AGRICULTURAL BIOLOGY,
2020, V. 55, № 6, pp. 1148-1158
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

UDC 636.5:591.16:611.013.11:57.04

doi: 10.15389/agrobiology.2020.6.1148eng
doi: 10.15389/agrobiology.2020.6.1148rus

EFFICIENCY OF USING A COMBINATION OF MONO- AND DISACCHARIDES IN A DILUENT FOR FREEZING ROOSTER SEMEN

Yu.I. SILYUKOVA ✉, O.I. STANISHEVSKAYA, N.V. PLESHANOV, A.A. KUROCHKIN

All-Russian Research Institute for Farm Animal Genetics and Breeding — Branch of Ernst Federal Science Center for Animal Husbandry, 55A, Moskovskoe sh., pos. Tyarlevo, St. Petersburg—Pushkin, 196625 Russia, e-mail svadim33@mail.ru (✉ corresponding author), olgastan@list.ru, Klaus-90@list.ru, kurochkin.anton66@gmail.com
ORCID:
Silyukova Yu.L. orcid.org/0000-0003-1905-6373
Stanishevskaya O.I. orcid.org/0000-0001-9504-3916
Pleshanov N.V. orcid.org/0000-0002-4634-7515
Kurochkin A.A. orcid.org/0000-0003-3171-8499
The authors declare no conflict of interests
Acknowledgements:
Research conducted on the topic AAAA-A18-118021590132-9.
Received September 10, 2020

Abstract

Different combination of saccharides can provide better semen protection during freezing/thawing cycle. Until now, the disaccharide maltose has not been used as a component of the medium for cryopreservation roosters' semen. Since maltose is not involved in carbohydrate metabolism of spermatozoa, there is an assumption about its role in strengthening the structure of the glycocalyx, which is a progressive evolutionary cellular structure that regulates specific cellular adaptations to a certain temperature, chemical and other paratypical effects. In this work, in order to increase the fertility of frozen/thawed semen, we tested a combination of saccharides in the diluent for cryopreservation of roosters' semen. For the first time, maltose has been proven to be effective in combination with fructose in a diluent to increase the fertility of frozen/thawed roosters' semen. The aim of the study was to determine the optimal concentration of the test component of maltose based on the diluent of the Leningrad Cryoprotective Medium (LCM) (1984) for freezing the semen of roosters and determining the time of maintaining the functional usefulness of frozen/thawed cock semen in the genital tract of the hen. The experiment was carried out in the Center for Collective Use Genetic collection of rare and endangered chicken breeds (the Russian Research Institute of Farm Animal Genetics and Breeding, 2020) on a breed of Russian white chickens (♂n = 10, ♀n = 30) at the age of 46-50 weeks. Three variants of media for cryopreservation of roosters' semen with different ratios of saccharides were evaluated. In each group, the Mal-10 (fructose 0.72%, maltose 0.166%), Mal-20 (fructose 0.64%, maltose 0.326%) and LCM-control (fructose 0.8%, maltose 0%), there were 10 hens for insemination. The results show not only an increase in the total percentage of fertilized eggs when using frozen/thawed semen in the Mal-10 (92.6%) and Mal-20 (86.3%) groups compared to the LCM-control group (74.7%), but also an increase in the duration of the functional usefulness of spermatozoa in the genital tract of hen within 5 days at the level of native sperm. Counting the points of interaction of spermatozoa with the perivitelline membrane of the yolk 5 days after the last insemination showed that the functional ability of spermatozoa is much higher when using experimental media containing maltose, 67.0 pcs/cm^2 for Mal-10 and 110.7 pcs/cm^2 for Mal-20 vs. 40.1 pcs/cm^2 in maltose-free LCM-control. The longest duration of the functional usefulness of spermatozoa was noted in the Mal-20 group. Even on day 15 after the last insemination, the fertilization capacity of frozen/thawed semen was recorded at the level of 20 %. A regression equation was drawn up for the relationship of egg fertilization with the points of interaction of spermatozoa with the perivitelline membrane of the yolk. To obtain an egg fertilization level of ≥ 80%, the functional usefulness of frozen-thawed spermatozoa (expressed through the number of points of interaction with the perivitelline membrane of the yolk) should be ≥ 60 pcs/cm^2. This was achieved by using an experimental diluent with 0.326% maltose. The results obtained open up the possibility of using cryopreserved semen not only in preserving the gene pool, but also in breeding programs.

Keywords: roosters, semen, fertility, cryopreservation, media, maltose, perivitelline membrane

Cryopreservation of avian semen is an important and widely used method of long-term storage of male reproductive cells, but its effectiveness in poultry

farming is not as high as in dairy cattle breeding, where it is used for genetic improvement of breeds. The search for new concepts and protocols in the cryopreservation of rooster semen will make it possible to create cryobanks of genetic material not only as a repository of samples of rare and endangered breeds but also as working reserve and insurance funds of reproductive cells [1-3]. Cryopreservation causes adverse changes in sperm cells, which leads to damage and total cell death and reduces the quality of sperm. Thus, there is partially irreversible damage to the morphological structures of the cell, disruption of the structure of organelles, changes in biochemical processes and the supramembrane structure — a dense carbohydrate layer (glycocalyx) [4]. All the listed changes in the sperm cell and its epigenetic modifications associated with DNA methylation, as well as protamine defect and disruption of cellular parameters, including membrane integrity, DNA stability, and mitochondrial activity, become the reasons for a decrease in sperm motility and fertility during freezing/thawing [5, 6].

