https://doi.org/10.15407/animbiol25.02.008
UDC 636.32/.38:612.616:620.3
Received 16.04.2023 ▪ Revision 18.05.2023 ▪ Accepted 25-06-2023 ▪ Published online 30.06.2023

The quality of ram spermatozoa after thawing with the addition of Mn^{2+}, Zn^{2+} and Cu^{2+} nanocitrate to cryopreservation diluent

O. Sharan[1], V. Stefanyk[1], M. Murawski[2]
oshaom737@gmail.com

[1]Stepan Gzhytskyi National University of Veterinary Medicine and Biotechnologies of Lviv, 50 Pekarska str. Lviv, 79010, Ukraine
[2]University of Agriculture in Kraków, Al. Mickiewicza, 21, Kraków, 31-120, Poland

ORCID:
O. Sharan https://orcid.org/0000-0002-6193-8727
V. Stefanyk https://orcid.org/0009-0002-9630-6540
M. Murawski https://orcid.org/0000-0002-0720-7755

Authors' Contributions:
SO: Conceptualization; Formal analysis; Investigation; Methodology; Writing — original draft.
SV: Conceptualization; Formal analysis; Investigation; Methodology; Project administration; Supervision; Review & editing.
MM: Formal analysis; Methodology; Supervision; Review & editing.

Declaration of Conflict of Interests:
None to declare.

Ethical approval:
During the experiment, all international, national and/or institutional principles of animal care and use were followed, in particular, Directive 2010/63/EU "On the protection of animals used for scientific purposes"..

Acknowledgements:
None.

The aim of the study was to find out the effect of adding nanocitrate of Mn, Zn and Cu to the diluent for ram spermatozoa cryopreservation on its quality and ability for fertilizing. The experiment was carried out on six clinically healthy breeder 2–4-year-old rams of the Texel breed. The received ejaculates of the rams were evaluated for the volume, sperm concentration and motility and then divided into control and experimental groups. Control sperm samples were diluted with lactose-yolk-tris-citrate-glycerin medium (LYTCGM). Nanocitrates of microelements were added to the medium in experimental samples of ram sperm in the following doses: Zn^{2+} and Mn^{2+} — 2.5, 5.0 and 7.5 μg/l, Cu^{2+} — 1.25, 2.5 and 3.75 μg/l. The diluted sperm was packaged in straws, equilibrated for 2.5 h and frozen. After thawing of sperm we determined motility, survival of sperm, activity of succinate dehydrogenase (SDH) and cytochrome oxidase (CO), activity of antioxidant protection enzymes superoxide dismutase (SOD), glutathione peroxidase (HPO) and catalase (CAT). A dose-dependent effect of Mn, Zn, and Cu nanocitrates upon their addition to LYTCGM was established. Addition of nanocitrates of Mn, Zn to LYTCGM at a dose of 5.0 μg/l increased sperm motility by 22.2% ($P<0.05$) and 26.0% ($P<0.01$), and sperm survival, respectively, by 12.6% on ($P<0.01$) and 5.9% ($P<0.05$) compared to the control. Nanocitrates of Mn, Zn at a dose of 5.0 μg/l as part of LYTCGM caused a probable increase in SDH ($P<0.001$) and CO ($P<0.05$–0.01), which indicates a high fertilizing ability of ram spermatozoa. Similarly, when Mn, Zn nanocitrates were added to LYTCGM at a dose of 5.0 μg/l, SOD activity decreased by 29.6% ($P<0.01$) and 38.8% ($P<0.01$) and HPO activity increased by 43.5% ($P<0.01$) and 39.1% ($P<0.01$), and CAT — by 40.0% ($P<0.05$) and 37.5% ($P<0.05$), respectively. At the same time, the addition of Cu nanocitrate to LYTCGM with an increase in the dose significantly reduces the activity, survival and fertilizing capacity of thawed ram spermatozoa, and also worsens their antioxidant protection.

Key words: ram, sperm, nanocitrate Mn, Zn, Cu, fertilizing ability, motility, antioxidant protection

Introduction

Artificial insemination is major biotechnics in assisted reproductive technology which had the greatest effect on fast increasing of the genetic value of farm animals all over the world. At the same time, artificial insemination requires the constant availability of cryopreserved sperm for breeders [23]. However, cryopreservation of the rams' sperm is still

not effective to be commonly used in insemination because of its high sensitivity for cooling and thawing after cryopreservation process. Because of that intensive research is carried out in many labs to solve that problems [2].

The process of sperm freezing causes ultrastructural, biochemical and functional changes in spermatozoa [29]. Sperm plasma and acrosome are especially cryosensitive, as a result of which the permeability of cell membranes increases and spermatozoa motility and their morphology are disturbed [30, 8]. Damage to plasma membranes is accompanied by the leakage of enzymes, including those that directly participate in fertilization processes. In addition, mitochondria, the main energy-generating organelles of germ cells, are impaired or destroyed [27].

To ensure adequate protection of spermatozoa from adverse factors in low temperatures, various diluents for cryopreservation are used, which effectiveness depends on their composition [37].

