CONSERVATION OF GENETIC RESOURCES IN HORSE BREEDING AND MAJOR STRUCTURAL DAMAGES OF SPERM DURING SEMEN CRYOPRESERVATION IN STALLIONS

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Assisted reproductive technologies have been used in zoos and breeding centres to preserve rare and endangered species of wild animals over the past three decades. The most common way to preserve the genetic material of farm and wildlife animals is to create cryobanks of semen producers and embryos. Studies of domestic species under controlled conditions provide an excellent opportunity to develop effective semen handling techniques for application to wild species of the genus *Equus*. The All-Russian Research Institute for Horse Breeding (ARRIH) began studies on the cryopreservation of genetic resources in the early 1950s. In 1954 the ARRIH got the world's first foal from the artificial insemination by cryopreserved sperm. The collection of genetic resources of ARRIH has been successfully working since 1972 and contains nowadays cryopreserved sperm from 56 stallions of 16 breeds of domestic and foreign selections. Freezing and subsequent thawing of stallion sperm leads to a decrease in progressive sperm motility and an increase in the number of sperm with structural pathologies. Electron microscopy is one of the most accurate methods used to assess the structural integrity of sperm. We conducted an electron microscopic study of native and cryopreserved sperm of 35 stallions of riding and trotting breeds, aged 4 to 22 years (mean age 11.3 ± 0.9 years). We found that organoids with a denser structure are more resistant to freezing and subsequent thawing of sperm. The use of stallion sperm cryopreservation according to the standard technology of ARRIH leads to a decrease in the number of sperms with intact heads by an average of 19.7%, and the share of sperm with intact heads in cryopreserved sperm is 49.1 ± 2.7% ($p < 0.001$). The most negatively cryopreservation of semen affects an acrosome of sperms. The main pathologies of acrosomes in sperm cryopreservation are acrosome hypoplasia and its degradation (secondary absence of acrosome). The number of spermatozoa with acrosome hypoplasia and the absence of internal content after cryopreservation increases by 20.9%, and the share of such sperms is 31.7% in thawed sperm ($p < 0.001$). The number of sperm with acrosome degradation during sperm freezing and thawing increases by 10.4%, and is 18.6 ± 2.4% in average. The share of spermatozoa with normal mitochondrial structure in native sperm is 89.3 ± 2.0% in average, in cryopreserved sperm – decreases by 6.5%. The share of spermatozoa with normal axoneme after cryopreservation decreases slowly, averaging by 4.4% and is 81.4 ± 2.0%. Some of the available assisted reproduction technologies will need to be further optimised in domestic horses before they can be applied to the endangered Przewalski's horse *Equus ferus przewalskii*.

Key words: acrosomes, electron microscopy, equids, *Equus ferus caballus*, mitochondria, progressive motility, ultrastructure

Introduction

Assisted reproductive technologies have been used in zoos and breeding centres to preserve rare and endangered species of wild animals over the past three decades. There are bioresource collections of genetic material of different species of farm animals and representatives of wild fauna in order to preserve the genetic diversity of the animal world in Russia.

Studies of domestic species under controlled conditions provide an excellent opportunity to develop effective semen handling techniques for application to wild species of the genus *Equus* Linnaeus, 1758. But, despite the progress that has been made in the field of assisted reproduction in domestic horses *Equus ferus caballus* Linnaeus, 1758, over the course of the last years, the work with Przewalski's horses *Equus ferus przewal-*

skii Poliakov, 1881, in zoos of Russia and neighbouring countries have not been carried out. An important problem on the conservation of the Przewalski's horse is that information about its reproductive biology is insufficient. This makes it difficult to apply even basic methods of assisted reproductive technologies, such as artificial insemination and embryo transfer, let alone more advanced technologies.

Due to the significant reproductive similarity between wild and domestic horses, the latter can be used to optimise cryopreservation and embryo production protocols for application to wild equids in future. Some of the available assisted reproduction technologies will need to be further optimised in domestic horses before they can be applied to the endangered Przewalski's horse.