The retention of the functional capacity of avian sperm after thawing depends on species, breed characteristics, the composition of the freezing medium, including cryoprotectants, sperm balancing procedures used, and freezing and thawing protocols. In addition, the starting quality of fresh sperm is important for successful cryopreservation of sperm [7].

To obtain good results when using frozen-thawed rooster semen, damage caused by water crystallization during freezing must be prevented. The formation of intracellular ice crystals, especially the three-vector form of crystallization [8, 9], is one of the main causes of cell death. In addition, researchers are looking for ways to increase the fertility of cryopreserved semen not only through freezing and thawing (fast/slow) protocols [10] but also by selecting the optimal cryopreservation media. Such media contain antioxidants that neutralize the effect of accumulated active oxygen on cells (l-carnitine, ellagic acid, cysteamine, ergothioneine, serine, catalase) [11-14], as well as energy components (saccharides) [15] that support the functionality of sperm cells, in particular due to the strengthening of the glycocalyx structure [4]. The presence of components that perform a dehydration function (saccharides, hyaluronic acid) allows the intracellular osmotic pressure to be optimized [16-18]. The protocol for cryopreservation of reproductive cells implies the mandatory use of cryoprotective agents that have endo- and exocellular [19, 20] or combined effects. As a rule, such agents are used directly in the creation of cryoprotective media, as well as in combination with external cryogenic agents – glycerol, dimethyl sulfoxide, and dimethylacetamide [21, 22]. In most cases, when freezing, Lake (Lake, Stewart, 1978), Beltsville [13] and LCM (Russian Research Institute of Farm Animal Genetics and Breeding — VNIIGRZH, Inventor's Certificate No. 1130339 dated December 23, 1984, Russia) media are used to dilute native roosters' semen with the addition of the necessary components.

According to the literature, a different combination of non-reducing and reducing sugars can provide better protection of the semen during freezing/thawing [23]. The influence of a combination of monosaccharides (fructose, galactose, glucose, xylose) and disaccharides (lactose, trehalose, maltose, sucrose) in the composition of diluents for semen cryopreservation was evaluated on various animal species. At the same time, the effectiveness of maltose in the composition of a diluent for rooster semen has hardly been studied, although its potential positive effect has been shown in other animal species [24, 25].

Research on cryopreservation of rooster semen is mainly carried out on 28-32-week-old livestock [10, 17, 26] and much less often on older birds, which is probably associated with a decrease in reproductive qualities and the effectiveness of cryopreservation. However, due to the tendency to an increase in the

period of productive use of chickens up to 80 weeks of life, it becomes necessary to conduct experiments on roosters at the age of 46 weeks.

In this work, for the first time, the effectiveness of the use of maltose in combination with fructose in the composition of a diluent has been proven to increase the fertilizing ability of frozen-thawed rooster semen.

The aim of the work was to determine the optimal concentration of maltose in the composition of the diluent for freezing the semen of roosters aged 44-50 weeks and to establish the time frame for maintaining the functional usefulness of the frozen-thawed semen in the genital tract of the hen.

Methods. Experiments were carried out in 2020 on birds (*Gallus gallus domesticus*) of the Russian White breed (♂n = 10, ♀n = 30) aged 44-50 weeks, kept in individual cages in accordance with the technology adopted at the Center for Collective Use, Genetic collection of rare and endangered chicken breeds (VNIIGRZH) (2020). Sperm was collected by qualified specialists, the ingress of contamination into the ejaculates was excluded or minimized. Sperm was obtained by the method of abdominal massage [27] twice a week (glass vials with a volume of 10 ml were used) for 4 weeks. For an individual assessment of the quality of each ejaculate, a CASA imaging system (Computer-Assisted Semen Analysis; Argus-CASA software, ArgusSoft LLC, Russia; Motic® BA310E microscope, ×200, Motic Instruments Inc., Canada) was used. The ejaculate volume (ml), sperm concentration (billion/ml) (Accuread Photometer, IMV Technologies, UK), their total and progressive motility (%), and the degree of agglutination (%) were determined. The evaluation was carried out in 5 replicates. To eliminate individual differences between individuals, semen samples after each assessment were pooled and divided into three aliquots according to the developed experimental design. The study was conducted in accordance with the principles of bioethics in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental or Other Scientific Purposes (ETS 123, 1986, Article 5, Chapter 2).

The composition of the LCM-control medium (Leningrad cryoprotective medium) for freezing sperm [28] was as follows (per 100 ml of distilled water): sodium glutamate (1.92 g), fructose (0.8 g), potassium acetate (0.5 g), polyvinylpyrrolidone (0.3 g), protamine sulfate (0.032 g). For experimental diluents Mal-10 and Mal-20, the composition was calculated with partial replacement of fructose by maltose (0.72 g fructose + 0.166 g maltose and 0.64 g fructose + 0.326 g maltose, respectively), the rest of the components remained unchanged.