An important role in the regulation of metabolic processes in sperm belongs to trace elements such as Zn^{2+}, Mn^{2+} and Cu^{2+}, which are cofactors of glycolysis enzymes, the respiratory chain of mitochondria and antioxidant protection, and also provide energy needs and utilization of cytotoxic metabolites of cells [26]. In particular, Zn^{2+} is included in the active centers of numerous enzymes of glycolysis and the pentose phosphate pathway of glucose oxidation, Cu^{2+} ensures the activity of enzymes of the respiratory chain and proteinases, and Mn^{2+} is a part of enzymes of the Krebs cycle. In addition, the indicated microelements as Cu^{2+}, Zn^{2+} and Mn^{2+} are part of the first link of enzymatic antioxidant protection which is superoxide dismutase (SOD), i.e. they are cofactors of the enzyme that converts O_2^- and by this inhibits the formation of active forms of oxygen [5, 19]. It is also known that SOD in sperm is present in three isoforms, which contain ions in the catalytic center: Mn^{2+} in mitochondria; Zn^{2+} and Cu^{2+} in cytoplasmic and exocellular matrix [33, 20]. These studies proved that the survival and, accordingly, the capacity of spermatozoa for fertilizing of germ cells depend on the activity of the specified enzyme and the ratio of its isozymes.

In the process of preparing ejaculates for cryopreservation, the concentration of these ions decreases, which leads to a decrease in enzyme activity and ultimately disrupts the process of substrate transformation and ATP resynthesis. Therefore, trace elements are added to ejaculate diluents to maintain high physiological characteristics and fertilizing ability of sperm. However, the use of inorganic salts of microelements as part of diluents is ineffective, which is due to their short-term contact with germ cells after sperm dilution, low permeability through membranes and the ability to be included in metabolism [28].

The disadvantages of using inorganic salts of trace elements in ejaculate diluents can be eliminated by using organic nanoforms of metals, in particular as nanocitrates, which will make easer their involvement in the metabolic processes of sperm [10, 15, 17, 22, 32, 36]. The investigation of Kosinov and Kaplunenko showed that toxic doses of nanoaquacitrates are 6 to 8 folds lower than their substitutes of mineral salts forms [17]. In this regard, it is of interest to study the influence of nanocitrate of Mn, Zn, and Cu as part of sperm diluents on the quality of ram spermatozoa. Therefore, the aim of the study was to find out the effect of adding nanocitrate of Mn, Zn and Cu to the diluent for cryopreservation of ram sperm on the quality and its ability for fertilization.

Materials and Methods

The study was conducted on six clinically healthy Texel rams, from 2- to 4-year-old, which were kept in three cages for two males in each one. Semen from rams was collected with artificial vagina (*Minitube*, Germany) and each ejaculate was evaluated separately for its volume, motility, concentration and total number of spermatozoa. Only ejaculates with spermatozoa concentration of at least 2.5 billion/ml were used for investigation. After evaluation, semen was diluted with a lactose-yolk-tris-citrate-glycerol medium (LYTCGM) to final spermatozoa concentration of 8×10^7/ml. Each diluted ejaculate was divided into one control and nine experimental groups. Three groups of diluents were prepared for each nanocitrate microelements of Zn, Mn and Cu in amount as follow: 2.5; 5.0; 7.5 μg/l and 1.25; 2.5; 3.75 μg/l, respectively. Nanocitrates of Mn, Zn and Cu were prepared by the method of erosion-explosive aqua nanotechnology by “Nanotechnologies and nanomaterials” LLC (Kyiv, Ukraine) [17].

Diluted semen was packed into 0.25 ml volume straws (*Minitube*, Germany) and cooled for 2.5 h at a temperature of +4°C. After that, straws were placed in nitrogen vapor for 30 min, then were put into liquid nitrogen. After thawing in a water bath at temperature of 40–42°C for 20 sec, sperm motility, survival of spermatozoa, activity of succinate dehydrogenase (SDH) and cytochrome oxidase (CO), activity of antioxidant protection enzymes superoxide dismutase (SOD), glutathione peroxidase (GPO) and catalase (CAT) were evaluated in each control and experimental group.

Semen analysis

The volume of ram ejaculate was determined with a graduated test tube, and the spermatozoa concentration was determined spectrophotometrically using a photometer SDM 6 with a touch display (*Minitube*, Germany). Germ cell motility, morphological abnormalities and the percentage of degenerate spermatozoa were determined by the computerized system CASA (*Computer Assisted Semen Analysis*) with activation of the *Sperm Vision* module [40].

After thawing semen was kept in refrigerator at temperature of +4°C and its survival was checked every hour under a microscope at magnification of 200-fold until the complete death of germ cells.

The activity of antioxidant protection enzymes was determined according to the methods described by Wirth and Mijal [38]. In particular, SOD activity was determined

by the amount of nitroformazan formed in the reaction between phenazinemetasulfate and NADH using a calibration curve, for which a standard solution of SOD (*Sigma*, USA; C1345) was used and expressed in IU/mg protein. Photometry of the samples was carried out at a wavelength of 540 nm on a spectrophotometer SF-46.

