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CHANGE OF BIOLOGICAL PARAMETERS OF POULTRY SEMEN AT CRYOPRESERVATION

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Abstract

The cryobanks of genetic material are an important element of assisted reproductive technologies. This technology allows more efficient use of genetic material, ensuring to obtain the maximum possible number of offspring for to preserve and restore of rare and endangered species. The most common biomaterial used in programs for the conservation and restoration of the genetic resources of agricultural poultry are spermatozoa. The spermatozoa undergo significant technological treatment during freezing and thawing. Some stages of this cycle are lead to the death of a large part of the cells, damage to their individual organelles or segments. The aim of the research was to study the effect of the freezing and thawing cycle on the biological parameters of spermatozoa in agricultural poultry. The objects of research were adult males of different types of agricultural poultry: roosters *Gallus gallus* (n = 6), quails *Coturnix coturnix* (n = 10), guinea fowls *Numida meleagris* (n = 6), turkeys *Meleagris gallopavo* (n = 3) and geese *Anser anser* (n = 4). Qualitative and quantitative indices of freshly received and frozen semen were studied. These indicators include the percentage of mobile spermatozoa, the proportion of spermatozoa with abnormal morphology, the percentage ratio of spermatozoa with abnormal morphology in the head, middle part and flagellum. Sperm was collected 3 times a week. The ejaculates were diluted with the medium for bird sperm dilution (1:1) followed by equilibration of samples at a temperature of 5 °C for 180 minutes. Before cryopreservation, dimethylacetamide was added to the samples as a cryoprotectant by gradually increasing its concentration to 8 %. The samples were frozen in straws (0.25 ml) with an automatic freezer Cryobath Biofreeze BV-65 (CONSARCTIC, Germany). Sperm quality was assessed using software Zoosperm 1.0 (LLC VideoTesT Goss) and Nikon microscope (Nikon Corporation», Japan) equipped with an image input system. To determine the proportion of viable spermatozoa, a supravital staining of a cell smear with 5 % eosin solution was used. The quality indicators of freshly obtained semen complied with the requirements. It was found that the biological value of spermatozoa decreases during the freeze-thaw cycle in all species of poultry conditioned by a decrease in the activity of germ cells. The sperm of the goose was more cryoresistant than the sperm of roosters, quails and turkeys. In geese, after thawing frozen sperm, the content of sperm cells with a straight-forward movement decreased by 30 %, while in roosters, quails and turkeys up to 40-44 %. During semen equilibration, in all poultry species the proportion of live spermatozoa decreased by 7.0-10.6 %. After the freeze-thaw cycle, the proportion of live spermatozoa in the ejaculate decreased compared to the values found for freshly sperm. In roosters, this indicator decreased by 41.6 % with an increase in the percentage of spermatozoa with an abnormal morphology by 22.0 %. In quails these indicators were 43.8 % and 21.8 % and in guinea fowls — 49.1 % and 28.8 %. The most visible disturbances in the morphology of spermatozoa were noted in the flagellum.

Keywords: *Gallus gallus* L., roosters, *Coturnix coturnix* L., quails, *Numida meleagris* L., guinea fowl, *Meleagris gallopavo* L., turkeys, *Anser anser* L., geese, cryopreservation, spermatozoa, abnormalities, freeze-thaw, equilibration, semen quality

Poultry products are among the main sources of proteins, fats, minerals, and vitamins among food products [1]. The rapid global rate of poultry industry development (from 2012 to 2016, the number of chickens in Russia increased by 14.6 %, in the world by 10.8 %) [2] is driven by its economic efficiency (the poultry is superior to other farm animals in maturity rate and feed conversion rate). The volume of poultry meat production is more than 300 million tons per year but the need for poultry products is only 34 % met. This fact, along with technological effectiveness of the industry, stimulates its development [3, 4].

Limiting factors are the lack of assisted reproductive technologies, including cryopreservation and artificial insemination, the methods to compensate for reproductive losses associated with increased animal productivity [5]. Cryopreservation of biological material is important in practical integration of reproductive technologies [6-8]. However, the use of cryopreserved sperm in the poultry industry is limited, which is largely due to the poor quality of thawed semen. Spermatozoa fertility decreases during freezing and thawing of generative plasma of birds [9, 10]. Spermatozoa of birds have a minimum volume of cytoplasm at a relatively large plasma membrane surface, the only cytoplasmic organoids are mitochondria [11, 12], the nucleus contains very condensed chromatin. In the freezing-thawing cycle, plasma and mitochondrial membranes are damaged mainly, as a result, the integrity of nuclear and mitochondrial DNA is violated, and the activity of spermatozoa is lost [13-15]. In birds, the structure of sperm is significantly different from that of mammals: the flagellum length is 90-100 μm, which is about 8 times longer than the head length [16-18].