Studies of cryopreservation of genetic resources began at the All-Russian Research Institute for Horse Breeding (ARRIH) in the early 1950s. In 1954 the Institute for Horse Breeding received the world's first foal from the artificial insemination by cryopreserved sperm. The collection of genetic resources of ARRIH has been successfully working since 1972 and nowadays contains cryopreserved sperm from 56 stallions of 16 breeds of domestic and foreign selections (Lebedeva et al., 2015). Scientists store cryopreserved sperm of many stallions in a biocollection for several decades and use it for artificial insemination of mares. Cryopreserved sperm of Tersk breed stallion Samotsvet (Symbol – Cima), born in 1959, has the longest period of storage, the sperm has been stored in the Cryobank since 1972 and still has a high fertilising ability (Naumenkova et al., 2013).

Indicators of quality and cryostability of stallion sperm depend on many factors, including hereditary and ecophysiological factors (Bagirov et al., 2017), the state of reproductive and somatic status of animal, etc. The greatest negative impact on sperm at all stages of work has its freezing and subsequent thawing (Atroshchenko et al., 2017). Reducing the number of sperm with progressive motility during freezing is one of the main problems in cryopreservation of stallion sperm. Thawing of frozen sperm leads to an increase in the number of sperms with structural damage of acrosomes, mitochondria, cell membranes in addition to reducing progressive motility (Atroshchenko & Bragina, 2011). Sperm with structural damage of these organelles is not able to fertilize the egg *in vivo*.

Light microscopy is one of the main methods of sperm quality evaluation. But determination of the integrity of sperm organoids requires the use of specialised evaluation methods. Electron microscopy is a method used to assess the structural integrity of sperm (Zamboni, 1987). Electron microscopic examination allows qualitative and quantitative assessment of the state of cellular organelles necessary for the sperm fertilising function (Pesch et al., 2006). The study of cryopreservation's effect on the structural integrity of germ cells allows objectively evaluating the loss from different organelles damage of sperm during the process of freezing.

The aim of this work is to study the structural integrity of spermatozoa in stallions using electron microscopy and to assess the damage of organoids during sperm cryopreservation.

Material and Methods

We examined 35 stallions of riding and trotting breeds, aged 4 to 22 years (mean age 11.3 ± 0.9 years). We evaluated each received sperm ejaculate according to the following parameters: ejaculate volume after filtration, concentration, progressive motility and sperm survival at a temperature of (T) +4°C. To dilute the sperm, we used lactose-chelate-citrate-yolk medium (LCHCY) in the ratio of volumes 1 : 3. Then we froze sperm in vapors of liquid nitrogen in 18 ml aluminum tubes following the standard technology worked out at the ARRIH and carried out thawing of the frozen sperm in a water bath at a temperature +40°C during 90 seconds.

To conduct electron microscopy we diluted samples of native and cryopreserved sperm with isotonic NaCl solution in a ratio of 1 : 10; added a fixator – 2.5% solution of glutaraldehyde («Ted Pella Inc.», USA), prepared on 0.1 M cacodilate buffer (pH 7.2) («Sigma», USA); centrifuged 15 min at 300 g; removed the sedimentary liquid, fixed the precipitate with the same fixator and additionally fixed with 1% solution of osmic acid («Serva», Germany) and poured into epon («Fluka», Germany). Then we obtained ultrathin sections on the microtome UltraCut III («Reichert Jung Optische Werke AG», Austria), stained it with an aqueous solution of uranyl acetate and lead citrate («Serva», Germany) and scanned in an electron microscope Hitachi 700 (Japan). Next we studied sperm with magnification 5000× (general viewing), and 16000×–25000× (the study of organelles); we evaluated 150 cells in each sample.

We performed the statistical processing using the program Statistica 8.0 («StatSoft Inc.», USA)

and presented the data as M ± SE, where M is the mean, SE is the standard error of the mean. We determined the statistical significance between the indicators using Student's t-test and considered differences statistically significant at $p \leq 0.05$.

Results and Discussion

The number of sperm with progressive motility decreased after sperm cryopreservation in average from 62.7% to 27.7% ($p < 0.001$) (Table). Sperm survival at +4°C also decreased from 117.0 hours to 48.5 hours ($p < 0.001$).