The activity of GPO was determined by using the Ellman reagent (0.01 M solution of 5,5-dithiobis-2-nitrobenzoic acid (*Acros Organics*, Belgium) The staining intensity was measured at 412 nm on a spectrophotometer SF-46. The sample was added to the control sample before protein precipitation.

The activity of CAT was determined by the method of Koroliuk et al. (1991). The staining intensity was measured at 410 nm on a spectrophotometer SF-46.

Statistical analysis

All obtained digital data were processed using the *Statistica* computer program using the method of variational statistics and the *Excel* program from the *Microsoft Office* 2007 and 2010 service packages. Differences between groups were considered statistically significant at P<0.05.

Results and Discussion

The highest motility of spermatozoa after thawing semen, 56.7%, was noticed in diluent with 5.0 μg/L of Zn nanocitrate, which was significantly higher (P<0.01), and the lowest one, 35%, was observed in diluent with 3.75 μg/L of Cu nanocitrate wich was significalntly lower (P<0.05) than in control group (table 1).

More pronounced changes in the motility of thawed ram spermatozoa were found with the addition of Zn nanocitrate to LYTCGM diluent. In particular, sperm motility increased by 12.9% (P<0.05), 26.0% (P<0.01) and 3.8% compared to the control, after adding it in amount of 2.5, 5.0 and 7.5 μg/l, respectively.

The addition of 1.25 μg/l Cu nanocitrate to the diluent for cryopreservation of ram semen caused increase in sperm motility in after thawing by 5.1%, but adding 2.5 and 3.75 μg/L of nanocitrate of Cu caused decrease in motility compared to the control by 12.9 and 35.0% (P<0.05), respectively. However, the highest time of spermatozoa survival, 105.3 h (P<0.01), was observed in diluent with 5.0 μg/l Mn nanocitrate and the lowest, 64.7 h (P<0.001), was in diluent with 3.75 μg/l of Cu nanocitrate which was lower than in diluent in control group (table 1).

The study of the activity of SDH and CO enzymes, which are markers of the fertilizing ability of spermatozoa revealed dose dependent differences in thawed ram semen. The highest activity of both SDH and CO enzymes were observed in spermatozoa in group with 5.0 μg/L in diluent of Mn nanocitrate on the level of 44 units (P<0.001) and 49.8 units (P<0.01) respectively than in control group (table 2).

The lowest activity of SDH and CO enzymes in comparison to control group were noticed in diluent with 3.75 μg/l on the level of 19.2 units (P<0.05) and 29 units (P<0.01) respectively.

Addition of nanocitrates of Mn, Zn and Cu to LYTCGM causes changes in the activity of antioxidant defense enzymes in thawed ram sperm. Thus, the addition of 2.5 μg/l Mn nanocitrate caused a decrease in the activity of SOD by 14.5% with a simultaneous increase in the activity of GPO by 15.2% and CAT by 7.5% compared to the control (table 3). At the addition of manganese nanocitrate in a dose of 5.0 μg/l, the difference in the activity of antioxidant protection enzymes in thawed spermatozoa was the highest compared to the control: the activity of SOD decreased by 29.6% (P<0.01), and the activity of GPO and CAT increased by 43.5% (P<0.01) and 40.0% (P<0.05), respectively. At the same time, with the addition of 7.5 μg/l of Mn nanocitrate, the difference in the activity of antioxidant defense enzymes in thawed spermatozoa of rams with the control was insignificant or absent: the activity of SOD was 7.1% lower than the control, and GPO and CAT were higher by 2.2% and 5.0%, respectively.

The similar changes in the activity of antioxidant defense enzymes in thawed spermatozoa were also found when Zn nanocitrate was added to the medium

Table 1. Motility and survival of ram spermatozoa after freezing in diluents with addition of Mn, Zn or Cu nanocitrate (M±SD, n=6)

Nanocitrate, dose, μg/l		Spermatozoa motility, %	Sperm survival, hours
Mn^{2+}	2.5	47.5±1.12	96.0±1.93*
	5.0	55.0±1.83*	105.3±2.04**
	7.5	44.2±1.54	101.3±2.90*
Zn^{2+}	2.5	50.8±1.54*	94.0±1.93
	5.0	56.7±1.67**	99.0±2.96*
	7.5	46.7±2.11	95.2±2.64
Cu^{2+}	1.25	47.3±2.67	83.7±2.99*
	2.5	39.2±3.01	75.3±2.78***
	3.75	35.0±1.83*	64.7±2.78***
Control		45.0±1.83	93.5±1.84

Table 2. Activity of SDH and CO in thawed ram spermatozoa in diluent with the addition of Mn, Zn or Cu nanocitrate (M±SD, n=6)

