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STUDY OF THE EFFECTS OF LOW TEMPERATURES ON THE MORPHOLOGICAL STATUS OF RAM SPERMATOZOA

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Abstract: The aim of the study was to evaluate how the process of cryopreservation influenced the morphological status of ram sperm. The experiment was carried out with 26 sexually matured, pure bred rams during their breeding campaign. The animals were divided in two groups according to the breed – 15 rams from Synthetic Population of Bulgarian Milk (SPBM) and 11 rams from Sofia breed (SB). The sperm morphology was evaluated under microscope (Nikon Eclipse E200, Japan) by staining with SpermBlue (Microptic, Spain), performed according to the manufacturer's protocol, and observed under bright field with magnification of ×40. Cryopreservation of semen straws was done by the method of Cassou (1964). After cryopreservation, the percentage of spermatozoa with normal morphology decreased and those with abnormal morphology increased. This difference was more pronounced at rams from SPBM compared to SB rams. After freezing, the percentage of spermatozoa with damage to the head at rams from both breeds increased. The percentage of the spermatozoa with damage in the midpiece and the tail was higher and significant ($P<0.001$) for the SPBM rams. The low temperatures of cryopreservation had a detrimental effect to the rams' sperm structures. This damaging effect was much stronger for the rams from SPBM breed compared to rams from Sofia breed.

Key words: ram, cryopreservation, spermatozoa, morphology status

Introduction

The ability to cryopreserve spermatozoa from all of the domestic species is challenging. Even though all of the cells must endure similar physical stresses

associated with the cryopreservation processes, sperm from the different species are very different in size, shape and lipid composition, all of which affect cryosurvival. Thus, when a cryopreservation protocol has been optimized for sperm of one species, it may not be ideal for sperm of other species (*Purdy, 2006*). Sperm preservation protocols vary among animal species owing to their inherent particularities that change extenders used for refrigeration and freezing. In small ruminants, individual variations in the quality of frozen semen have been observed, suggesting specific differences in sperm susceptibility to freezing methods, (*Barbas and Mascarenhas, 2009).*

During the freeze-thawing process, mammalian sperm are exposed to temperature changes that lead to physical and chemical stress, changes in the plasma membrane lipid composition (*Pérez-Pé et al., 2002*) and externalization of phosphatidylserine residues (*Paasch et al., 2004*). These sperm alterations are dependent on the maturity of the cell (*Host et al., 1999*), the cryoprotectant used (*Soylu et al., 2007*), and the cooling and freeze-thawing rates (*Glander and Schaller, 1999*). Many aspects of sperm protection in the freezing-thawing process, e.g. sperm motility, viability and membrane stabilisation of the sperm cells during relative cryopreservation, are the key factors in determining the preservation of sperm function (*Uysal and Bucak, 2007*) The cryopreservation process acts as an apoptotic inducer in ram semen; some cryoprotectants allowed apoptosis to some extent, with negative effects on sperm morphology and DNA integrity (*Nur et al., 2010*). Semen cryopreservation had little or no effect on the susceptibility of ram sperm DNA to denature in situ when measured immediately at thawing or after 3 hours of incubation, but significant DNA damage appeared later in physiological conditions (*Peris et al. 2004).*

The aim of the study was to evaluate how the process of cryopreservation influenced the morphological status of ram spermatozoa.

Materials and Methods

The experiment was carried out with 26 sexually matured, pure bred rams during their breeding campaign. The animals were divided in two groups according to the breed – 15 rams of Synthetic Population of Bulgarian Milk (SPBM) breed and 11 rams of Sofia breed. The animals had normal sexual performance, aged between 2 – 4 years. Rams from SPBM were raised in experimetal base of Institute of Animal Science –Kostinbrod. They were fed with meadow hay ad libitum and concentrated mix (bought from forage company with 12% crude protein) in dose 700 g per ram per day. Rams from Sofia breed were raised in private farm, located in village Mirokovo, Sofia district. They were fed with meadow hay ad libitum and barley grain in dose 500 g. per ram per day. The ejaculates were obtained by the artificial vagina method.