Chemical and physical effects during freezing-thawing inevitably lead to changes in the ultrastructure of sperm and affect its biological full-grade. Cryopreservation causes damage to organelles and cell segments. The minor damages in the cells increase during freezing and thawing, and the cells lose their biological quality. Sperm cryoresistance and the ability to resist the damaging effect of ultra-low temperature depend on the states of membranes, their permeability, lipid composition, and fluidity [19, 20]. Due to such changes, the efficiency factor of insemination of thawed sperm is much lower than that of fresh and chilled sperm [21-24].

The cryoresistance of spermatozoa of birds depends on the species, despite the similarity of morphology [25]. The intraspecific variability of cryostasis depending on the linear accessory of males roosters was noted [26]. In the musky drake, sperm is more cryostable than in the Peking duck, sperm of guinea fowl, when compared to a rooster and a turkey, is very sensitive to cryopreservation [27]. The biological value of sperm is influenced by abiotic factors during processing. The main of them is the composition of diluents and cryoprotectors, the freezing and thawing mode [28, 29]. The reaction of sperm to these factors depends on the species characteristics of birds. With the increase in shelf life, the proportion of dead and abnormal sperm increases as well. It also depends on the breed and species characteristics: for example, in the sperm of guinea fowl, the process is more intense than in roosters [30].

In this paper, the biological parameters of sperms in the main species of poultry (roosters, quails, guinea fowls, turkeys, geese) were compared at different technological stages in the freezing-thawing cycle and the data was obtained confirming that in the process of equilibration the content of dead sperm in the generative plasma increases significantly regardless of the bird species.

The work objective was to study the effect of the freezing and thawing cycle on the biological parameters of sperm in different poultry species.

Techniques. Full-grown roosters *Gallus gallus* L. (n = 6), quails *Coturnix*

coturnix L. (n = 10), guinea fowls *Numida meleagris* L. (n = 6), turkeys *Meleagris gallopavo* L. (n = 3) and geese *Anser anser* L. (n = 4) selected for the experiment were kept in individual cages (the physiological yard of Vivarium of Ernst Federal Science Center For Animal Husbandry, 2017-2018). The poultry diet complied with the standards provided for each species.

Sperm was collected 3 times a week using spinal and peritoneal massage. For dilution, storage, and cryopreservation, a synthetic medium was prepared (bidistilled water 100 ml; fructose 1.0 g; glucose 1.0 g; Tris-HCl 0.195 g; disodium phosphate 1.1 g; sodium glutamate 3.0 g). The ejaculates were diluted with the medium for bird sperm dilution (1:1) followed by equilibration of samples at 5 °C for 180 min. Before cryopreservation, dimethylacetamide was added as a cryoprotectant by gradually increasing its concentration to 8 %. The samples were frozen in 0.25 ml straws. An automatic freezer Biofreeze BV-65 (Consarctic Entwicklung und Handels GmbH, Germany) was used.

Sperm quality was assessed using software Zoosperm 1.0 (LLC VideoTesT Goss, Russia) and Nikon microscope (Nikon Corporation», Japan) equipped with an image input system. The proportion and morphology were scored for straightforward moving and non-linearly moving spermatozoa, as well as for motionless cells. To determine the proportion of viable spermatozoa, supravital staining of smears with 5 % eosin solution was used.

The obtained data were processed in the Microsoft Excel software. The mean values (M) and standard errors of means (±SEM) are presented in the tables. The significance of differences was assessed according to Student's t-criterion. Differences were considered statistically significant at $p < 0.05$.

Results. Quality indicators of freshly obtained semen met the established requirements (Table 1). The content of mobile spermatozoa in the ejaculates varied depending on the bird species. The highest rate was in quails (87.5 %), the minimum in geese (64.5%). The average sperm activity in fresh semen of geese was lower than that of roosters, quails, guinea fowls, and turkeys, respectively by 21.4; 23.0; of 17.0 and 17.0 %. The differences between the average sperm motility in geese and other studied species were statistically significant ($p \leq 0.001$). Percent of spermatozoa with abnormal morphology was also higher in geese (from 10.9 to 18.6 %, 14.6±1.2 % on average). In quails, guinea fowls, roosters, and turkeys, it was lower by 1.8; 1.1; 5.4, and 5.0 %, respectively ($p \leq 0.001$). In guinea fowls, spermatozoa with morphological abnormalities were more common than in roosters and quails respectively by 4.3 and 3.9% ($p \leq 0.01$).

