

MORPHOLOGICAL AND MORPHOMETRIC CHARACTERISTICS OF FRESH AND CRYOPRESERVED SPERMATOZOA OF SAANEN BUCKS

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Background. Cryopreservation of spermatozoa with the best morphofunctional characteristics may allow their use for assisted reproductive technologies in goat breeding throughout the year to effectively increase livestock. **Aim.** To evaluate the influence of seasons on morphological and morphometric characteristics of fresh and cryopreserved goat sperm. **Methods.** The ejaculate samples of three sexually mature bucks of the Saanen breed were obtained during the breeding and non-breeding seasons. To assess the morphological characteristics, the smears of sperm were stained and then visualized under a light microscope with a magnification of $\times 1,000$. For morphometric measurements, sperm micrographs were taken and analyzed using the ImageJ software. The cryopreservation of sperm was performed in a medium with 10 % glycerol and 20 % egg yolk. The samples were thawed in a water bath, then the morphological and morphometric characteristics of the cells were evaluated. **Results.** After the cryopreservation, the number of sperm with abnormal morphological structures was significantly higher compared to fresh sperm, both in the breeding and non-breeding seasons ($p \leq 0.05$). After the cryopreservation, the length and width of the head decreased significantly ($p \leq 0.05$) compared to the corresponding sperm sizes before the cryopreservation. Analyzing the morphological characteristics of the tail part, we revealed the fact of its reduction after the cryopreservation, probably due to twisting, loop formation, and detachment of its part. **Conclusions.** It was found that in the non-breeding season, the number of spermatozoa with damaged morphological structures increases, compared to the breeding season, which must be taken into account when using reproductive technologies. The morphological and morphometric characteristics of Saanen buck spermatozoa change after the cryopreservation, regardless of the season, which indicates the need to choose the fertilization tactics that will ensure the selection of spermatozoa without any morphological abnormalities.

Key words: cryopreservation, goat sperm, breeding season, morphological characteristics, morphometric characteristics.

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INTRODUCTION

Goat breeding is currently developing both in Ukraine and globally. And though goat milk is only about 1.6 % (Pulina et al, 2018) of the entire milk production, the demand for goat milk products is increasing since it has a number of advantages as compared to cow milk (Sepe et al, 2019). This is a reason why the effective increase in the livestock of goats with the consideration of their milk productivity is required to meet the consumers' demand. Successful reproductive management requires

improvement of biotechnological methods of breeding, based on the assisted reproductive technologies (ART) (Amiridis et al, 2012), including the methods of cryopreservation of gametes and embryos (Kopeika et al, 2019). For instance, the cryopreservation of spermatozoa with the best morphofunctional characteristics may promote their use for the purposes of the ART throughout the entire year to achieve a faster increase in the livestock of valuable breeds.

The reproductive characteristics of bucks depend both on their genetically determined features and environmental factors (Redden et al, 2020). The condi-

tions of their management, including the feeding ratio (Zaragaza et al, 2009), grazing, and such climatic factors as the changes in humidity, temperature, and length of the photoperiod may impact spermatogenesis, changing the spermiologic characteristics of the ejaculate (Kunavongkrit et al, 2005). The conventional methods of forecasting ejaculate fertility are based on the assessment of such sperm parameters as the volume of the ejaculate, the concentration, and motility of spermatozoa (Rodríguez-Martínez, 2003). However, it is known that the morphological characteristics of spermatozoa are a relevant index of the decrease in the fertility of animals (Rodríguez-Martínez, 2017). It was shown in our previous works that the breeding season impacts both the quantitative and qualitative characteristics of the spermatozoa of Saanen bucks (concentration, motility, viability) (Bogdaniuk et al, 2022). In addition, it was found that cryopreservation increased the level of DNA fragmentation of spermatozoa in the non-breeding season considerably. Unfortunately, the methods of assessing the DNA state require expensive equipment and disposables, and the method is time-consuming. Thus, it is more urgent to search for more affordable methods of assessing the quality of spermatozoa, including the analysis of the morphological specificities of their structure, which would be used as an instrument for cryopreservation efficiency screening. The methods of spermatozoa cryopreservation are known to be suboptimal (Todorovic et al, 2022). It was proven regarding many species of animals that the spermatozoa survival rate after thawing depended on their initial morphofunctional characteristics (Dorado et al, 2010; Casas et al, 2009; Núñez-Martínez et al, 2007; Pavlovych et al, 2020). It is reasonable to determine whether the seasonal dynamics can also impact the distribution of the subpopulations of the morphological forms of spermatozoa. At present, there are scarce scientific publications about the seasonal fluctuations in the morphological and morphometric characteristics of spermatozoa and the impact of cryopreservation on these characteristics of goat spermatozoa, depending on the season. Thus, the aim of this study was to evaluate the influence of seasons on the morphological and morphometric characteristics of fresh and cryopreserved goat sperm.