The sperm morphology was evaluated under microscope (Nikon Eclipse E200, Japan) by staining with SpermBlue (Microptic, Spain), performed according to the manufacturer's protocol, and observed under bright field with magnification of ×40. The study was performed before freezing and after thawing of the ejaculates in a specialized laboratory of the Institute of Biology and Immunology of Reproduction.

The cryopreservation was performed at the Institute of Biology and Reproduction Immunology. Cryopreservation of semen straws was done by the method of *Cassou (1964).* After assessment, the ejaculates normozoospermia ware diluted with colloid diluent (6AG), containing cryoprotectant glycerine and then equilibrated. After equilibration the semen were divided in plastic straws (tubes, 133 mm long, 2 mm in diameter and volume of 0, 25 cm^3). The one end of the straw is closed with powder of polyvinyl alcohol, placed between two layers cotton-paper filter, and the other end was sealed. The cryopreservation of the straws was conducted on the vapor of nitrogen, and after that they are placed in containers with liquid nitrogen (-196°C) for long term preservation. Before their usage, the straws were thawed by pulling them off the container with liquid nitrogen and placing them directly in horizontal position on water bath, with temperature of + 34°C.

All data were calculated by statistical program IBM SPSS 19. The results of the studied parameters were compared with Paired T-test. The significance of the differences between groups was evaluated by t-criterion of Student. Findings were considered statistically significant if $P< 0.05$.

Results and Discussion

The results for normal and pathological spermatozoa were presented in Table 1. The data for the two tested breeds before freezing and after defrosting were compared for the percentage ratio between sperm with normal and abnormal morphology.

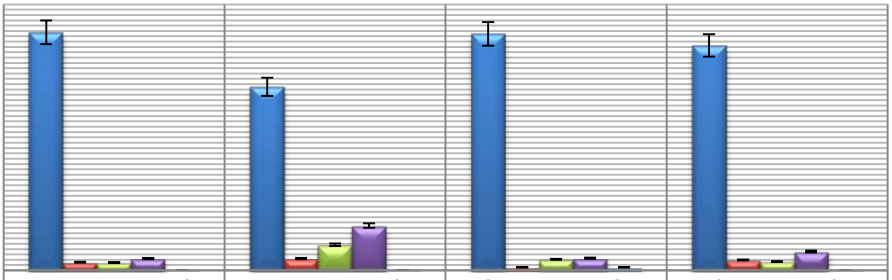
Table 1. Percentage ratio between sperm with normal and abnormal morphology

	Normal % Mean ± SE	Abnormal % Mean ± SE
SPBM breed before freezing	89,47 ± 1,47	10,53 ± 1,47 ***
SPBM breed after freezing	69,00 ± 2,45	31,00 ± 2,45 ***
Sofia breed before freezing	88,91 ± 1,36	11,09 ± 1,36 NS
Sofia breed after freezing	84,55 ± 1,90	15,45 ± 1,90 NS

Note. Significant differences *** at P<0,001, NS – non significant

The data in the table showed that, after cryopreservation, the percentage of spermatozoa with normal morphology decreased and those with abnormal increased. These differences were more pronounced and significant (P<0.001) for rams from SPBM breed and less pronounced and non-significant for rams from Sofia breed.

Percentage ratio between normal sperm and sperm with damage in head, midpiece and tail is shown in Figure 1. The data presented for both tested breeds are also before and after cryopreservation. Pictures of the morphological status of the spermatozoa before and after freezing were presented at Fig. 2 and Fig. 3 (for SPBM rams) and Fig.4 and Fig.5 (for SB rams).



	SPBM breed befor freezing / %	SPBM breed after freezing / %	Sofia breed before freezing / %	Sofia breed after freezing / %
Normal	89,47	69	88,91	84,55
Head	3,07	4,47	1,18	3,91
Midpiece	2,93	9,67	4,18	3,36
Tail	4,47	16,8	4,55	7,09
Cytoplasmic droplets	0,07	0,07	1,18	0,09

Figure 1. Percentage ratio of spermatozoa with damage in head, midpiece, tail, with the presence of cytoplasmic droplets and normal spermatozoa

The percentages of spermatozoa with damage to the head at rams from both breeds were increased after freezing, but the differences were not significant (Fig. 1). The percentage of affected spermatozoa with damage of the midpiece and tail was higher and significant (P<0.001) after cryopreservation at SPMB breed (2.93% vs. 9.67 for midpiece and 4.47 vs. 16.80 for tail). The same tendency was observed for SB rams, but the differences were not significant (Fig.1).

