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COMPARATIVE STUDY OF THE STRUCTURAL INTEGRITY OF SPERMATOZOA IN EPIDIDYMAL, EJACULATED AND CRYOPRESERVED SEMEN OF STALLIONS

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Abstract

Cryopreservation of semen is an important way to preserve genetic resources. It is the most actual in horse breeding than in other livestock industries, as currently in many horse breeds, especially unique domestic breeds, the number is approaching a critical level. For many native breeds due to a specific management, year-round outdoors in herds, it is impossible to obtain sperm for cryopreservation by a traditional method using an artificial vagina, and the only cost-effective way to create cryobanks is getting epididymal semen. The technology for cryopreservation of stallion semen includes some critical steps that are characterized by a decrease in sperm quality. These are procedure for semen donation, dilution, temperature shock during freezing and thawing. For the first time a comparative transmission electron microscopy study of the ultrastructural integrity of spermatozoa was done for ejaculated and epididymal sperm in the same stallions thus avoiding the influence of different individuals on the compared parameter. Structural damage caused by cryoconservation of epididymal and ejaculated sperm was studied. It was found that acrosomes were the most susceptible and undergone the greatest impact. Its predominant pathology is the absence of internal contents (acrosome hypoplasia), resulted in enzyme deficiency. The frequency of this pathology was 12.4 % and 14.0 % for fresh epididymal and ejaculated sperm, respectively, and increased almost twofold after freezing and thawing, reaching 26.5 and 27.4 %, respectively. The rate of spermatozoa with the second most common pathology, acrosome degradation (premature release of sperm enzymes that dissolve the oocyte membrane, resulting in the loss of the ability to fertilize), after cryopreservation increased by 5.9 % ($p < 0.05$) and 8.9 % ($p < 0.01$) for epididymal and ejaculated semen, respectively. The nucleus of the sperm is one of the most resistant to cryopreservation among the organelles, though in two stallions we observed changes in the shape of the nucleus and vacuolation of chromatin after cryopreservation with a frequency of 1.6 to 6.1 %. Less than 10 % of the sperm had pathology of mitochondria. The axoneme of sperm is sufficiently resistant to cryopreservation. Outer dense fibers and fibrous membrane are almost not damaged during semen collecting as well as under semen freezing and thawing. Higher rates of ultrastructural integrity found for epididymal semen were not statistically significant. Thus, the sperm collecting results in minimal ultrastructural damage, whereas the main pathology is caused by cryopreservation. The ultrastructural integrity of spermatozoa in epididymal semen allow us to recommend this sperm collecting technique to organize cryobanks in case of impossibility of sperm collecting from stallions in traditional ways, or in need for early castration of stallions, in particular in sporting and productive horse breeding.

Keywords: stallion, electron microscopy, epididymal semen, ejaculated semen, cryopreservation

Artificial insemination of mares with freshly diluted, chilled and cryopreserved sperm makes it possible to make the most of the genetic potential of stallions [1]. The method of cryopreservation of sperm and technology to increase the reproductive status of stallions is important in maintaining genetic diversity and horse breeding. The advantages of cryopreservation are obvious: Its

use makes it possible to create sperm banks of high-value producers, for a long time to preserve genetic material for breeding, to transport semen over long distances [2].

Various technologies have been developed and successfully used for cryoconservation of the stallion ejaculated sperm [3-5]. Nevertheless, obtaining and cryopreservation of sperm from stallions includes a number of critical stages in which the quality of sperm is reduced [6]. These are the procedure for taking, dilution, temperature shock during freezing and thawing. In a number of cases, there is an objective need for cryopreservation of the epididymal semen, for example, when the death of highly valuable stallions or traumas with an unfavorable prognosis for recovery [7-9]. For some unique indigenous breeds with winter herd grazing, the use of epididymal semen is the only economically reasonable way to the cryobank formation.

The main problem with cryopreservation of sperm is its quality after thawing [10]. Basically, it worsens due to a decrease in the number of active spermatozoa with rectilinear movement, a decrease in the survival of spermatozoa and their structural disorders. The morphology of spermatozoa, along with mobility and survival, is one of the most important indicators of sperm quality [11]. The increase in the proportion of abnormal germ cells in the ejaculate is to a different extent due to the less fertility of stallions [12-14]. Due to a number of biological features of such spermatozoa, their fertility is reduced [15]. Usually, semen is studied by-light microscopy, which does not allow to reveal a significant part of ultrastructural damage in spermatozoa. At low stallion fertility it is recommended to conduct additional studies of sperm [16], in particular electron microscopy [17]. To date, this is the most accurate method of ultrastructural analysis [18] used to determine the etiopathogenesis of pathozoospermia, the spermatozoa function, and the diagnosis of disorders in the reproductive cells due to cryopreservation.