MATERIALS AND METHODS

All the manipulations with the animals were done in compliance with the ethical norms according to the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific

Purposes (Strasbourg, 1986), the Law of Ukraine “On Protection of Animals from Cruelty” No. 1759-VI dated 15.12.2009 (Ukraine, 2006) and the decision of the Bioethics committee of the Institute for Problems of Cryobiology and Cryomedicine of the NAS of Ukraine, Minutes No. 1 dated 28.01.2021 (Kharkiv, 2021).

Three sexually mature bucks of the Saanen breed, kept at the “Tetiana 2011” farm, were chosen for the study (Cherevky village, Kyiv region, Ukraine). The ejaculate samples were obtained using an artificial vagina twice a month during the breeding season (September-December, $n = 24$) and non-breeding season (February-July, $n = 36$).

Once the ejaculate was obtained, its volume was determined with the subsequent calculation of the spermatozoa concentration and their motility in the Makler chamber (“Sefi Medical Instrument”, Israel) using the light microscope AmScope B120C (AmScope, USA). To assess the morphological characteristics of spermatozoa, the smears of ejaculate were stained using the Spermac Stain kit (“FeriPro”, Belgium) according to the manufacturer’s instructions and then visualized under a light microscope with a magnification of $\times 1,000$. One hundred spermatozoa from each ejaculate sample were analyzed, and the number of cells with abnormal head, tail, and flagellum structures was assessed.

For morphometric measurements, sperm micrographs were taken and analyzed using the ImageJ software (version 1.51j8, NIH, USA).

The cryopreservation of sperm was performed after the removal of the seminal fluid in the medium with HEPES buffer (WASH, “IVF Bioscience”, United Kingdom), 10 % glycerol, and 20 % egg yolk. The suspension of spermatozoa with the cryoprotectant was transferred to cryostraws of 0.25 ml (“Minitube”, Germany), equilibrated for 30 min at room temperature ($+20\text{ }^{\circ}\text{C}$), 2.5 h at $+5\text{ }^{\circ}\text{C}$, 15 min in the nitrogen vapor at a distance of 4 cm from the surface of liquid nitrogen and submerged into the liquid nitrogen. The samples were thawed in a water bath ($+37\text{ }^{\circ}\text{C}$) for 30 s. The cryoprotectant was removed by centrifuging the suspension of cells, isolating the supernatant, and depositing the cultivation medium on the precipitate. Then the motility and morphological characteristics of cells were evaluated.

The data were statistically processed in Graph Pad Prism (GraphPad Software, USA). The data were presented as mean values \pm standard deviation. The Fro-

sini test was used to check the data in terms of normal distribution. The Mann-Whitney U-test was used to compare two samplings; the difference was considered reliable if $p \leq 0.05$.

RESULTS

Our previous studies demonstrated no differences between the volumes of goat ejaculates during the breeding and non-breeding seasons (850.00 ± 373.05) and (727.78 ± 294.86) mcl, respectively) (Bogdaniuk et al, 2022). However, the concentration and motility of spermatozoa were significantly ($p \leq 0.05$) higher in the breeding season – (3.09 ± 0.69) $\cdot 10^9$ /ml and (70.43 ± 11.01) %, respectively, compared to the non-breeding season – (1.64 ± 0.87) $\cdot 10^9$ /ml and (52.22 ± 17.16) %. After the cryopreservation, the motility of spermatozoa was significantly ($p \leq 0.05$) decreased during both seasons, amounting to (56.57 ± 10.06) % in the breeding season and (37.22 ± 3.15) % – in the non-breeding season.

To determine the impact of seasonal dynamics on the distribution of subpopulations of the morphological spermatozoa forms, we analyzed the stained smears, where the following types of anomalies in the morphological characteristics of cells were found (Fig. 1), comparing with normal morphology (Fig. 2, a): the deformation of the heads of spermatozoa (Fig. 2, b), damages to the middle part (Fig. 2, c), damages to the tail (Fig. 2, d).

The damages to the spermatozoa heads were mainly found as macro- and microforms.