Nanocitrate in diluent, μg/l		Enzyme activity, units	
		SDH	CO
Mn^{2+}	2.5	33.7±2.16**	43.2±2.77
	5.0	44.0±3.24***	49.8±2.26**
	7.5	39.8±4.18**	39.7±1.69
Zn^{2+}	2.5	38.7±2.70**	41.3±2.57
	5.0	41.7±3.04***	48.3±1.80*
	7.5	39.2±4.04**	40.2±1.94
Cu^{2+}	1.25	31.5±1.89*	41.0±2.25
	2.5	29.2±2.93*	35.5±1.75*
	3.75	19.2±3.67*	29.0±1.83**
Control		25.5±1.59	40.0±2.52

Note. In this and the following tables *, ** and *** — superscripts indicate statistical differences P<0.05, P<0.01 and P<0.001, respectively.

for cryopreservation of ram sperm. In particular, the addition of zinc nanocitrate in doses of 2.5, 5.0 and 7.5 μg/l caused a decrease in SOD activity in thawed ram sperm by 18.3% (P<0.05), 33.8% (P<0.01) and 10.4%, respectively, and GPO activity, on the contrary, increased by 13.0%, 39.1% (P<0.01) and 4.3%, respectively. At the same time, CAT activity in thawed sperm of rams significantly increased compared to the control at doses of Zn nanocitrate 5.0 μg/l by 37.5% (P<0.05), and at other doses only by 10.0 and 5.0%.

Addition of Cu nanocitrate to LYTCGM caused opposite changes in the activity of antioxidant defense enzymes in thawed ram sperm. Thus, with the addition of 1.25 μg/l of cuprum nanocitrate, SOD activity increased by 6.2%, while GPO and CAT, on the contrary, decreased by 10.9% and 2.5%, respectively, compared to the control. With an increase in the dose of Cu nanocitrate, the difference became more significant: with the addition of 2.5 μg/l, the activity of SOD increases by 18.8% (P<0.05), and the activity of GPO and CAT decreases by 28.5% (P<0.05) and 12.5%, respectively. The difference in the activity of antioxidant protection enzymes in thawed spermatozoa of rams with the control at doses of Cu nanocitrate 3.75 μg/l is even greater: the activity of SOD is higher by 30.3% (P<0.01), the activity of GPO and CAT is lower by 37.0% (P<0.001) and 25.0% (P<0.05) , respectively.

The use of frozen-thawed ram semen is important in modern sheep breeding, so effective cryopreservation of ram spermatozoa is an important tool for developing of this branch of breading and for the whole reproductive technology [3]. It is known that diluents, dilution-cooling-freezing and thawing methods play an important role in the success of ram sperm cryopreservation [34]. To maintain high physiological characteristics of sperm, trace elements are added to ejaculate thinners. At the same time, literature data indicate a negative effect of an excess of trace elements on the physiological characteristics and fertilizing ability of sperm [31, 40]. With an excess of certain elements, it is possible to disrupt the functions of mitochondria, which leads to a decrease in the physiological characteristics and fertilizing ability of spermatozoa [24]. In view of the above, many authors conduct research on the effect of metals in the form of nanosized forms or nanoparticles on the quality of mammalian sperm [6, 9, 12].

Today, an important role in ensuring the health and productivity of animals is played by new directions of obtaining and using biologically active substances, in particular nanotechnology and nanomaterials [1, 4, 11, 13, 14, 18, 35].

In experiments with the sperm of bulls, the effectiveness of using nanosuccinate of Mn, Zn, and Cu as part of the diluent for cryopreservation of semen of breeding bulls was determined, where the optimal doses of nanosuccinate of manganese and zinc were established, which have a positive effect on the activity, movement parameters, survival, and fertilizing ability of spermatozoa [16, 40]. At the same time, the addition of Cu nanosuccinate to the medium for cryopreservation of spermatozoa has a slight positive effect on bull sperm only at a low dose, with an increase in the dose, the quality and fertilizing ability of germ cells significantly decreases.

In view of the above, we conducted a study to study the effect of adding nanocitrate of Mn, Zn and Cu to the composition of the diluent for cryopreservation of ram semen on quality parameters and fertilizing ability of spermatozoa. It was found experimentally that the addition of Mn and Zn nanocitrate at an optimal dose of 5.0 μg/L to LYTCGM probably increases the motility of ram spermatozoa after thawing, and also reduces the percentage of sperm with morphological disorders. In contrast, the addition of Cu nanocitrate in increasing doses significantly reduces sperm motility in thawed ram semen, simultaneously increasing the percentage of degenerate spermatozoa. This is confirmed by the research of Leahy et al., which was established that an excess of Cu^{2+} in diluted ram semen causes sperm agglutination due to the oxidation of free sulfhydryl groups to disulfide ones and educed its motility [20, 21, 39].

In our studies, the addition of Mn and Zn nanocitrate at a dose of 5.0 μg/L to the medium for freezing ram sperm increases spermatozoa survival, and the addition of Cu nanocitrate in increasing doses significantly reduces the survival time, which confirms the results of studies with bull sperm [16, 39].

Scientists have proven that SDH and CO are markers for determining the fertilizing ability of spermatozoa of male farm animals [25]. In our studies, the addition of Mn and Zn nanocitrate at a dose of 5.0 μg/L to LYTCGM increases the activity of SDH and CO in ram sperm after thawing, and the addition of Cu nanocitrate to diluent in Cu1 and Cu2 groups significantly reduces the activity of these enzymes.