The diluted semen samples were cooled from 18 to 5 °C for 40 minutes. Then, dimethyl-acetamide (DMA) (Sigma-Aldrich, USA) was added to each sample to a final concentration of 6%. After the addition of DMA, the samples were incubated at 5 °C for 1 min. The pellets were frozen by dropping the seed into liquid nitrogen. The initial position of the pipette with the semen was controlled by a hand-held digital temperature indicator with a sensor (AHLBORN® THERM 24201L, Ahlborn Mess- und Regelungstechnik GmbH, Germany), in the area where the pipette was placed, the temperature was –15...–20 °C, –135 °C on the surface of liquid nitrogen. The average rate of semen supply to liquid nitrogen was ~ 1.4 drops per second. The frozen semen was stored in liquid nitrogen in Dewar flasks for 30 days. The granules were thawed according to the fast protocol on a heated metal plate at 60 °C (equipment developed by VNIIGRZH, 1989).

Locomotor activity (total and progressive motility) of frozen-thawed spermatozoa was recorded in each extender using a CASA imaging system (Motic® BA310E, ×200). Each sample was evaluated twice.

Virgin hens aged 46-50 weeks were used for artificial insemination (in total n = 30, n = 10 in each experimental group). Hens were inseminated intravaginally

with single daily doses of 0.04-0.07 ml of frozen-thawed semen (at least 70-80 million progressively moving active spermatozoa) [29]: during the first 2 days, one insemination, then one insemination every 2 days. Insemination time — after 14^{00}, in total, five inseminations were performved. Eggs for incubation were collected daily for 9 days, starting from the second day after the first insemination. Eggs (n = 239) were incubated for 6 days to assess fertility.

The eggs collected in each experimental group on days 5, 10, and 15 after the last insemination were broken and the fertilization was assessed by the state of the blastodisc.

The dynamics of the functional activity of frozen-thawed spermatozoa in the genital tract of hens on days 5, 10, and 15 from the last insemination was determined by the method of Bakst et al. [30] by the number of holes in the vitelline membrane of the yolk (sample size n = 55) formed as a result of interaction with spermatozoa. The yolks were carefully separated from the egg white. A filter paper ring was placed on the yolk in the blastodisc region, then the membrane was cut out with curved medical scissors with a sharp end along the outer diameter of the ring. The separated membrane was grasped with tweezers and washed several times with 0.9% NaCl solution at 4-5 °C until the yolk was completely removed, then carefully placed on a glass slide. For better visualization, the preparation was stained according to the following protocol. On the vitelline membrane with a micropipette, 30 μL of an alcoholic 10% formalin solution was evenly applied. After 15-20 s, formalin was decanted and placed on the membrane with ~ 30-40 μl of Schiff's reagent using a micropipette with a tip wrapped in aluminum foil (to minimize light exposure to Schiff's reagent). After the vitelline membrane acquired a purple hue (~ 30 s), the excess of Schiff's reagent was washed with distilled water, the preparations were dried in air for 5 min before microscopy. The area inside the filter paper ring (S = 1 cm^2) was determined using an Axio Imager microscope (Carl Zeiss Microscopy GmbH, Germany) in a dark field at ×200 magnification; the number of holes in the vitelline membrane was counted.

For statistical data processing and regression analysis, the Microsoft Excel 2013 and Statistica 7.0 software applications (StatSoft, Inc., USA) were used. Data were presented as means (M) and standard errors of means (±SEM). The samples according to the assessment of the native semen corresponded to the normal Gaussian distribution with the parameters $\chi^2_{theoretic} = 12.6 > \chi^2_{empiric} = 6.5$ in terms of assessing sperm volume and sperm motility. The differences between the samples were assessed by Student's t-test and were considered significant at $p < 0.05$.

Results. The total motility of freshly obtained semen averaged from 79.43 to 83.93%, the proportion of spermatozoa with rectilinear translational movement was from 63.22 to 70.90% (Table 1). The volume of individual ejaculates on different days varied from 0.3 ml to 1.1 ml; the concentration of spermatozoa was at least 3.2 billion/ml, agglutination did not exceed 10%.

1. Native semen quality indicators in Russian White roosters (*Gallus gallus domesticus*) (n = 50, M±SEM, the Center for Collective Use, Genetic collection of rare and endangered chicken breeds, 2020)

Rooster No.	Ejaculate volume, ml	Total motility, %	Progressive motility, %
1	0.54±0.03	82.88±2.50	70.07±3.52
2	0.36±0.06	79.43±5.30	64.37±6.21
3	0.05±0.05	83.93±1.27	70.12±1.82
4	0.45±0.07	83.93±1.05	70.90±0.54
5	0.60±0.04	78.25±5.39	63.26±5.78
6	0.46±0.02	81.35±2.41	67.49±2.10
7	0.73±0.07	81.15±1.17	66.22±1.23
8	0.54±0.04	82.85±2.44	67.34±1.02
9	0.36±0.04	81.45±1.48	63.95±2.49
10	0.39±0.07	82.83±2.18	67.19±3.01

2. Quality indicators (min-max according to the dates of the experiment) **of frozen-thawed semen of Russian White roosters (*Gallus gallus domesticus*) and egg fertilization in the first 9 days of collection, depending on the composition of the medium for cryopreservation** (♂n = 10, ♀n = 30, the Center for Collective Use, Genetic collection of rare and endangered chicken breeds, 2020)

Diluent	Total motility, %	Progressive motility, %	Egg number	Egg fertilization, %
LCM-control	51-54	45-50	83	74,7
Mal-10	51-54	45-50	81	92,6
Mal-20	51-54	45-50	75	86,3

N o t e. For the composition of media for freezing semen, see the Methods section.