According to electron microscopy data acrosome is present in 91.8 ± 1.6% of spermatozoa in native sperm of stallions, respectively, the number of spermatozoa with acrosome degradation (acrosome reacted) is 8.2%. Acrosome is present in 81.4 ± 2.4% of spermatozoa ($p < 0.001$) after cryopreservation of stallion sperm. The number of sperm with acrosome degradation during sperm freezing and thawing increases by 10.4%. Assessment of acrosome integrity is a reasonable alternative to the experimental penetration tests. With the help of electron microscopy we can reliably determine the acrosome integrity, the state of its enzyme apparatus and the post-acrosomal segment involved in the attachment of the sperm to the egg.

One of sperm acrosomes pathologies is the primary or secondary absence of acrosomes. The primary absence of acrosome includes globulozoospermia – a disease of genetic nature. The secondary loss of an acrosome is a result of premature acrosome reaction, in other words destruction of an existing acrosome. We can see in this case a destruction of plasma membrane and the appearance of nuclear envelope on the surface of sperm in the acrosomal zone. In a physiological acrosomal reaction intact sperm retains a postacrosomal region and plasma membrane of the postacrosomal segment (Bragina & Bocharova, 2014). According to Bragina & Bocharova (2014) the number of sperm cells with reacted acrosome does not exceed 20% in ejaculate of fertile men, and the presence in the ejaculate of increased (> 20%) sperm content with a reacted acrosome (with acrosome degradation) may cause a decrease in sperm fertility. Pesch et al. (2006) established a significant negative correlation between the number of sperm with acrosomes located far from nucleus and fertility of stallions.

Electron microscopic examination also allows diagnosing another acrosome pathology – acrosome hypoplasia and the lack of internal content (acrosome enzyme deficiency). The population of sperm with a compact content of acrosome in native sperm is 89.2 ± 1.1%, after cryopreservation of sperm this data reduces to 68.3 ± 2.3%. The number of spermatozoa with acrosome hypoplasia and the absence of internal content after cryopreservation increases by 20.9%, and is 31.7 ± 2.3% ($p < 0.001$).

Spermatozoa with a normal shape of nucleus make up 94.7 ± 1.4% in native stallion semen, and their number reduces by 2.4% after cryopreservation. The number of sperm cells with nuclear chromatin vacuolation after freezing-thawing increases by an average of 1.5%, and is 2.7% ($p < 0.05$).

Table. The effect of cryopreservation on sperm quality and the integrity of sperm organelles (n = 35)

Indicator	Sperm	
	native	thawed
The volume of the ejaculate, ml	54.5 ± 3.88	–
Concentration, million/ml	216.8 ± 15.27	–
Progressive mobility, %	62.7 ± 2.69	27.7 ± 1.73**
The survival rate at T +4°C, hour	117.0 ± 12.35	48.5 ± 5.74**
The acrosome is present, %	91.8 ± 1.6	81.4 ± 2.4**
Acrosomal hypoplasia, %	10.8 ± 1.1	31.6 ± 2.3**
Normal core shape, %	94.7 ± 1.4	92.3 ± 1.6
Vacuolated chromatin of the nucleus, %	1.2 ± 0.4	2.7 ± 0.5*
Destroyed chromatin, %	0.5 ± 0.2	1.1 ± 0.4
Spermatozoa with intact heads, %	68.8 ± 2.5	49.1 ± 2.7**
Spermatozoa with normal shape and structure of mitochondria, %	89.3 ± 2.0	82.8 ± 2.5*
Spermatozoa with normal shape and structure of axoneme, %	85.8 ± 2.0	81.4 ± 2.0

Note: * $p < 0.05$; ** $p < 0.001$.

One of the main indicators of sperm quality, determined by the method of electron microscopy, is a number of sperms with intact heads in the ejaculate or in a dose for artificial insemination. Intact sperm heads are heads with normal acrosomes, shape and chromatin of the nucleus. In native stallion sperm the population of sperms with intact heads averages 68.8 ± 2.5%. That is, even in freshly obtained sperm on average 31.2% of sperms are diagnosed with various pathologies of the head, and these sperms are not suitable for fertilisation of the egg *in vivo*. In case of cryopreservation of sperm according to the standard technology of ARRIH, the number of sperm with intact heads decreases by an average of 19.7% and is 49.1 ± 2.7%.

