 72 and 96 hours of storage ($P < 0.05$).

However, the addition of ZnGlu at a concentration of 1 μg led to an increase in the DC level at the initial time of investigation compered to the control and remained at the same level as the control group at 96 hours.

The content of MDA in boar sperm was significantly lower with higher concentrations of ZnGlu (2 and 5 μg/ml) compared to the control group ($P < 0.05$; $P < 0.01$) (Fig. 2b). Furthermore, the MDA content was significantly lower in the group supplemented with the highest concentration of ZnGlu ($P < 0.01$). Meanwhile, the concentration of MDA product in sperm of the control and 1st experimental groups remained almost the same during investigation time of storage.

The addition of ZnGlu significantly increased the the activity of CAT in all experimental groups during incubation (Fig. 3a). This trend was most pronounced when higher doses of the compound were used. Regarding SOD, exposure to ZnGlu resulted in an insignificant increase in SOD activity in experimental groups at 24–48 hours (Fig. 3b). SOD enzyme activity decreased at 72 hours but increased until the end of the measured period in experimental groups with the addition of 2 and 5 μg ZnGlu compared to the control group. Thus, the addition of ZnGlu modulated the redox homeostasis and protected sperm from ROS stress.

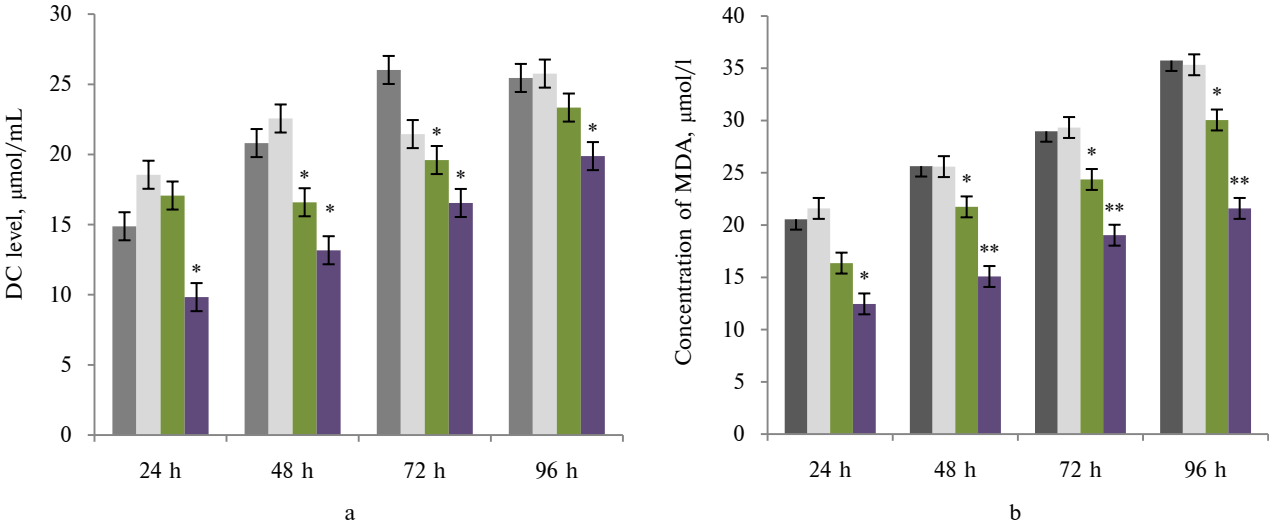

Figure 2: Levels of diene conjugates (DC) (a) and malondialdehyde (MDA) (b) in boar semen after treatment with ZnGlu during 96 hours of incubation at 18 °C: ■ – control, ■ – ZnGlu 1.0 μg/ml, ■ – ZnGlu 2.0 μg/ml, ■ – ZnGlu 5.0 μg/ml. Samples were supplemented with different concentrations of ZnGlu immediately after ejaculation and were assessed every 24 hours of storage. Experimental groups were compared pairwise with the control. Data are presented as mean values ± SEM; * – $P < 0.05$, ** – $P < 0.01$ vs. the control group