The prevailing types of damages to the acrosome were the presence of vacuolization in the acrosomal

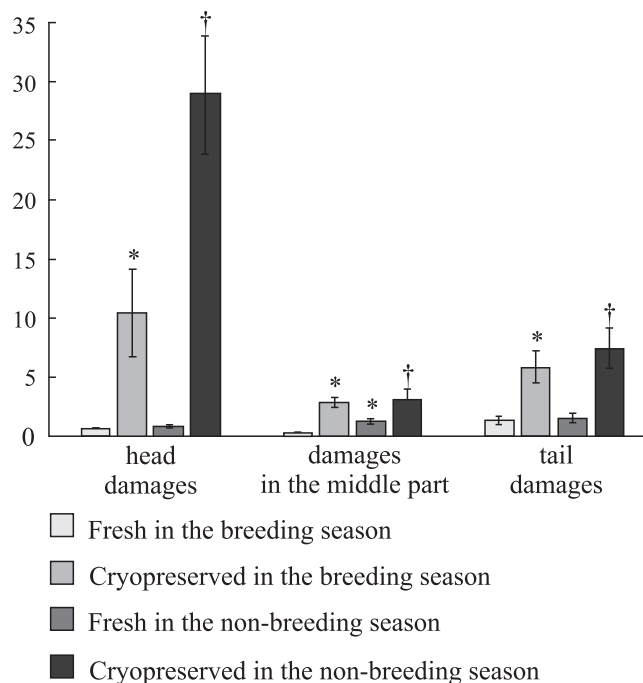


Fig. 1. The frequency of morphological anomalies in the fresh and cryopreserved spermatozoa of bucks in different seasons. Note: * – significant difference ($p \leq 0.05$) compared to the group of fresh spermatozoa, obtained in the breeding season, † – significant difference ($p \leq 0.05$) compared to the group of fresh spermatozoa, obtained in the non-breeding season

region and the detachment of acrosomal membranes (Fig. 2, c).

After the cryopreservation, the number of sperm with abnormal morphological structures was significantly higher compared to fresh sperm, both in the breeding and non-breeding seasons ($p \leq 0.05$).

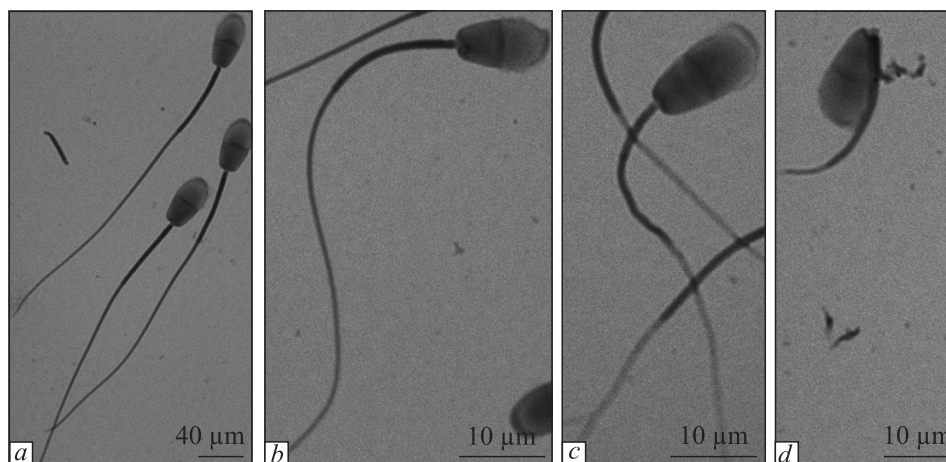


Fig. 2. The fresh spermatozoa, obtained in the non-breeding season from Saanen bucks: a – with normal morphological characteristics, b – with a deformed head, c – with the damage in the middle part and the detachment of the acrosomal membrane, d – with a reduced tail and the abnormal middle part

The morphometrical characteristics of fresh and cryopreserved spermatozoa, obtained in different periods of reproduction

Spermatozoa	Characteristics		
	Head length, μm	Head width, μm	Tail length, μm
Breeding season			
Fresh	8.33 ± 0.05	3.69 ± 0.03	40.23 ± 0.25
Cryopreserved	$8.20 \pm 0.04^*$	3.63 ± 0.04	$39.5 \pm 0.20^*$
Non-breeding season			
Fresh	8.31 ± 0.06	3.65 ± 0.05	40.25 ± 0.21
Cryopreserved	$8.15 \pm 0.08^\dagger$	$3.55 \pm 0.03^\dagger$	$38.7 \pm 0.20^\dagger$

Note. * – significant difference ($p \leq 0.05$) compared to the group of fresh spermatozoa, obtained in the breeding season, † – significant difference ($p \leq 0.05$) compared to the group of fresh spermatozoa, obtained in the non-breeding season.