Sperm has an effective enzymatic system of antioxidant protection (SAP), which destroys the excess of formed active forms of oxygen and improves the quality of semen. The main enzymes of SAP are SOD, GPO and CAT [7]. In our experiment, the addition of Mn and Zn nanocitrates to the diluent for cryopreservation of ram semen intensifies

Table 3. Activity of antioxidant defense enzymes in thawed spermatozoa of rams at the addition of micronutrient nanocitrate (M±SD, n=6)

Micronutrient nanocitrate, dose, μg/l		SOD, IU/mg of protein	GPO μmol/min×mg of protein	KAT, μmol/min×mg of protein
Mn^{2+}	2.5	47.3±2.11	0.53±0.040	0.43±0.027
	5.0	38.5±1.77**	0.66±0.039**	0.56±0.037*
	7.5	50.8±1.88	0.47±0.045	0.42±0.029
Zn^{2+}	2.5	44.7±2.39*	0.52±0.028	0.44±0.024
	5.0	36.2±1.85**	0.64±0.038**	0.55±0.043*
	7.5	49.0±1.75	0.48±0.038	0.42±0.031
Cu^{2+}	1.25	58.1±2.44	0.41±0.024	0.39±0.033
	2.5	65.0±2.88*	0.32±0.037*	0.35±0.040
	3.75	71.3±2.68**	0.29±0.036***	0.30±0.027*
Control		54.7±2.58	0.46±0.026	0.40±0.025

the activity of antioxidant defense enzymes in thawed spermatozoa, which indicates their higher quality at the optimal dose of 0.5 μg/l. At the same time, the addition of Cu nanocitrate LYTCGM in higher doses increases the activity of SOD and decreases the activity of GPO and CAT, which indicates a decrease in sperm quality.

Thus, the addition of Mn and Zn nanocitrate at an optimal dose of 5.0 μg/L to the medium for cryopreservation of ram sperm probably increases the motility of thawed spermatozoa, their survival, the activity of SDH and CO in germ cells, and also intensifies the activity of antioxidant defense enzymes in sperm.

In summary, in our study we observed the highest survival time of semen and highest activity of SDH, CO, GPO and CAT enzymes in ram spermatozoa after thawing in diluent supplemented with 5.0 μg/l Mn and values of those parameters in that group were much better than in control group. Also, the addition of 5.0 μg/l Zn citrate to the extender resulted in a significantly longer sperm survival time after thawing and had a significantly beneficial effect on increasing the SDH, CO, SOD, GPO and CAT enzymatic activity of ram semen after thawing, and the semen survival time was significantly higher ($P<0.05$) compared to the control group. Although the addition of 1.25 μg/l Cu the sperm survival time was significantly shorter than in the control group, an increase in SOD activity was observed ($P>0.05$), while the activity of other enzymes did not change significantly compared to the control group.

The obtained results indicate that the addition of Mn, Zn or Cu nanocitrates has a positive effect on all or some parameters of semen suitability for fertilization and also extends its survival (Mn, Zn nanocitrate) after thawing. Due to the multifactorial effect of the addition of Mn, Zn and Cu nanocitrates on semen parameters, research should be continued in order to explain their impact on ram sperm metabolism and survival in the cryopreservation process.

1. Addition of Mn and Zn nanocitrate in amount of 5.0 μg/l to LYTCGM increases the motility of ram sperm ($P<0.05–0.01$) after thawing, and with the addition of Cu nanocitrate in increasing doses, sperm motility significantly decreases in thawed sperm of rams.

2. Addition of Mn and Zn nanocitrate at a dose of 5.0 μg/L to the extender for freezing ram semen increases sperm survival ($P<0.05–0.01$) but addition of Cu nanocitrate in amount used in our experiment significantly reduces its survival time.

3. The addition of Mn and Zn nanocitrate at a dose of 5.0 μg/l to LYTCGM increases the activity of SDH ($P<0.01–0.001$) and TSO ($P<0.05–0.001$) in thawed ram sperm, and the addition of nanocitrate Cu in higher amount significantly reduces the activity of these enzymes.

4. Addition of Mn and Zn nanocitrates in amount of 0.5 μg/l to the extender for cryopreservation of ram semen intensifies the activity of antioxidant defense enzymes in thawed spermatozoa, which indicates their important role for spermatozoa protection during cryopreservation.