Differences in the functional state of frozen-thawed rooster semen between the control and experiment were assessed by artificial insemination of chickens with subsequent incubation of eggs. The fertility rates of eggs (Table 2) differed depending on the composition of the diluent for semen cryopreservation. In the Mal-10 and Mal-20 groups, when maltose was used as a component of the medium for cryopreservation, a significant (by 11.6-17.9%) increase in egg fertilization was noted as compared to the control ($p < 0.05$). However, it should be noted that when studying frozen-thawed semen by optical microscopy, no differences were revealed between the control and experimental groups in terms of motility and the proportion of spermatozoa with rectilinear translational movement.

The highest percentage of egg fertilization on the 5th day of insemination was observed in the Mal-20 group (100% versus 50% in the LCM-control group), on the 10th day in the Mal-10 and Mal-20 groups, the fertilization was 60 and 100%, respectively, against 40% in control. On the 15th day from the last insemination in the LCM-control and Mal-10 groups, no fertilized eggs were obtained, however, in the Mal-20 group, their share was 20% (Fig. 1).

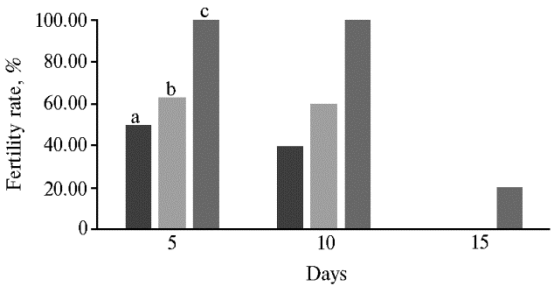

Fig. 1. Fertilization of eggs of Russian White hens (*Gallus gallus domesticus*) on days 5, 10, and 15 from the last insemination, depending on the composition of the medium for semen cryopreservation: a — LCM-control, b — Mal-10, c — Mal-20; for the composition of media for freezing semen, see the Methods section (n = 55, the Center for Collective Use, Genetic collection of rare and endangered chicken breeds, 2020).

Determination of the number of points of interaction of spermatozoa with the vitelline membrane of the yolk (Fig. 2) showed that the functional capacity of spermatozoa was significantly higher when using experimental media containing maltose.

Semen diluted with Mal-20 medium differed from other samples in increased viability in the genital tract of the hen: even on the 15th day after the last insemination, the number of points of interaction of spermatozoa with the vitelline membrane in the Mal-20 group was 45.8% higher than in control (Table 3).

To reveal the relationship between the number of points of interaction of spermatozoa with the vitelline membrane of the yolk and the fertilization of eggs, a regression equation was drawn up and the approximation coefficient was determined (Fig. 3). The result obtained in the Mal-20 group was confirmed by a positive linear relationship (the reliability of the regression equation was determined by the coefficient of determination $R^2 = 0.88$) of two signs on the interval of variability of the variable Y, where the variable X took values from 10 to 80. On the part of the curve where X took values > 80, the value of the variable Y was constant.