The share of spermatozoa with normal shape and structure of mitochondria in native sperm is 89.3 ± 2.0%. The number of sperm with normal mitochondria decreases by 6.5% during cryopreservation. The number of sperms with normal axoneme slightly decreases in the process of freezing-thawing, on average by 4.4%, and is 81.4 ± 2.0%.

Conclusions

After the study we can conclude that organoids with denser structure are more resistant to freezing and subsequent thawing of sperm. At cryopreservation of stallion sperm according to the standard technology of the ARRIH the number of sperms with intact heads decreases on average by 19.7% and makes 49.1 ± 2.7% in cryopreserved sperm. Stallion sperm cryopreservation has the greatest negative effect on sperm acrosomes. The main pathology of an acrosome during sperm cryopreservation is acrosome hypoplasia and its degradation (secondary absence of an acrosome).

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СОХРАНЕНИЕ ГЕНЕТИЧЕСКИХ РЕСУРСОВ В КОНЕВОДСТВЕ И ОСНОВНЫЕ СТРУКТУРНЫЕ ПОВРЕЖДЕНИЯ СПЕРМАТОЗОИДОВ ПРИ КРИОКОНСЕРВАЦИИ СПЕРМЫ ЖЕРЕБЦОВ

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В последние три десятилетия в зоопарках и питомниках начали использовать вспомогательные репродуктивные технологии для сохранения редких и исчезающих видов диких животных. Самым распространённым способом сохранения генетического материала сельскохозяйственных животных и представителей дикой фауны является создание криобанков семени производителей и эмбрионов. Для представителей рода *Equus* исследования домашних видов в контролируемых условиях дают прекрасную возможность разработать эффективные методы обработки спермы для применения у диких видов. Исследования по криоконсервации генетических ресурсов начались в институте коневодства в начале 1950-х гг. И в 1954 г. сотрудники института коневодства получили первого в мире жеребенка от искусственного осеменения криоконсервированной спермой. Коллекция генетических ресурсов ВНИИ коневодства успешно работает с 1972 г. и в настоящее время содержит криоконсервированную сперму от 56 жеребцов 16 пород отечественной и зарубежной селекции. Замораживание и последующее оттаивание спермы жеребцов ведет к снижению прогрессивной подвижности сперматозоидов и увеличению количества сперматозоидов со структурными патологиями. Одним из наиболее точных методов используемых, для оценки структурной целостности сперматозоидов, является электронная микроскопия. Мы провели электронно-микроскопическое исследование нативной и криоконсервированной спермы 35 жеребцов верховых и рысистых пород, в возрасте от 4 до 22 лет (средний возраст 11.3 ± 0.9 лет). Мы установили, что органоиды с более плотной структурой наиболее устойчивы к воздействию замораживания и последующего оттаивания спермы. Применение криоконсервации спермы жеребцов по стандартной технологии ВНИИ коневодства ведет к снижению количества сперматозоидов с интактными головками в среднем на 19.7%, таким образом, доля сперматозоидов с интактными головками в криоконсервированной сперме составляет 49.1 ± 2.7% ($p < 0.001$). Наиболее негативно криоконсервация спермы жеребцов влияет на акросомы сперматозоидов. Основными патологиями акросом при криоконсервации спермы являются гипоплазия акросомы и её деградация (вторичное отсутствие акросомы). Количество сперматозоидов с гипоплазией акросомы и отсутствием внутреннего содержимого после криоконсервации увеличивается на 20.9%, и составляет 31.7% в оттаянной сперме ($p < 0.001$). Численность сперматозоидов с деградацией акросомы при замораживании-оттаивании спермы увеличивается на 10.4%, и составляет в среднем 18.6 ± 2.4%. Сперматозоиды с нормальной структурой митохондрий в нативной сперме составляют в среднем 89.3 ± 2.0%, при криоконсервации их количество уменьшается на 6.5%. При криоконсервации количество сперматозоидов с нормальными аксонемами снижается незначительно, в среднем на 4.4% и составляет 81.4 ± 2.0%. Некоторые из доступных методов вспомогательных репродуктивных технологий необходимо будет дополнительно оптимизировать у домашних лошадей, прежде чем их можно будет применять к лошади Пржевальского *Equus ferus przewalskii*.

Ключевые слова: *Equus ferus caballus*, акросомы, митохондрии, прогрессивная подвижность, ультраструктура, эквиды, электронная микроскопия