1. Quality of fresh semen of different species of poultry

Species	Sample size, n	Motile spermatozoa, %	Spermatozoa with abnormal morphology, %	Live spermatozoa, %
Roosters	6	86.1±6.4	9.2±2.7	89.2±8.1
Quails	10	87.5±3.8	9.6±1.2	92.5±6.1
Guinea fowls	6	82.1±3.5	13.5±2.7*	91.6±5.4
Geese	4	64.5±8.2**	14.6±1.2**	76.5±8.1
Turkeys	3	81.5±3.6	12.8±1.6	87.2±4.2

* The differences between guinea fowls, roosters, and quails are statistically significant at $p \leq 0.01$.

** The differences between geese and other species are statistically significant ($p \leq 0.001$).

The share of live spermatozoa varied from 76.5 to 92.5 % depending on the bird species. During equilibration it decreased. The number of dead spermatozoa in semen samples of roosters' seed after equilibration increased by 7.0 %, quails by 9.5%, guinea fowls by 10.2%, geese by 10.6% and turkeys by 9.8% (Fig. 1). The differences between the live spermatozoa content in all the studied samples were statistically significant at $p \leq 0.001$.

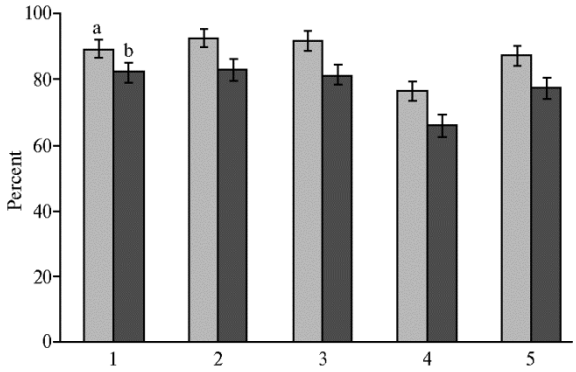

Fig. 1. The proportion of live spermatozoa in the fresh (a) **and equalized** (b) **semen of roosters** (1), **quails** (2), **guinea fowls** (3), **geese** (4), **and turkeys** (5).

After equilibration, a slight decrease in the number of spermatozoa with the straight-forward movement was noted. During further cryopreservation and subsequent thawing, this indicator decreased significantly. After thawing, the share of spermatozoa with the straight-forward movement in roosters decreased by 40 %, in quails by 43 %, in guinea fowls by 41 %, in geese by 30 %, and in turkeys by 44 % (Fig. 2).

The cycle of freezing and thawing adversely affected not only the activity but also the share of live spermatozoa. In roosters, this indicator dropped from 89.2 up to 47.6 %, in quails from 92.5 up to 48.7 %, in guinea fowls from 91.6 up to 45.2 %, in geese from 76.5 up to 38.2 %, in turkeys from 87.2 up to 48.5 %.

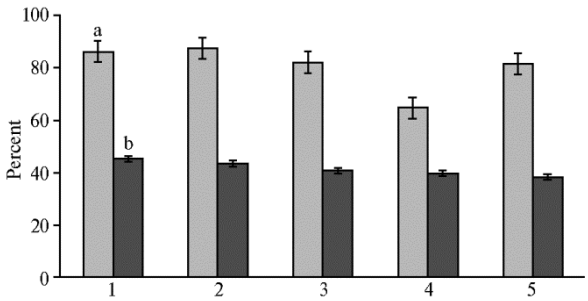

Fig. 2. The proportion of live spermatozoa with the straight-forward movement in the fresh (a) **and frozen-thawed** (b) **semen of roosters** (1), **quails** (2), **guinea fowls** (3), **geese** (4), **and turkeys** (5).