References

1. Ali A, Ijaz M, Khan YR, Sajid HA, Hussain K, Rabbani AH, Shahid M, Naseer O, Ghaffar A, Naeem MA, Zafar MZ, Malik AI, Ahmed I. Role of nanotechnology in animal production and veterinary medicine. *Trop. Anim. Health Prod.* 2021; 53 (5): 508. DOI: 10.1007/s11250-021-02951-5.
2. Alvarez M, Anel-Lopez L, Boixo JC, Chamorro C, Neila-Montero M, Montes-Garrido R, de Paz P, Anel L. Current challenges in sheep artificial insemination: A particular insight. *Reprod. Domest. Anim.* 2019; 54 (S4): 32–40. DOI: 10.1111/rda.13523.
3. Benson JD, Woods EJ, Walters EM, Critser JK. The cryobiology of spermatozoa. *Theriogenol.* 2012; 78 (8): 1682–1699. DOI: 10.1016/j.theriogenology.2012.06.007.
4. Borisevich VB, Kaplunenko VG, Kosinov MV. *Nanomaterials in Biology. Fundamentals of nano-veterinary medicine*. Kyiv, Avicenna, 2010: 416 p. (in Ukrainian)
5. Eghbali M, Alavi-Shoushtari SM, Rezaii SA. Effects of copper and superoxide dismutase content of seminal plasma on buffalo semen characteristics. *Pakistan J. Biol. Sci*. 2008; 11 (15): 1964–1968. DOI: 10.3923/pjbs.2008.1964.1968.
6. Falchi L, Khalil WA, Hassan M, Marei WFA. Perspectives of nanotechnology in male fertility and sperm function. *Int. J. Vet. Sci. Med*. 2018; 6 (2): 265–269. DOI: 10.1016/j.ijvsm.2018.09.001.
7. Ford WCL. Regulation of sperm function by reactive oxygen species. *Human Reprod.* 2004; 10 (5): 387–399. DOI: 10.1093/humupd/dmh034.
8. Gandini L, Lombardo F, Lenzi A, Spanò M, Dondero F. Cryopreservation and Sperm DNA Integrity. *Cell Tiss. Bank.* 2006; 7: 91–98. DOI: 10.1007/s10561-005-0275-8.
9. Iftikhar M, Noureen A, Uzair M, Jabeen F, Daim MA, Cappello T. Perspectives of nanoparticles in male infertility: evidence for induced abnormalities in sperm production. *Int. J. Environ. Res. Publ. Health.* 2021; 18 (4): 1758. DOI: 10.3390/ijerph18041758.
10. Iskra RY, Vlizlo VV, Fedoruk RS, Antonyak GL. *Chromium in Animal Nutrition*. A monograph. Kyiv, Agrarian Science Publ., 2014: 312 p. (in Ukrainian)
11. Kareem EH, Dawood TN, Al-Samarai FR. Application of nanoparticle in the veterinary medicine. *Magna Scientia Adv. Res. Rev.* 2022; 4 (1): 27–38. DOI: 10.30574/msarr.2022.4.1.0082.
12. Khalil W, El-Harairy MA, Zeidan AEB, Hassan MAE. Impact of selenium nanoparticles in semen extender on bull sperm quality after cryopreservation. *Theriogenol*. 2019; 126: 121–127. DOI: 10.1016/j.theriogenology.2018.12.017.
13. Kondrasiy LA, Yakubchak ON, Maliuk NO, Kaplunenko VH. The quality variation of raw milk under preparation based on citrate Zn and Ge. *Sci. Rep. NULES Ukraine*. 2017; 3 (67): 19–32. DOI: 10.31548/dopovidi2017.03.019. (in Ukrainian)
14. Kondratska OA, Grushka NG, Kaplunenko VG, Pavlovych SI, Sribna VO, Yanchii RI. Protective effect of germanium citrate in endotoxin-induced ovarian dysfunction in mice. *Med. Perspect*. 2018; 23 (1/1): 71–77. DOI: 10.26641/2307-0404.2018.1(part1).127240. (in Ukrainian)
15. Kornyat S, Sharan M, Ostapiv D, Korbeckij A, Jaremchuk I, Andrushko O. Quality of deconserved bull sperm for the action of nanosuccinates Zn, Cu and Mn in the diluents. *Bìol. Tvarin*. 2021; 23 (1): 23–29. DOI: 10.15407/animbiol23.01.023. (in Ukrainian)
16. Kornyat S, Yaremchuk I, Andrushko O, Ostapiv D, Sharan M, Chajkovska O. The intensity of the oxidation processes in the sperm of the boar at the add of metal nanosuccinates to the Ecosperm medium. *Sci. Tech. Bull. SSRCIVMPFA.* 2019; 20 (2): 352–357. DOI: 10.36359/scivp.2019-20-2.46. (in Ukrainian)
17. Kosinov MV, Kaplunenko VG. Method for metal carboxylates obtaining “Nanotechnology of obtaining metal carboxylates”. Patent of Ukraine no. 38391 from 12.01.2009. Available at: https://base.uipv.org/searchINV/search.php?action=viewdetails&IdClaim=128062 (in Ukrainian)
18. Kovalchuk II, Kykish IB, Kaplunenko VH. *Influence of citrate microelements on the reproductive capacity of queen bees. Actual problems of natural sciences: modern scientific discussions.* A collective monograph. Riga, Baltija Publishing, 2020: 87–110. DOI: 10.30525/978-9934-26-025-4-6. (in Ukrainian)