Spermatozoa from the deferent canals enter the epididymis, where their final maturation takes place resulting in the ability to fertilize [19-22]. A total of 15 to 25 billion spermatozoa are contained in the epididymis [23, 24]. Therefore, extraction of spermatozoa from the epididymis allows obtaining a sufficient number of gametes for artificial insemination of both freshly diluted and chilled and cryopreserved sperm.

Mobility, morphology, DNA integrity, viability of spermatozoa in ejaculated and epididymal sperm have been studied by many researches [7, 24-26]. The methodological approaches used by us for the first time made it possible to carry out a comparative analysis of the ultrastructure of germ cells (obtained by different methods and after cryopreservation) on biological material from the same stallions, which eliminated the influence of the individual characteristics of different individuals on the feature to be compared. This gives new knowledge about the state and safety of organoids in the freezing-thawing of epididymal and ejaculated spermatozoa.

The purpose of this study was to compare the ultrastructure of spermatozoa from epididymis and ejaculate subjected to cryopreservation and to diagnose the main pathologies of organoids.

Methods. Ejaculated sperm of 5 stallions of different breeds aged 5 to 8 years was obtained at 48 hour intervals using artificial vagina. In the data processing, the sperm indices of the two ejaculates produced after sexual rest were cutoff. The ejaculate volume, spermatozoa concentration, mobility, morphology [27], survival at 2-4 °C, and the time of mobility [3] were assessed. When diluting sperm, lactose-chelate-citrate-yolk (LCCY) medium (1:3, v/v) was used [3]. The sperm was frozen in liquid nitrogen vapor as per the standard technology of

the All-Russian Research Institute of Horse Breeding and stored in liquid nitrogen at –196 °C. After ejaculated semen collection, the stallions were castrated to obtain epididymal sperm which was diluted and frozen according to the same technology.

In electron microscopy, after a dilution (1:10) of native and cryopreserved samples of ejaculated and epididymal semen with isotonic NaCl, 2.5 % glutaraldehyde (Ted Pella Inc., USA), prepared with 0.1 M cacodylate Buffer (pH 7.2) (Sigma, USA), was added. The samples were centrifuged for 15 min at 1000 rpm, the supernatant was removed, The precipitate was treated with glutaraldehyde as described above, additionally fixed with 1 % osmic acid (Serva, Germany) and poured into epon (Fluka, Germany). Ultrathin sections were prepared on an UltraCut III microtome (Reichert Jung Optische Werke AG, Austria), dyed with an aqueous solution of uranyl acetate and lead citrate (Serva, Germany), and viewed in an electron microscope Hitachi 700 (Japan). General view of spermatozoa was studied at ×5000 magnification, acrosome, chromatin nucleus and mitochondria — at ×16000-18000, axonemal anomalies on transverse sections of flagella — at ×20000-25000. In each sample, at least 150 germ cells were analyzed.

The data was processed by conventional methods of variational statistics. The table shows the mean (X) and standard errors of the mean (x). The reliability of the differences was determined using Student's t-test. The differences were statistically significant when $p < 0.05$.

Results. On average, the ejaculate volume was 54.8±8.8 ml at spermatozoa concentration (184.5±23.7)×10^6/ml; the activity was 4.4±0.3 points, and a survival period at 2-4 °C was 125.2±8.4 hours. After cryopreservation the activity decreased to 1.8±0.2 points, the survival time — to 59.2±14.7 hours. The proportion of morphologically normal spermatozoa reached 71.4±4.4 % in native sperm and 68.4±4.0 % in cryopreserved sperm.

The proportion (%) of spermatozoa with normal morphology in freshly received and cryocon-served ejaculated and epididymal sperm of stallions ($n = 5$, $X \pm x$)

Morphological sign	Epididymal sperm		Ejaculated sperm	
	at collection	after thawing	at collection	after thawing
Intact head	76.1±4.2	64.4±6.1	73.6±4.2	57.2±7.3
The presence of the acrosome	98.5±0.5	92.6±1.3	98.0±0.7	89.1±1.9
Normal position of the acrosome	99.2±0.8	98.2±1.6	98.4±0.7	91.8±3.7
Normal form of the acrosome	94.4±1.6	87.8±2.9	91.6±1.5	87.0±2.9
Compact contents of the acrosome	87.6±3.3	73.5±5.2	86.0±3.7	72.6±6.7
Normal form of the nucleus	99.6±0.2	97.3±1.2	98.3±1.3	93.7±3.3
Normal mitochondria	97.1±1.3	90.3±1.4	93.7±2.3	88.0±2.1
Normal axoneme	91.5±2.3	84.7±4.5	88.3±2.9	84.8±4.1
Normal outer dense fibrils	99.5±0.5	98.9±1.1	98.8±1.2	98.7±1.3
Normal fibrous membrane	100	99.0±1.1	100	98.0±2.0