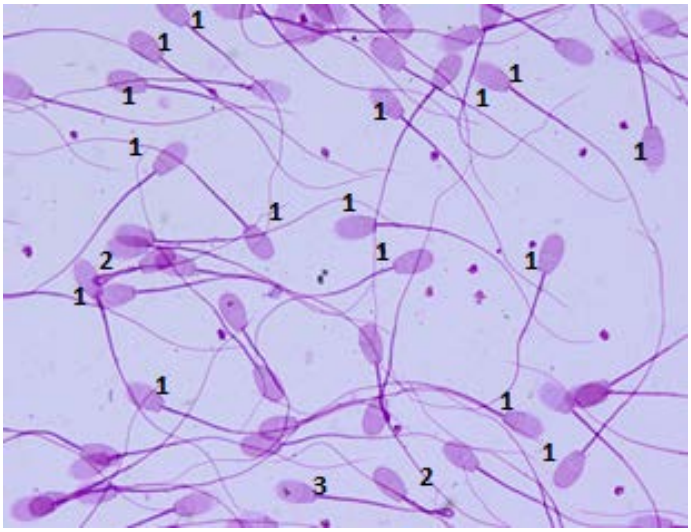


Figure 2. Morphological status of SPBM breed before freezing

1 .Normal spermatozoa
2 Damage in midpiece
3. Damage in tail

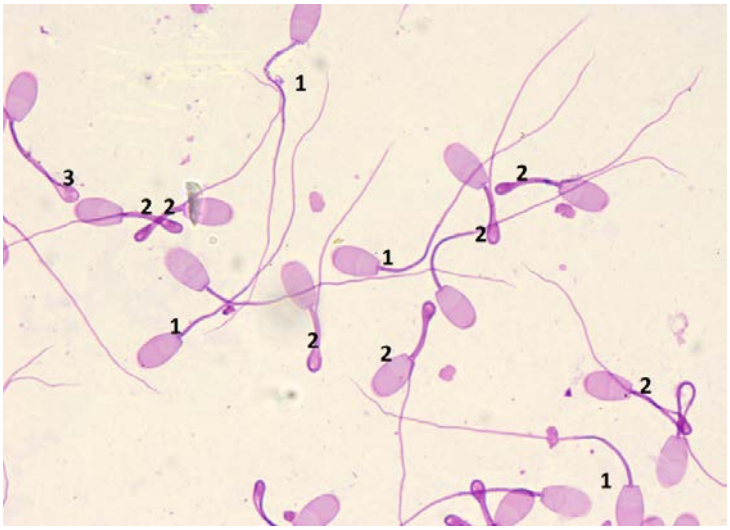


Figure 3. Morphological status of SPBM breed after freezing

1 .Normal spermatozoa
2 Damage in midpiece
3. Damage in tail

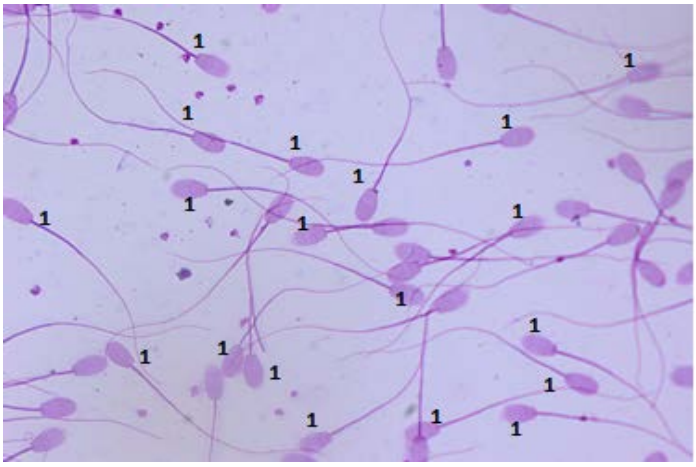


Figure 4. Morphological status of Sofia breed before freezing

1 .Normal spermatozoa
2 Damage in midpiece
3. Damage in tail

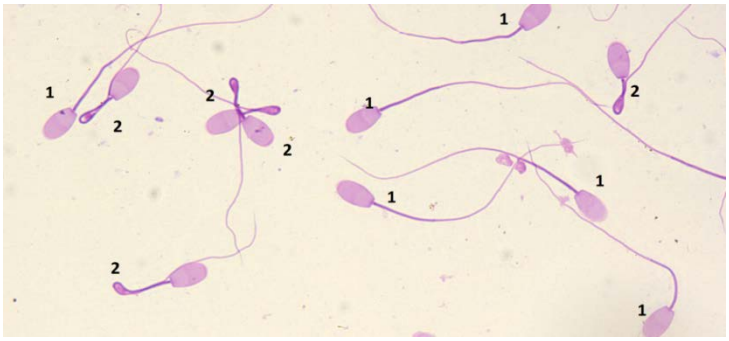


Figure 5. Morphological status of Sofia breed after freezing

1 .Normal spermatozoa
2 Damage in midpiece
3. Damage in tail

According to O'Connell (2002), who have conducted human sperm studies, freeze-thawing caused a 37% reduction in normal morphological forms of sperm. In our study, the reduction of normal sperm after cryopreservation was lower - 20% for SPBM rams and below 4% for SB rams (Table 1).

In other species of animals such as bulls it was found that, the percentage of spermatozoa with normal acrosomes remained higher after dilution, cooling, or equilibration (73.2% ± 2.4%) than after freezing and thawing (61.8% ± 2.4%; $P < .05$) (*Rasul et al., 2001*).

Kim et al. (2011) also found the damaging effect of low temperatures. In their study with semen from boar, they found that freezing–thawing may induce reductions in sperm function with increase in membrane damage.

Conclusion

The low temperatures of cryopreservation had a detrimental effect to the rams' sperm structures. This damaging effect was much stronger for the rams from SPBM breed compared to rams from Sofia breed.