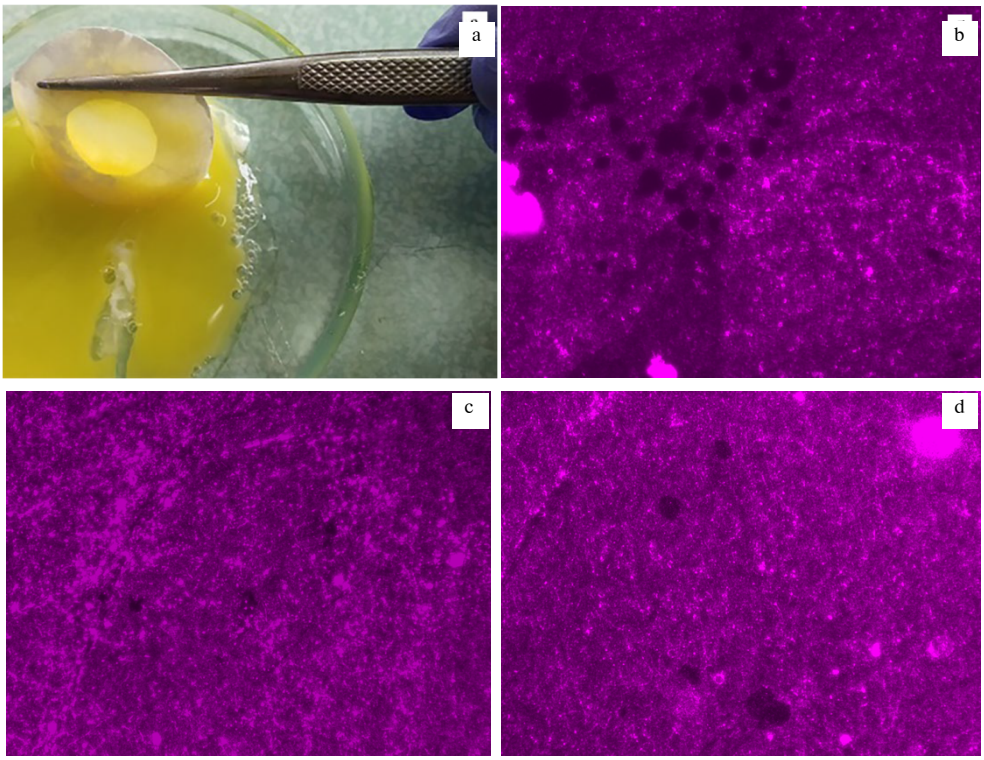

Fig. 2. Holes (dark points) **formed during the interaction of frozen-thawed spermatozoa of Russian White roosters (*Gallus gallus domesticus*) with the yolk vitelline membrane, depending on the composition of the medium for semen cryopreservation:** a — separation of the vitelline membrane; b — Mal-20 medium, c — Mal-10 medium, d — LCM-control (Axio Imager microscope, Carl Zeiss Microscopy GmbH, Germany, magnification ×200; staining with Schiff's reactive); for the composition of media for freezing semen, see the Methods section (the Center for Collective Use, Genetic collection of rare and endangered chicken breeds, 2020).

3. Interactions (number per cm^2) **of frozen-thawed spermatozoa of Russian White roosters (*Gallus gallus domesticus*) with the yolk vitelline membrane, depending on the composition of the medium for semen cryopreservation** ($n = 55$, $M \pm$SEM, the Center for Collective Use, Genetic collection of rare and endangered chicken breeds, 2020)

Diluent	Days from the last insemination		
	5	10	15
LCM-control	40.1±17.9[a]	36.4±15.2	26.6±6.1
Mal-10	67.0±5.5	67.0±18.7	27.8±5.8
Mal-20	110.7±15.8[b]	86.7±28.2	38.8±21.8

N o t e. For the composition of media for freezing semen, see the Methods section.
[a, b] Differences between the noted indicators are statically significant at $p < 0.05$.

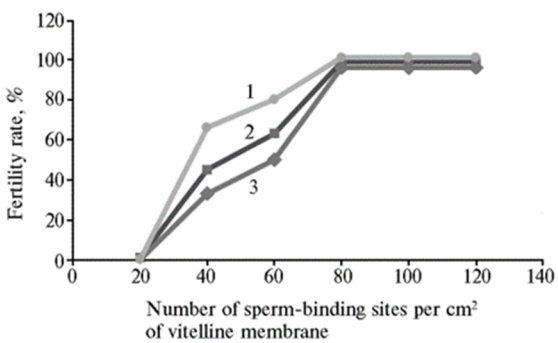

Fig. 3. Egg fertilization vs. the number of interaction points of frozen-thawed spermatozoa of roosters (*Gallus gallus domesticus*) with the yolk vitelline membrane, depending on the composition of the medium for semen cryopreservation: 1 — Mal-20 ($y = 1.585x - 17.5$, $R^2 = 0.8807$), 2 — Mal-10 ($y = 1.585x - 28.5$, $R^2 = 0.9913$), 3 — LCM-control ($y = 1.525x - 31.5$, $R^2 = 0{,}9701$); for the composition of media for freezing semen, see the Methods section (the Center for Collective Use, Genetic collection of rare and endangered chicken breeds, 2020).

Continuing the research of past years, we found that the decrease in the fertility of rooster semen in the freezing and thawing cycle was largely due to the high damage to the plasma membranes of spermatozoa [31]. Therefore, it is important not only to regulate the osmotic balance of the cell but also to strengthen the membrane itself by strengthening the structure of the glycocalyx. This problem can be solved due to the ability of disaccharides to attach to the heads of lipids and proteins on the outer surface of the cell membrane. Glycocalyx, reinforced with disaccharides dissolved in the medium for cryopreservation of rooster semen, stabilizes the sperm membranes and not only works as a cryo-resistant structure but also increases the period of preservation of the functional usefulness of spermatozoa in the genital tract of the hen. According to Tecle and Gagneux [32], the sperm glycocalyx mediates numerous functions of the female reproductive tract, including protection against innate and adaptive female immunity, and masks sperm proteins involved in fertilization. The glycocalyx of spermatozoa is modified during their movement in the genital tract of the hen and in crypts and represents the primary interface between the male gamete and the environment [33].