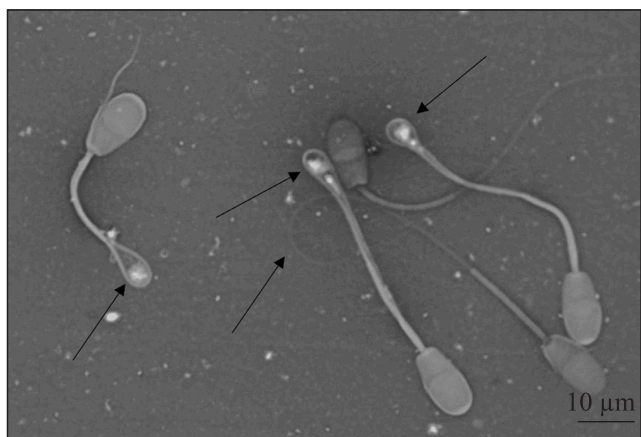


Fig. 3. The spermatozoa of Saanen bucks after the cryopreservation, the reduction of tails is observed due to their twisting. The arrows indicate the places of loop formation in the twisted tails of spermatozoa

In the non-breeding season, after the cryopreservation there was a considerably higher number of spermatozoa with head anomalies, compared to the breeding season ($p \leq 0.05$).

The analysis of spermatozoa morphometry results demonstrated some differences in the characteristics of spermatozoa, obtained in different periods of reproduction, after the impact of cryopreservation factors (**Table**).

After the cryopreservation, the length and width of the head decreased significantly ($p \leq 0.05$) compared to the corresponding sperm sizes before the cryopreservation. The indicated changes were observed during both the breeding and non-breeding seasons. Analyzing the morphological characteristics of the tail part, we revealed the fact of its reduction after the cryopreservation, probably due to twisting, loop formation, and detachment of its part (**Fig. 3**).

Regardless of the season, the tail length of obtained spermatozoa was significantly smaller after the cryopreservation compared to the fresh cells ($p \leq 0.05$).

DISCUSSION

We determined the impact of seasons on the morphological characteristics of spermatozoa; in particular, the percentage of morphologically abnormal spermatozoa increased in the non-breeding season. Similar data were obtained by the group of G.V. Aguiar (Aguiar et al, 2013), who found a higher number of spermatozoa with abnormal characteristics in the non-breeding season of Saanen bucks in Brazil. A similar tendency was also found in Nubian bucks (Skalet et al, 1988) and Murciano-Granadina bucks (Roca, 1992).

The season-induced changes in the morphological characteristics of spermatozoa may be related to heat shock. As the non-breeding season takes place in spring and summer, high environmental temperatures may have a negative impact on spermatogenesis (Thundathil et al, 2012) and induce the occurrence of a high number of spermatozoa with abnormal morphological characteristics. Infrared radiation may induce cellular death during which the reactive oxygen intermediaries are released, which may damage the structures of spermatozoa (González-Marín et al, 2012, Yurchuk et al, 2021) and cause their morphological anomalies.

We also evaluated the impact of cryopreservation on the morphological characteristics of spermatozoa during thawing. In the non-breeding season, the negative impact of cryopreservation was more vivid, which is evidenced by the presence of a higher number of spermatozoa with damaged morphological structures. It may be related to the fact that in the specified season, there is a decrease in the cholesterol concentration in the membranes of spermatozoa (Argov-Argaman et al,

2013), which leads to the lesser resistance of the membranes to temperature changes, and there is a decrease in the expression of proteins, responsible for DNA repair (lysine-specific demethylase 5D) (van Tilburg et al, 2015).

The morphometric analysis of spermatozoa demonstrated that the sizes of heads and tails decreased after the cryopreservation, compared to the indices prior to the cryopreservation. Similar results were obtained by the group of F. Marco-Jiménez (Marco-Jiménez et al, 2006) and M. Hidalgo (Hidalgo et al, 2007). The decrease in the length and width of the spermatozoa heads may occur due to the dehydration of cells during the cryopreservation and due to the damaged membrane of the acrosome or a complete loss of the acrosome.