19. Kuzmina NV, Ostapiv DD. SOD isozymes in diluted ejaculates of bulls. *Anim. Breed. Genet.* 2010; 44: 107–108. Available at: http://nbuv.gov.ua/UJRN/rgt_2010_44_37 (in Ukrainian)
20. Leahy T, Rickard JP, Aitken RJ, de Graaf SP. D-penicillamine prevents ram sperm agglutination by reducing the disulphide bonds of a copper-binding sperm protein. *Reprod.* 2016; 151 (5): 491–500. DOI: 10.1530/REP-15-0596.
21. Maulana T, Said S. Kinematics motility of frozen-thawed X and Y sperm of Sumba Ongole bull. *IOP Conf. Ser. Earth Environ. Sci.* 2019; 387: 012030. DOI: 10.1088/1755-1315/387/1/012030.
22. Nagata MPB, Egashira J, Katafuchi N, Endo K, Ogata K, Yamanaka K, Yamanouchi T, Matsuda H, Hashiyada Y, Yamashita K. Bovine sperm selection procedure prior to cryopreservation for improvement of post-thawed semen quality and fertility. *J. Anim. Sci. Biotechnol*. 2019; 10: 91. DOI: 10.1186/s40104-019-0395-9.
23. Nakada K, Sato A, Yoshida K, Morita T, Tanaka H, Inoue SI, Yonekawa H, Hayashi JI. Mitochondria-related male infertility. *PNAS*. 2006; 103 (41): 15148–15153. DOI: 10.1073/pnas.0604641103.
24. Nischemenko N, Kaplunenko V, Emelianenko A. Embryonic development of quails in the incubating eggs processing solution aquachelate germany. *Sci. Bull. LNUVMBT. Ser. Vet. Sci.* 2014; 16 (2/2): 258–264. Available at: http://nbuv.gov.ua/UJRN/nvlnu_2014_16_2%282%29__44. (in Ukrainian)
25. Pal RP, Mani V, Mir SH, Singh RK, Sharma R. Importance of trace minerals in the ration of breeding bull — a review. *Int. J. Curr. Microbiol. App. Sci.* 2017; 6 (11): 218–224. DOI: 10.20546/ijcmas.2017.611.026.
26. Rokotyanska VO. The influence of nanoaquachelates on the biological quality of sperm. *Bull. Agr. Sci. Black Sea Region*. 2018; 3 (99): 56–60. DOI: 10.31521/2313-092X/2018-3(99)-9. (in Ukrainian)
27. Rowe MP, Powell JG, Kegley EB, Lester TD, Rorie RW. Effect of supplemental tracemineral source on bull semen quality. *Appl. Anim. Sci.* 2014; 30 (1): 68–73. DOI: 10.15232/S1080-7446(15)30085-1.
28. Salamon S, Maxwell WMC. Frozen storage of ram semen I. Processing, freezing, thawing and fertility after cervical insemination. *Anim. Reprod. Sci.* 1995; 37 (3–4): 185–249. DOI: 10.1016/0378-4320(94)01327-I.
29. Salamon S, Maxwell WMC. Storage of ram semen. *Anim. Reprod. Sci.* 2000; 62 (1–3): 77–111. DOI: 10.1016/S0378-4320(00)00155-X.
30. Sengupta P. Environmental and occupational exposure of metals and their role in male reproductive functions. A review. *Drug Chem. Toxicol*. 2013; 36 (3): 353–368. DOI: 10.3109/01480545.2012.710631.
31. Serdyuk AM, Gulich MP, Kaplunenko VG, Kosinov MV. Nanotechnologies of micronutrients: problems, prospects and ways to eliminate the deficiency of macro- and microelements. *J. NAMS Ukraine*. 2010; 16 (1): 107–114. (in Ukrainian)
32. Skrzycki M, Czeczot H. Extracellular superoxide dismutase (EC–SOD) — structure, properties and functions. *Adv. Hygiene Exp. Med*. 2004; 24 (58): 301–311. PMID: 15280800 (in Polish)
33. Tekin N, Uysal O, Akçay E, Yavaş İ. Effects of different taurine doses and freezing rate on freezing of row semen. *Ankara Üniv. Vet. Fak. Derg*. 2006; 53 (3): 179–184. Available at: http://vetjournal.ankara.edu.tr/en/pub/issue/47514/599969 (in Turkish)
34. Uysal O, Bucak MN. Effects of oxidized glutathione, bovine serum albumin, cysteine and lycopene on the quality of frozen-thawed ram semen. *Acta Vet. Brno*. 2007; 76 (3): 383–390. DOI: 10.2754/avb200776030383.
35. Vlizlo VV. (ed.). *Laboratory Methods in Biology, Stockbreeding and Veterinary Medicine*. Lviv, Spolom Publ., 2012: 764 p. (in Ukrainian)
36. Vlizlo V, Bashchenko M, Iskra R, Fedoruk R, Zhukorskyi O, Mezentseva L. Nanotechnologies and their application in animal husbandry and veterinary medicine. *Bull. Agr. Sci.* 2015; 93 (11): 5–9. DOI: 10.31073/agrovisnyk201511-01. Available at: https://agrovisnyk.com/index.php/agrovisnyk/article/view/178 (in Ukrainian)
37. Vlizlo VV, Fedoruk RS, Iskra RJ. Biological effect of functional nanomaterials in various species of animals. *Bull. Agr. Sci*. 2018; 96 (11): 80–86. DOI: 10.31073/agrovisnyk201811-11. (in Ukrainian)
38. Wirth JJ, Mijal RS. Adverse effects of low level heavy metal exposure on male reproductive function. *Syst. Biol. Reprod. Med.* 2010; 56 (2): 147–167. DOI: 10.3109/19396360903582216.
39. Yaremchuk I, Kuzmina N, Ostapiv D, Sharan M, Kava S. Oxidative processes intensity and quallity of bull semen when adding microelements nanosuccinate compounds. *Sci. Bull. LNUVMBT Ser. Vet. Sci*. 2017; 19 (77): 185–189. DOI: 10.15421/nvlvet7740. (in Ukrainian)
40. Yaremchuk IM, Sharan MM. Modern analysis capabilities sperm quality and sperm dose calculation. *Biol. Tvarin*. 2012; 14 (1–2): 697–703. Available at: http://aminbiol.com.ua/index.php/archive?catid=1:2013-02-15-09-09-13&id=203:2013-03-09-12-31-38 (in Ukrainian)