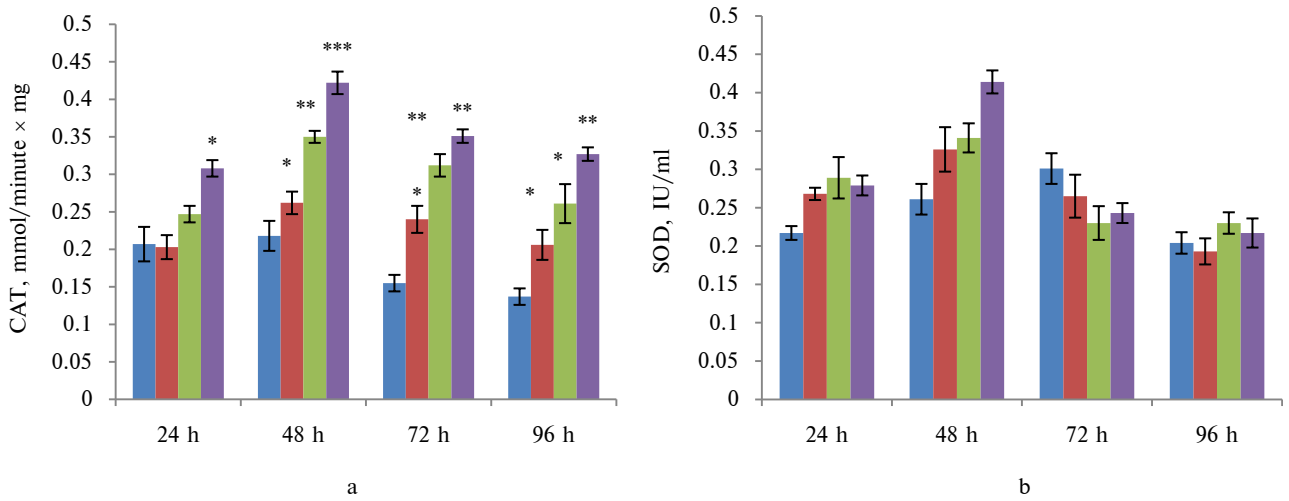

Figure 3: Activity of antioxidant enzyme in boar sperm after treatment with ZnGlu**:** catalse (CAT) (a) and superoxide dismutase (SOD) (b) activity: ■ – control, ■ – ZnGlu 1.0 μg/ml, ■ – ZnGlu 2.0 μg/ml, ■ – ZnGlu 5.0. Sperms were exposed to 1.0, 2.0 and 5.0 μg/mL ZnGlu for 24, 48, 72, and 96 h. Experimental groups were compared pairwise with the control. Data are presented as mean values ± SEM. * – $P < 0.05$; ** – $P < 0.01$; *** – $P < 0.001$ vs. the control group

Discussion

The essential role of zinc on the spermatogenesis is well-known. Zinc plays an essential role in sperm cell maturation and motility and pre-fertilization process, including sperm capacitation, binding of the sperm zona pellucida (ZP), penetration at the site of the ZP, and zygote activation [4, 24]. An unadequate supply of zinc can negatively affect male fertility [25] leading to decreased sperm quality, as zinc levels in seminal fluid are related to sperm concentration and motility [26]. Our research has shown a strong association between zinc levels and sperm quality. The addition of Zinc glutamate (ZnGlu) *in vitro* has a positive effect on the viability and motility of boar sperm when storedat 18 °C. This effect is dependent on the concentration of the compound – ZnGlu, at concentrations of 2 and 5 μg, leads to an increase in the percentage of vital spermatozoa during 48–96 hours of storage. Other studies have reported that the addition of $ZnCl_2$ to Sus extender for storage and transport of liquid boar sperm indicates a protective effect of zinc on mitochondrial sheath and acrosomal membranes; higher percentage of progressive and rapid sperm in doses supplemented with 2 mM $ZnCl_2$ [27]. Zinc deficiency leads to a depletion of total germ cell mass due to impaired spermatogenesis and increased apoptosis of the testicular cell sub-population. On the other hand, some researchers have reported a negative effect of high zinc levels in seminal plasma or sperm tails on sperm motility [2, 28, 29]. Therefore, maintaining the homeostasis of Zn^{2+} is crucial, as both deficiency and excessive zinc absorption can disrupt normal reproductive function.