Saccharides (sucrose, lactose, trehalose, glucose, fructose) were used as energy sources and cryoprotectants as components of a semen diluent for cryopreservation of spermatozoa of various animals (dogs, Japanese black bears, goats, red jungle chicken) [34-37]. The study of the structure and properties of maltose disaccharide [38] gives grounds to predict its successful use in combination with a monosaccharide in the composition of a diluent for cryopreservation of rooster semen. The size of the maltose molecule does not allow crossing the cell membrane; in addition, this disaccharide has a low fermentation rate [39] and can enhance the “sugar coat” on the glycocalyx construct. An analysis of the available publications has shown that maltose has not yet been used as a component of a medium for cryopreservation of rooster semen. In cryopreservation of sturgeon semen, the use of maltose was equally successful with other disaccharides (lactose, trehalose, and lactulose) [40]. When added to a diluent for native rooster semen, maltose is slightly involved in the carbohydrate metabolism of spermatozoa [41]. Therefore, it can be assumed that it joins the glycocalyx, an evolutionarily progressive structure that provides the cell with the ability to specifically adapt to temperature, chemical, and other paratypical influences.

In our experiment, the task was not set to investigate the cellular mechanisms of the influence of the composition of experimental media on the viability of spermatozoa, but some hypotheses can be proposed. Maltose and fructose in the composition of Mal-10 and Mal-20 media in combination with DMA, in terms of their effect on the protective properties of the cell at low temperatures, represent a combination of three cryoprotectants of penetrating and non-penetrating action. DMA is a cryoprotectant of the amide group that promotes the formation of hydrogen bonds, which creates conditions for the formation of compounds that prevent the crystallization of water. Fructose, as an insignificant molecular unit, freely penetrates through the membrane into the cell plasma and functions both as an energy structure and as a component that reduces the osmotic load on the membrane during freezing/thawing. Maltose, according to our hypothesis, is fixed on the supra membrane shell (glycocalyx) of the cell, creating a stronger carbohydrate scaffold that protects the cell from cold shocks and prevents its destruction. This entire system of components allows minimizing the negative impact of the freezing and thawing processes on spermatozoa and maintaining their functional usefulness directly in the genital tract of the hen, since the integrity of the sperm glycocalyx becomes a critical factor when interacting with chicken gametes [4].

Thus, our experiments revealed an increase in the total fertility of chicken

eggs when inseminated with frozen-thawed semen, if maltose was used as a component of the medium for freezing rooster semen. With the addition of 0.166 and 0.326 g of maltose per 100 ml to the diluent, the fertilization of eggs was 92.6 and 86.3%, respectively, vs. 74.7% in the control. In addition, in the genital tract of the hen, spermatozoa retained their functional usefulness for 5 days, which is comparable to the indicators when using native semen for artificial insemination. Indicators of progressive motility, characterizing the integrity of the kinetic apparatus of spermatozoa, did not differ between the groups, but the functional usefulness of sperm, assessed by the number of interactions with the vitelline membrane, differed significantly. For acceptable fertilization of eggs (at least 80%), such functional usefulness of frozen-thawed spermatozoa is sufficient, at which the number of points of interaction with the vitelline membrane of the yolk is at least 60 pcs/cm^2. This was achieved using an experimental maltose diluent at a concentration of 0.326%. The results obtained open up prospects for the use of cryopreserved bird semen not only in programs for preserving the gene pool but also in breeding.

REFERENCES

1. Thélie A., Bailliard A., Seigneurin F., Zerjal T., Tixier-Boichard M., Blesbois E. Chicken semen cryopreservation and use for the restoration of rare genetic resources. *Poultry Science,* 2018, 98(1): 447-455 (doi: 10.3382/ps/pey360).
2. Silyukova Yu., Stanishevskaya O., Dement'eva N. *Vavilovskii zhurnal genetiki i selektsii*, 2020, 24(2): 176-184 (doi: 10.18699/VJ20.611) (in Russ.).
3. Di Iorio M., Rusco G., Iampietro R., Colonna M.A., Zaniboni L., Cerolini S., Iaffaldano N. Finding an effective freezing protocol for turkey semen: benefits of ficoll as non-permeant cryoprotectant and 1:4 as dilution rate. *Animals*, 2020, 10(3): 421 (doi: 10.3390/ani10030421).
4. Peláez J., Long J.A. Characterizing the glycocalyx of poultry spermatozoa: II. In vitro storage of Turkey semen and mobility phenotype affects the carbohydrate component of sperm membrane glycoconjugates. *Journal of Andrology*, 2008, 29(4): 431-439 (doi: 10.2164/jandrol.107.004259).
5. Salehi M., Mahdavi A.H., Sharafi M., Shahverdi A. Cryopreservation of rooster semen: evidence for the epigenetic modifications of thawed sperm. *Theriogenology*, 2020, 142: 15-25 (doi: 10.1016/j.theriogenology.2019.09.030).
6. Silyukova Y., Pleshanov N., Stanishevskaya O. The influence membranes damage and activity of roosters' sperm on the fertilization of eggs when using cured cryopreserved sperm. *Reproduction in Domestic Animals*, 2019, 54(S3): 101 (doi: 10.1111/rda.13528).
7. Partyka A., Łukaszewicz E., Niżański W. Flow cytometric assessment of fresh and frozen-thawed Canada goose (*Branta canadensis*) semen. *Theriogenology*, 2011, 76(5): 843-850 (doi: 10.1016/j.theriogenology.2011.04.016).
8. Huebinger J., Han H.-M., Hofnagel O., Vetter I.R., Bastiaens P.I.H., Grabenbauer M. Direct measurement of water states in cryopreserved cells reveals tolerance toward ice crystallization. *Biophysical Journal*, 2016, 110(4): 840-849 (doi: 10.1016/j.bpj.2015.09.029).
9. Askarianzadeh Z., Sharafi M., Torshizi M.A.K. Sperm quality characteristics and fertilization capacity after cryopreservation of rooster semen in extender exposed to a magnetic field. *Animal Reproduction Science*, 2018, 198: 37-46 (doi: 10.1016/j.anireprosci.2018.08.043).
10. Zaniboni L., Cassinelli C., Mangiagalli M.G., Gliozzi T.M., Cerolini S. Pellet cryopreservation for chicken semen: effects of sperm working concentration, cryoprotectant concentration, and equilibration time during in vitro processing. *Theriogenology*, 2014, 82(2): 251-258 (doi: 10.1016/j.theriogenology.2014.04.007).
11. Fattah A., Sharafi M., Masoudi R., Shahverdi A., Esmaeili V., Najafi A. L-Carnitine in rooster semen cryopreservation: flow cytometric, biochemical and motion findings for frozen-thawed sperm. *Cryobiology*, 2017, 74: 148-153 (doi: 10.1016/j.cryobiol.2016.10.009).
12. Amini M.R., Kohram H., Zare-Shahaneh A., Zhandi M., Sharideh H., Nabi M.M. The effects of different levels of catalase and superoxide dismutase in modified Beltsville extender on rooster post-thawed sperm quality. *Cryobiology*, 2015, 70(3): 226-232 (doi: 10.1016/j.cryobiol.2015.03.001).
13. Najafi A., Taheri R.A., Mehdipour M., Farnoosh G., Martínez-Pastor F. Lycopene-loaded nanoliposomes improve the performance of a modified Beltsville extender broiler breeder roosters. *Animal Reproduction Science*, 2018, 195: 168-175 (doi: 10.1016/j.anireprosci.2018.05.021).
14. Thananurak P., Chuaychu-noo N., Thélie A., Phasuk Y., Vongpralub T., Blesbois E. Different concentrations of cysteamine, ergothioneine, and serine modulate quality and fertilizing ability of cryopreserved chicken sperm. *Poultry Science*, 2020, 99(2): 1185-1198 (doi: 10.1016/j.psj.2019.10.040).