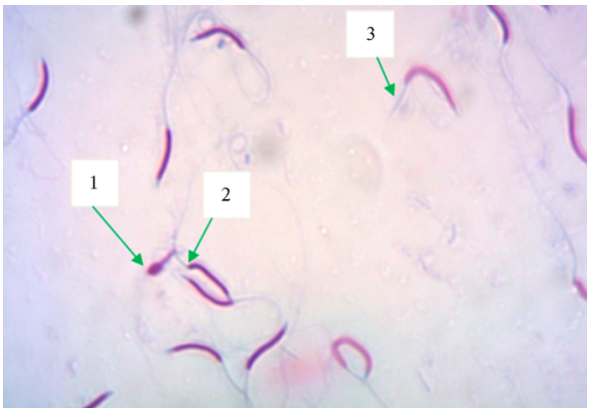

Fig. 3. Pathology of the head (1), **neck** (2), **and flagellum** (3) **in guinea fowls spermatozoa** (eosin staining).

As a result of freezing-thawing, the share of sperm with abnormal morphology increased (Fig. 3). The frequency of abnormalities of different segments changed, and the number of sperm with flagellum pathology increased (Table 2). For example, in the frozen-thawed samples of roosters' sperm in comparison with indicators for the fresh ejaculate, the percentage of sperm with the pathology of heads, middle part and flagellum increased by 0.4, 0.4, and 1.3 %, respectively ($p \leq 0.001$). Other studied species showed a similar trend.

The freezing-thawing cycle significantly changes the biological parameters of the spermatozoa, which are influenced by many factors. Several studies have confirmed the dependence of cryoresistance of poultry sperm on species peculiarities (for example, the cryoresistance of guinea fowl and geese sperm is lower than that of other types) [30, 31]. The sperm structure in these species is common but some properties on which the biological value of sperm depends are species-specific. For example, the differences in the sperm cell subfractions, length, the

number of mitochondria, flagellum fibrous membranes, and metabolic capabilities are species-specific. Therefore, the technology of cryopreservation, successfully used for one species, is unacceptable for others. Spermatozoa of guinea fowl and rooster have many morphological and morphometric similarities but differ in biochemical composition (i.e. in the ratio of cholesterol and phospholipids), which is reflected in the biophysical properties of the membrane. The membrane of the spermatozoa of guinea fowl compared to rooster is rigid, therefore, they are inferior to the latter in cryoresistance. When cryopreserving the sperm of guinea fowl by the technology used for rooster sperm, the fertility rate is only 15 %. The biological value of sperm is influenced by the methods of packing (in granules or packets), and the rates of freezing and thawing.

2. Spermatozoa with abnormal morphology (%) in freshly obtained semen (FOS) and the samples after freezing-thawing (FT) in different poultry species

Species	Abnormal area					
	head		middle part		flagellum	
	FOS	FT	FOS	FT	FOS	FT
Roosters	2.4±0.02	2.8±0.03*	2.6±0.02	3.0±0.03*	4.2±0.03	5.5±0.03*
Quails	2.5±0.01	2.8±0.05*	2.3±0.03	3.1±0.02*	4.8±0.03	5.8±0.03*
Guinea fowls	3.6±0.01	3.9±0.01*	3.7±0.02	4.6±0.03*	6.2±0.08	8.9±0.03*
Geese	4.8±0.02	5.2±0.06*	3.9±0.03	4.2±0.01*	5.9±0.02	7.4±0.02*
Turkeys	4.3±0.02	4.9±0.02*	3.3±0.05	3.9±0.02*	4.6±0.03	5.8±0.04*

* The differences between average values for FOS and FT are statistically significant at $p \leq 0.001$.

The proportion of spermatozoa with abnormal morphology depends on species characteristics, used diluents, and shelf life. Ultrastructural studies confirm a high correlation of sperm activity with ultrastructural lesions in the flagellum and the middle part of the spermatozoa, on which the kinematics of sperm depends [32]. The obtained data of light microscopy on violations of the morphology of the middle part and flagellum, as well as a decrease in activity after freezing-thawing indirectly indicate the accumulation of ultrastructural damages in the middle part and flagella of spermatozoa.

Thus, in cryopreserving semen of different poultry species, the biological value of spermatozoa deteriorates because of a decrease in semen activity. The equilibration of semen before cryopreservation decreases the share of live spermatozoa in all poultry species studied. The proportion of cells with abnormal morphology increases. During freezing and thawing, these indicators increase significantly. In comparison with the fresh sperm, the number of live spermatozoa after freezing-thawing in the ejaculates of roosters decreased by 41.6 %, with a 22.0 % increase in spermatozoa with abnormal morphology. In quails these indicators were 43.8 and 21.8 %, respectively, and in guinea fowls 49.1 and 28.8 %, respectively. The greatest abnormalities in the morphology of sperm occur in flagellum.

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