The significantly higher percentage of motility in semen samples after the addition of ZnGlu could be attributed to the protective effect of zinc on the microfilaments and mitochondrial and acrosomal membranes of spermatozoa. Cellular membranes have been previously shown to be stabilized by zinc [27], and this effect may be responsible for the improved sperm motility and vitality observed in samples supplemented with zinc. Another study has shown the protective effect of adding zinc to semen samples before the freeze-thaw process, which reduces freeze-thaw-induced DNA damage and helps maintain sperm post-thaw motility at higher levels [30, 31]. Additionally, zinc efflux from sperm activates matrix metalloproteinases (MMP2 and MMP9), which can contribute to increased acrosomal stability [32, 33]. Both MMPs contain two Zn^{2+} sites and four (MMP2) and three (MMP9) Ca^{2+} sites [34, 35].

Our results can also be explained by the higher bioavailability of ZnGlu as an organic zinc compound, which enhances semen characteristics. Since zinc is known to be crucial for reproduction, the use of organic zinc compounds helps compensate for the high endogenous zinc loss during spermatogenesis, thus maintaining a healthy zinc homeostasis. The bioavailability of trace elements is high when they are chelates of peptides and amino acids, resulting in augmentation of the reproductive function and overal health [36].

Meta-analysis data indicate that the bioavailability of different organic mineral compounds can depend on the nature of the ligand, the strength of chelation, and the solubility and stability of the compounds [15]. Huang *et al.* found that the bioavailability of zinc from Zn-amino acid or Zn-proteinate complexes was closely related to their chelation strength, represented by the stability constant, in starter broilers [38]. In another study comparing the bioavailability of zinc from zinc-glycine (Zn-Gly) and zinc-methionine (Zn-Met) with zinc sulfate ($ZnSO_4$), it was found that zinc in Zn-Met had higher bioavailability than Zn-Gly [39]. Organic zinc supplementation increased semen volume, sperm concentration, and improved the spermatozoal membrane integrity in bulls and bucks [40–42].

To investigate the biochemical impact or as a model for testing potential toxic effects, cell cultures are widely used. Compared to animal models, *in vitro* models are more consistent in predicting clinical outcomes and more suitable for identifying mechanisms relevant for *in vivo* toxicity [43]. Beside this, the use of spermatozoa to find out the direct influence of the substance under study on their motile function and viability is the most expedient and practically justified. Due to their high differentiation and specific function, spermatozoa depend on and are deeply influenced by the external environment, making them ideal for testing trace elements, including antioxidant compounds [44]. Therefore, in this work, boars sperm as an *in vitro* model, has been used to assess the efficacy of zinc.

Oxidative stress is another factor that may account for defective sperm function and lead to infertility. At physiological levels, ROS are known to support the sperm's fertilization ability by promoting chromatin compaction in maturing spermatozoa, increasing intracellular cAMP, motility, hyperactivation, sperm capacitation, acrosome reaction, and oocyte interaction [45, 46]. However, excessive ROS generation and oxidative stress can compromise gamete function by causing damage to cellular lipids, organelles, DNA strand breakage, alteration in enzymatic function, and apoptosis. Spermatozoa are particularly susceptible to oxidative stress-induced damage due to the high concentration of polyunsaturated fatty acids in their plasma membranes and the low concentrations of antioxidant enzymes [47]. Zinc has been proven to play a key role in spermatogenesis as a critical component of the antioxidant defense system [4, 48], which serves to inhibit the generation of ROS and enhance the activity of antioxidant pathways [8, 10]. One of the most active antioxidant enzyme in semen is copper-zinc superoxide dismutase (CuZnSOD) [5]. In our investigation, supplementing zinc glutamic amino acid chelate demonstrated a protective effect on sperm against oxidative stress. The activities of superoxide dismutase and catalase improved after adding ZnGlu to boar semen, particularly at concentrations of 2 and 5 μg/mL ($P < 0.05$). Mitochondria are rich in zinc-dependent SOD, which may help prevent free radical-induced membrane damage, loss of mitochondrial potential, and subsequently, loss of motility. Furthermore, as DC and MDA content decreased with ZnGlu treatment, this protective effect can also be attributed to the antioxidant property of zinc. Similar trends were observed with addition of zinc chloride to the preservation diluent for storing boar sperm at 18 °C. The results indicated that the addition of $ZnCl_2$ improved the integrity of the sperm membrane, total antioxidant capacity, and CuZnSOD activity, while reducing MDA content and sperm aggregation [49]. It was also shown that adding Zinc oxide to dilute ram semen significantly improved superoxide dismutase and glutathione peroxidase activities during cooling. [50]. In the present study, the addition of ZnGlu increased CAT and SOD activity and reduced the DC and MDA content of boar semen ($P < 0.05$), demonstrating that supplementation with organic complexes of Zn^{2+} with L-glutamic acid can protect sperm from oxidative stress.