15. Thananurak P., Chuaychu-Noo N., Thélie A., Phasuk Y., Vongpralub T., Blesbois E. Sucrose increases the quality and fertilizing ability of cryopreserved chicken sperms in contrast to raffinose. *Poultry Science*, 2019, 98(9): 4161-4171 (doi: 10.3382/ps/pez196).
16. Lotfi S., Mehri M., Sharafi M., Masoudi R. Hyaluronic acid improves frozen-thawed sperm quality and fertility potential in rooster. *Animal Reproduction Science*, 2017, 184: 204-210 (doi: 10.1016/j.anireprosci.2017.07.018).
17. Donoghue A.M., Wishart G.J. Storage of poultry semen. *Animal Reproduction Science*, 2000, 62(1-3): 213-232 (doi: 10.1016/s0378-4320(00)00160-3).
18. Mavrodina T., Stanishevskaya O., Cherepanov S., Silyukova Y. Influence of osmolality of the media for dilution and cryopreservation of turkey toms' sperm on fertilization ability of thawed sperm. *Reproduction in Domestic Animals*, 2018, 53(Suppl. 2): 164.
19. Gloria A., Toscani T., Robbe D., Parrillo S., De Amicis I., Contri A. Cryopreservation of turkey spermatozoa without permeant cryoprotectants. *Animal Reproduction Science*, 2019, 211: 106-218 (doi: 10.1016/j.anireprosci.2019.106218).
20. Shahverdi A., Sharafi M., Gourabi H., Yekta A.A., Esmaeili V., Sharbatoghli M., Janzamin E., Hajnasrollahi M., Mostafayi F. Fertility and flow cytometric evaluations of frozen-thawed rooster semen in cryopreservation medium containing low-density lipoprotein. *Theriogenology*, 2015, 83(1): 78-85 (doi: 10.1016/j.theriogenology.2014.07.044).
21. Mosca F., Madeddu M., Sayed A.A., Zaniboni L., Iaffaldano N., Cerolini S. Data on the positive synergic action of dimethylacetamide and trehalose on quality of cryopreserved chicken sperm. *Data in Brief*, 2019, 9: 1118-1121 (doi: 10.1016/j.dib.2016.11.059).
22. Menezes G.F.O., Bittencourt R.F., Ribeiro Filho A.L., Cardoso F.L., Silva M.A.A., Santos E.S. Dimetilacetamida associada ou não ao glicerol para criopreservação de sêmen ovino [Dimethylacetamide associated or not to glycerol for criopreservation of sheep semen]. *Ciência Animal Brasileir*, 2018, 19: e-48026 (doi: 10.1590/1809-6891v19e-48026).
23. Xi M.D., Li P., Du H., Qiao X.M., Liu Z.G., Wei Q.W. Disaccharide combinations and the expression of enolase3 and plasma membrane Ca^{2+} ATPase isoform in sturgeon sperm cryopreservation. *Reproduction in Domestic Animals*, 2018, 53(2): 472-483 (doi: 10.1111/rda.13134).
24. Yildiz C., Kaya A., Aksoy M., Tekeli T. Influence of sugar supplementation of the extender on motility, viability and acrosomal integrity of dog spermatozoa during freezing. *Theriogenology*, 2000, 54(4): 579-585 (doi: 10.1016/S0093-691X(00)00373-3).
25. Golshahi K., Aramli M.S., Nazari R.M., Habibi H. Disaccharide supplementation of extenders is an effective means of improving the cryopreservation of semen in sturgeon. *Aquaculture*, 2018, 486: 261-265 (doi: 10.1016/j.aquaculture.2017.12.045).
26. Khiabani A.B., Moghaddam G., Kia H.D. Effects of adding different levels of glutamine to modified Beltsville extender on the survival of frozen rooster semen. *Animal Reproduction Science*, 2017, 184: 172-177 (doi: 10.1016/j.anireprosci.2017.07.013).
27. Burrows W.A., Quinn J. P. A method of obtaining spermatozoa from the domestic fowl. *Poultry Science*, 1935, 14(4): 251-254.
28. Tselyutin K.V., Tur B.K. *Iskusstvennoe osemenenie i kriokonservatsiya spermy sel'skokhozyaistvennoi ptitsy (petukhi, indyuki, gusaki, selezni)* [Artificial insemination and cryopreservation of poultry sperm (roosters, turkeys, geese, drakes)]. St. Petersburg—Pushkin, 2013 (in Russ.).
29. Popov I.I. *Voprosy iskusstvennogo osemeneniya domashnikh ptits* [Artificial insemination of poultry]. St. Petersburg—Pushkin, 2000 (in Russ.).
30. Bakst M., Eastridge J., Malecki I. The inner perivitelline layer sperm hole assay: use of filter paper rings for the isolation of the perivitelline layer overlying the germinal disc and new observations on its morphology. *Journal of Applied Poultry Research*, 2014, 23(1): 121-128 (doi: 10.3382/japr.2013-00873).
31. Pleshanov N., Stanishevskaya O. Evaluation of the cocks spermatozoa membranes' damaging during cryopreservation with use of Sperm VitalStain colorant. *Reproduction in Domestic Animals*, 2018, 53(S2): 183.
32. Tecle E., Gagneux P. Sugar-coated sperm: unraveling the functions of the mammalian sperm glycocalyx. *Mol. Reprod. Dev.*, 2015, 82(9): 635-650 (doi: 10.1002/mrd.22500).
33. Long J.A. Avian semen cryopreservation: what are the biological challenges? *Poultry Science*, 2006, 85(2): 232-236 (doi: 10.1093/ps/85.2.232).
34. Ponglowhapan S., Essén-Gustavsson B., Forsberg C.L. Influence of glucose and fructose in the extender during long-term storage of chilled canine semen. *Theriogenology*, 2004, 62(8): 1498-1517 (doi: 10.1016/j.theriogenology.2004.02.014).
35. Okano T., Murase T., Yayota C., Komatsu T., Miyazawa K., Asano M., Tsubota T. Characteristics of captive Japanese black bears (*Ursus thibetanus japonicus*) semen collected by electroejaculation with different voltages for stimulation and frozen-thawed under different conditions. *Animal Reproduction Science*, 2006, 95(1-2): 134-143 (doi: 10.1016/j.anireprosci.2005.10.002).
36. Naing S.W., Wahid H., Mohd Azam K., Rosnina Y., Zuki A.B., Kazhal S., Bukar M.M., Thein M., Kyaw T., San M.M. Effect of sugars on characteristics of Boer goat semen after cryopreservation. *Animal Reproduction Science*, 2010, 122(1-2): 23-28 (doi: 10.1016/j.anireprosci.2010.06.006).
37. Rakha B.A., Ansari M.S., Akhter S., Akhter A., Blesbois E., Santiago-Moreno O. Effect of