Ispitivanje uticaja efekata niskih temperatura na morfološki status spermatozoida ovnova

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Rezime

Cilj rada bio je da se proceni kako je proces krioprezervacije uticao na morfološki status spermatozoida ovnova. Ogled je sproveden na 26 reproduktivno zrelih ovnova čiste rase tokom sezone parenja. Životinje su, na osnovu genotipa, podeljene u dve grupe: 15 ovnova iz Bugarske mlečne sintetičke populacije (Synthetic Population of Bulgarian Milk - SPBM) i 11 ovnova iz Sofijske rase (Sofia breed - SB). Morfologija spermatozoida je procenjena pod mikroskopom (Nikon Eclipse E200, Japan) bojenjem sa SpermBlue (Microptic, Španija), izvedenim prema protokolu proizvođača, i posmatrano pod svetlosnim mikroskopom sa uvećanjem od 40 puta. Krioprezervacija pajeta izvršena je metodom po Cassou (1964). Posle krioprezervacije, procenat spermatozoida sa normalnom morfologijom se smanjio, a onih sa abnormalnom se povećao. Ova razlika je bila izraženija kod ovnova iz SPBM u poređenju sa SB ovnima. Posle zamrzavanja, povećan je procenat spermatozoida sa oštećenjem glave kod ovnova obe rase. Procenat spermatozoida sa oštećenjem u sredini i repu bio je veći i značajniji ($P < 0,001$) za SPBM ovnove. Niske temperature krioprezervacije imale su štetan efekat na strukturu spermatozoida ovnova. Ovaj štetni efekat bio je mnogo jači za ovnove SPBM rase u odnosu na ovnove SB rase.

Ključne reči: ovnovi, krioprezervacija, spermatozoidi, morfološki status

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