Conclusions

The present study confirms the crucial function of zinc in spermatogenesis and demonstrates that supplementation of organic zinc compound – ZnGlu improves the semen quality and metabolic activity of boar semen *in vitro*. ZnGlu at concentrations of 2 and 5 μg, significantly increased the total motility of boar sperm during 96 hours of storage at 18 °C. Furthermore, the addition of zinc glutamic amino acid chelate enhances the antioxidant defence system of sperm. Our results show that ZnGlu can increase the activities of SOD and CAT while reducing the MDA content of boar semen, thus playing a protective role in preserving sperm.

Interests disclosure

The authors declare no conflict of interests.

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ВПЛИВ ДОДАВАННЯ ЦИНКУ ДО РОЗРІДЖУВАЧА СПЕРМИ КНУРІВ НА ХАРАКТЕРИСТИКУ СПЕРМІЇВ І АКТИВНІСТЬ АНТИОКСИДАНТНИХ ФЕРМЕНТІВ

Проблематика. Цинк має важливе значення для чоловічої репродуктивної функції, оскільки необхідний для дозрівання сперматозоїдів, процесу капацитації, акросомної реакції та запліднення. Встановлено, що використання хелатних сполук металів з амінокислотами, нуклеотидами, пептидами, вуглеводами є більш ефективним та економічно обґрунтованим.

Мета. Метою роботи було дослідити та порівняти вплив різних концентрацій хелату цинку з глутаміновою амінокислотою (ZnGlu) на рухливість, життєздатність спермії в та формування прооксидантно-антиоксидантного гомеостазу в спермі кнурів-плідників за умов *in vitro*.

Методика реалізації. Свіжоотримані еякуляти кнурів-плідників після додавання розбавника “Екосперм” розділяли на групи: контрольну та три дослідні. До дослідних зразків додавали глутамат цинку в кількості 1,0; 2,0; 5,0 мкг/мл відповідно. Зразки сперми зберігалася за 18 °С протягом 4-х діб. Кожні 24 год оцінювали рухливість, життєздатність спермії в та визначали антиоксидантний статус за рівнем дієнових кон’югатів (ДК), концентрацією малонового діальдегіду (МДА), активністю каталази та супероксиддисмутази.

Результати. Встановлено, що рухливість спермії в упродовж інкубування знижується, тоді як додавання 2 і 5 мкг ZnGlu істотно покращило загальну рухливість і сприяло підвищенню відсотка виживаності спермії в на 48–92 год інкубування. Наші дослідження також демонструють, що ZnGlu чинить захисну дію на сперму кнурів *in vitro* у відповідь на виникнення оксидативного стресу. Додавання ZnGlu істотно знижувало вміст МДА та ДК у зразках еякулятів усіх дослідних груп упродовж інкубування порівняно з показником контрольної групи. Водночас активність супероксиддисмутази та каталази в інкубованій спермі кнурів дослідних груп при додаванні ZnGlu зростала впродовж інкубування, особливо за концентрацій 2 та 5 мкг/мл ($P < 0,05$).

Висновки. Отримані дані демонструють, що додавання 2 та 5 мкг/мл глутамат цинку сприяє активації системи антиоксидантного захисту та покращує якість сперми кнурів *in vitro*.

Ключові слова: якість сперми; рухливість сперматозоїдів; цинк; сперма кнура; антиоксиданти.