Якість спермiїв баранів після розморожування за додавання наноцитрату Mn^{2+}, Zn^{2+} та Cu^{2+} до середовища для кріоконсервування

О. Шаран[1], В. Стефаник[1], М. Муравскі[2]
oshaom737@gmail.com

[1]Львівський національний університет ветеринарної медицини та біотехнологій імені С. З. Ґжицького, вул. Пекарська, 50, м. Львів, 79010, Україна
[2]Краківський сільськогосподарський університет, ал. Міцкевича, 21, м. Краків 31-120, Польща

Метою роботи було з'ясувати вплив додавання наноцитрату Mn, Zn та Cu до середовища для кріоконсервування сперми баранів на якість та запліднювальну здатність спермiїв. Експеримент проводили на шести клінічно здорових баранах-плідниках віком 2-4 роки породи тексель. Отримані еякуляти баранів оцінювали за об'ємом, концентрацією та рухливістю спермiїв і ділили на контрольну і дослідні групи. Контрольні зразки сперми розбавляли лактозо-жовтково-тріс-цитрато-гліцериновим середовищем (ЛЖТЦГС). У дослідних зразках сперми баранів до середовища додавали наноцитрати мікроелементів у дозах: Zn^{2+} і Mn^{2+} — 2,5, 5,0 та 7,5 мкг/л, Cu^{2+} — 1,25, 2,5, та 3,75 мкг/л. Розбавлену сперму фасували у соломинки, еквілібрували впродовж 2,5 год. і заморожували. Після розморожування сперми визначали рухливість, виживання спермiїв, активність сукцинатдегідрогенази (СДГ) та цитохромоксидази (ЦО), активність ензимів антиоксидантного захисту супероксиддисмутази (СОД), глутатіонпероксидази (ГПО) і каталази (КАТ). Встановлено дозозалежну дію наноцитратів Mn, Zn та Cu за додавання їх до ЛЖТЦГС. Додавання наноцитратів Mn, Zn до ЛЖТЦГС у дозі 5,0 мкг/л активність спермiїв підвищилася на 22,2% ($P<0,05$) та 26,0% ($P<0,01$), а виживання спермiїв — відповідно, на 12,6% ($P<0,01$) та 5,9% ($P<0,05$) порівняно з контролем. Наноцитрати Mn, Zn у дозі 5,0 мкг/л у складі ЛЖТЦГС спричинили вірогідне зростання СДГ ($P<0,001$) і ЦО ($P<0,05–0,01$), що вказує на високу запліднювальну здатність спермiїв баранів. Аналогічно, за додавання наноцитратів Mn, Zn до ЛЖТЦГС у дозі 5,0 мкг/л активність СОД знизилася на 29,6% ($P<0,01$) та 38,8% ($P<0,01$), активність ГПО підвищилася, відповідно, на 43,5% ($P<0,01$) та 39,1% ($P<0,01$), КАТ — відповідно, на 40,0% ($P<0,05$) та 37,5% ($P<0,05$). Водночас додавання наноцитрату Cu до ЛЖТЦГС зі збільшенням дози вірогідно знижує активність, виживання та запліднювальну здатність деконсервованих спермiїв баранів, а також погіршує показники їх антиоксидантного захисту.

Ключові слова: баран, сперма, наноцитрат Mn, Zn, Cu, запліднювальна здатність, рухливість, антиоксидантний захист

Sharan O, Stefanyk V, Murawski M. The quality of ram spermatozoa after thawing with the addition of Mn^{2+}, Zn^{2+} and Cu^{2+} nanocitrate to cryopreservation diluent. *Biol. Tvarin*. 2023; 25 (2): 8–13. DOI: 10.15407/animbiol25.02.008.