The use of ejaculated and epididymal sperm from the same stallions made it possible to compare the damage to germ cells after cryopreservation, depending on the method of sampling. One of the main indicators of sperm quality, determined by electron microscopy, is the number of spermatozoa with intact heads (Fig. 1), normal shape, acrosomes and chromatin [18]. In epididymal sperm, such spermatozoa amounted to 76.1 %, after ejaculation their number decreased by 2.5 % (Table). During freezing-thawing of ejaculated sperm, the number of spermatozoa with intact heads additionally decreased by 16.4 % and averaged 57.2 %. In cryopreserved epididymal sperm, the number of spermatozoa with intact heads (64.4 %) was 7.2 % higher than that in frozen ejaculates.

Of the pathologies of spermatozoa heads in collected and cryopreserved sperm, we noted an increase in the number of spermatozoa with acrosome degradation due to a premature acrosomal reaction, acrosome hypoplasia (incompact

acrosome contents), changes in the nucleus shape, and chromatin vacuolization.

Basically, during cryoconservation of both ejaculated and epididymal stallion sperm, the acrosome was damaged (Fig. 2, 3). In epididymal sperm, the acrosome was present in 98.5 % of spermatozoa, in the ejaculates and after freezing-thawing the proportion of spermatozoa with the reacted acrosome (with acrosome degradation) increased by 9.4 %. Upon freezing and subsequent thawing, the number of spermatozoa with acrosome degradation increased by 5.9 % ($p < 0.05$) in epididymal sperm, and by 8.9 % ($p < 0.01$) in the ejaculated sperm.

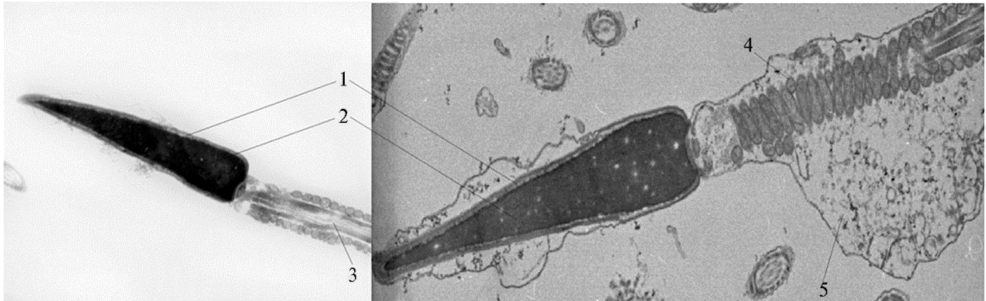

Fig. 1. Normal stallion spermatozoon (left) **and spermatozoon with asymmetric cytoplasmic droplet** (right)**:** 1 — acrosome, 2 — chromatin, 3 — longitudinal section through axonema flagellum, 5 — **cytoplasmic droplet**. Hitachi 700 (Japan), ×5000-25000 magnification.

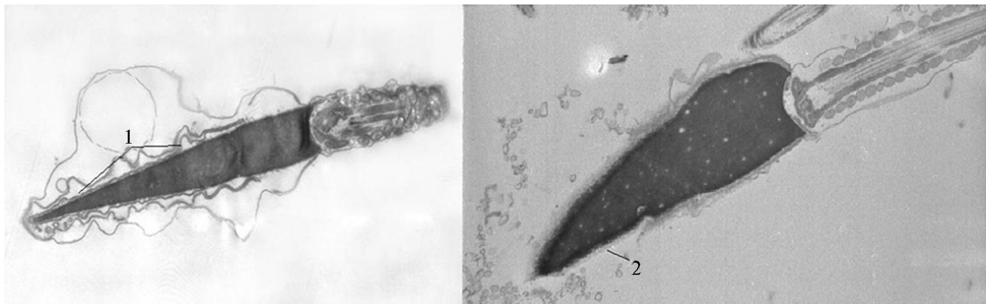

Fig. 2. Stallion spermatozoons with "empty" acrosome (left) **and reacted acrosome (after acrosomal reaction)** (right)**:** 1 — acrosome with transparent contents ("empty") and uneven contours, 2 — residual material of the reacted acrosome. Hitachi 700 (Japan), ×16000-18000 magnification.