Also, the morphometric parameters of the sperm head may reflect the structure and degree of chromatin condensation, which takes a larger part of the sperm head (Lange-Consiglio et al, 2010). It was found that sperm heads of small size have a higher index of DNA fragmentation (Kumaresan et al, 2020). It shows that the form and size of the sperm head are relevant criteria during the classification of germ cells from the standpoint of their fertilization ability (Itoi et al, 2022).

According to the data of many researchers, the sizes of spermatozoa tails of most mammals define the variation in the spermatozoa activity (Cardullo et al, 1991, Noorafshan et al, 2010, Magdanz et al, 2019). In their publications, most scientists come to the conclusion that spermatozoa with longer tails have better motility, high fertilization ability, and longer viability period (Yanagimachi, 2022). The reduction in the tail part of spermatozoa after the cryopreservation should be taken into consideration while selecting the technique of isolating the spermatozoa prior to fertilization (Holt et al, 2010), the method and time of fertilization since it may impact its efficiency (García-Vázquez et al, 2015).

A group of scientists, who conducted the studies in Portugal (Barbas et al, 2018), analyzed the ultrastructural characteristics of spermatozoa of Portuguese Serrana bucks and found the negative impact of cryopreservation on different organelles, including irreversible changes in the acrosome, neck, and tail parts, which confirms our results.

Our study promotes obtaining of new knowledge about the morphometric changes which take place in the spermatozoa structures in breeding and non-breeding seasons and under the action of cryopreservation. However, the consequences of changes in the spermological characteristics on the embryologic

characteristics and the frequency of live birth in goats are our intended objects of study in our further research.

CONCLUSIONS

It was determined that the morphological and morphometric characteristics of Saanen bucks change after the cryopreservation regardless of the season of their obtaining. In the non-breeding season, compared to the breeding season, there are much more damages to the morphological structures both in fresh and cryopreserved spermatozoa. The changes in the morphological and morphometric characteristics should be considered while planning the ART with cryopreserved sperm. It is important to choose the tactics of fertilization which will ensure the selection of spermatozoa without morphological anomalies.

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Морфологічні та морфометричні характеристики свіжеотриманих і кріоконсервованих сперматозоїдів цівів зааненської породи

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Актуальність. Кріоконсервування сперматозоїдів з найкращими морфофункціональними характеристиками дає можливість використовувати їх для допоміжних репродуктивних технологій у козівництві впродовж усього року для ефективного збільшення поголів'я. **Мета.** Оцінити вплив сезонності на морфологічні та морфометричні характеристики свіжеотриманих і кріоконсервованих сперматозоїдів цапів. **Методи.** Зразки еякулятів трьох статевозрілих цапів зааненської породи отримували протягом парувального та непарувального сезонів. Оцінку морфологічних характеристик сперматозоїдів здійснювали після забарвлення мазків та їх візуалізації під світловим мікроскопом зі збільшенням $\times 1000$. Для проведення морфометричних вимірів мікрофотографії сперматозоїдів аналізували за допомогою програми ImageJ. Кріоконсервування сперматозоїдів здійснювали у середовищі з 10%-вим гліцерином та 20%-вим яєчним жовтком. Відтаювали зразки на водній бані, після чого оцінювали морфологічні та морфометричні характеристики клітин. **Результати.** Після кріоконсервування кількість сперматозоїдів з аномальними морфологічними характеристиками значущо збільшувалась, порівняно зі свіжеотриманими, як в парувальний, так і в непарувальний сезони ($p \leq 0,05$). Після кріоконсервування довжина та ширина голівки значущо ($p \leq 0,05$) зменшувалися порівняно з відповідними розмірами сперматозоїдів перед кріоконсервуванням. При аналізі морфологічних характеристик хвостової частини нами було виявлено факт його скорочення після кріоконсервування, імовірно, внаслідок закручування, формування петель і відриву його частини. **Висновки.** Встановлено, що в непарувальний сезон збільшується кількість сперматозоїдів з пошкодженням морфологічних структур, порівняно з парувальним сезоном, що потрібно враховувати при використанні репродуктивних технологій. Морфологічні та морфометричні характеристики сперматозоїдів цапів зааненської породи змінюються після кріоконсервування, незалежно від сезону отримання, що свідчить про необхідність обирати таку тактику запліднення, яка забезпечить відбір сперматозоїдів без морфологічних аномалій.

Ключові слова: кріоконсервування, сперматозоїди цапів, парувальний сезон, морфологічні характеристики, морфометричні характеристики.

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