dimethylformamide on sperm quality and fertilizing ability of Indian red jungle fowl (*Gallus gallus murghi*). *Theriogenology*, 2020, 149: 55-61 (doi: 10.1016/j.theriogenology.2020.03.023).
38. Feofilova E.P., Usov A.I., Mysyakina I.S., Kochkina G.A. *Mikrobiologiya*, 2014, 83(3): 271-283 (doi: 10.7868/S0026365614020074) (in Russ.).
39. Ganatsios V., Koutinas A.A., Bekatorou A., Kanellaki M., Nigam P. Promotion of maltose fermentation at extremely low temperatures using a cryotolerant *Saccharomyces cerevisiae* strain immobilized on porous cellulosic material. *Enzyme and Microbial Technology*, 2014, 66: 56-59 (doi: 10.1016/j.enzmictec.2014.08.007).
40. Golshahi K., Aramli M.S., Nazari R.M., Habibi E. Disaccharide supplementation of extenders is an effective means of improving the cryopreservation of semen in sturgeon. *Aquaculture*, 2018, 486: 261-265 (doi: 10.1016/j.aquaculture.2017.12.045).
41. Harris C.C., Wilcox F.H. The carbohydrate metabolism of chicken semen. *Poultry Science*, 1962, 41(2): 409-416 (doi: 10.3382/ps.0410409).