The most common pathologies in cryopreservation were also the increase in the number of spermatozoa with acrosome hypoplasia and lack of content (acrosomes with electronically transparent contents). The percentage of the spermatozoa with incompact acrosome contents increased by 13.4 % in cryopreserved ejaculates, and by 14.1 % after freezing and thawing of the epididymal semen.

The effect of cryopreservation on the nucleus of spermatozoa was minimal. Changes in the shape of the nucleus were found in fresh and cryopreserved semen in not more than 5.9 % cells on average. In fresh sperm, spermatozoa with vacuolated chromatin did not occur, after cryopreservation, they were detected in a small amount (from 1.6 to 6.1%) in samples from two stallions.

The spermatozoa with normal mitochondria amounted to 97.1 % in epididymal sperm and 93.7% in the ejaculate sperm. Thus, at sperm collection and primary processing, the number of spermatozoa with damaged mitochondria increased by 3.4 %, after cryopreservation —by additional 5.7 %, as a result mitochondria were damaged in 9.1 % of the spermatozoa. The increase in the number of mitochondrial abnormalities in the cryopreservation of epididymal sperm compared to fresh one produced quite high ($p < 0.05$) significance of differences.

Morphological anomalies of the axoneme are closely related to low fertility and infertility [27]. Therefore, the study of axoneme integrity is one of the

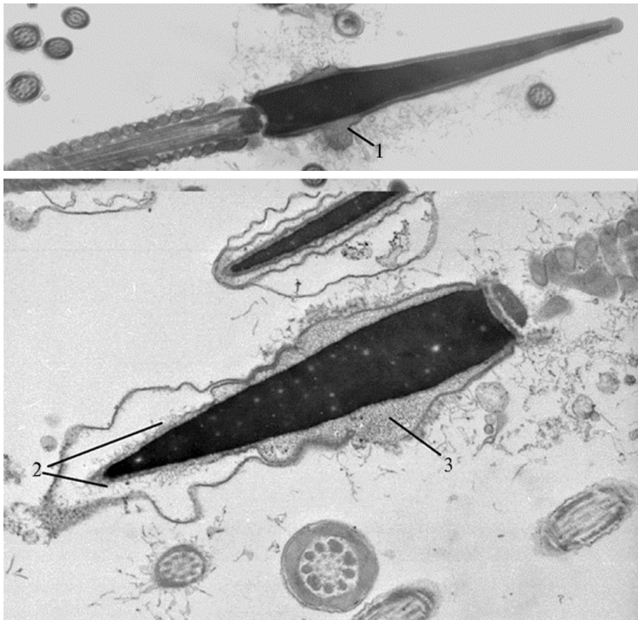

Fig. 3. The initial stage of stallion spermatozoa acrosome damage: 1 — diffuse expanded zones of an acrosome with fibrous contents, 2 — section of the expanded acrosome with electronically transparent contents ("empty" acrosome), 3 — the expanded acrosome with fibrous contents. Hitachi 700 (Japan), ×16000-18000 magnification.

mandatory stages in the electron microscopic study of ejaculate. The axoneme in both epididymal and ejaculated spermatozoa proved to be very resistant to cryopreservation. From the sperm collection to freezing and thawing, the number of spermatozoa with an abnormal flagellum increased by 6.7 %, which was significantly lower than the analogous index for mitochondrial and especially acrosomal damage.

External dense fibrils and fibrous membrane of the spermatozoa axoneme in stallions are also resistant to external influences. It was shown that they were practically not damaged either at sperm collection, or at freezing-thawing.

In general, the results of diagnostics of ultrastructural abnormalities in epididymal and ejaculated spermatozoa demonstrate that when semen is produced on an artificial vagina, the damage to germ cell organoids is minimal, and the main structural changes occur in cryopreservation. Confirmation of the high safety of epididymal spermatozoa, as well as their resistance to cryopreservation, allows us to make a positive forecast for the development and application of the cryopreservation technology for epididymal sperm of stallions.

So, the ultrastructural integrity of organoids in the germ cells of stallions in epididymal sperm is higher than in ejaculated one, but these differences are unreliable. To a lesser extent, ultrastructural damage to spermatozoa occurs when collecting sperm, and is mostly due to cryopreservation. When freezing and thawing both epididymal and ejaculated sperm, the spermatozoa acrosome is most vulnerable, mitochondria are less sensitive, whereas the axoneme, outer dense fibrils and fibrous shell of the axoneme are fairly stable, and the effect on the sperm nucleus is minimal.

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