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STUDY ON THE CRYOPRESERVED RAM SPERM CELL ULTRASTRUCTURE AFTER THE VARIATION OF THAWING TIME AND TEMPERATURE

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Abstract
The study aimed to determine the ultrastructural changes of ram spermatozoa after freezing-thawing at different rates (temperature, time) and the correlations between them and the cytological parameters. The thawing variants tested were: thawing at 90° C for 2 seconds, thawing at 75° C for 5 seconds, thawing at 75° C for 10 seconds, thawing at 50° C for 30 seconds, and thawing at 39° C for 120 seconds. The results of this research on the degree of damage to the plasma membrane after freezing-thawing at different rates show that the best thawing rates are obtained when the fine straws were thawed at 39° C and 50° C as compared with other thawing temperatures.
Key words: : ram, cryopreservation, sperm cell ultrastructure, T.E.M.

Introduction

Semen cryopreservation is a widely used method for artificial insemination because it facilitates the dissemination of valuable genetic material, even in small herds, leading to the increase of the improvement of the gene pool.

While the basic cryogenic damage can be morphological and often lead to dysfunction, physical stress suffered by the sperm membrane during the freezing process must be considered as the most limiting factor [8]. Even though sperm motility and morphology assessments can be used as a quick analysis of semen samples, these tests do not reveal morphological changes in nanometer-sized defects [3].

Different regions of the plasma membrane of sperm cells plays different roles in sperm cell function and survival. Structural components of the head, intermediate piece and flagellum respond differently to factors such as heat and osmotic shock during storage in a liquid or frozen state [7].

Using TEM (Transmission Electron Microscopy) as a complementary diagnostic for qualitative assessment of semen lead to more accurate determination of its fertilizing ability.

The study aimed to determine the ultrastructural changes of ram spermatozoa after freezing-thawing at different rates (temperature, time) and the correlations between them and the cytological parameters.

Materials and methods

Animals: sperm samples were collected from five adult Merinos de Palas rams with known fertility. Collection was made with an artificial vagina, 2 times per week. Sperm samples from each animal were analyzed separately in order to take into account the variability in the individual. For each male 1-2 ejaculates were collected (every 15-30 minutes), which were subsequently mixed and subjected to experiments. A total of 86 ejaculate were processed. Semen was cryopreserved in 0.25 ml fine straws.

The activity of freezing ram semen was performed according to the freezing technology developed in the Laboratory of Biotechnology of Reproduction, Institute of Research-Development for Sheep and Goats Breeding of Palas, Constanta [12], [13]. Experiments were conducted in the normal breeding season, during October 2012 - December 2012. Thawing and testing semen samples was performed in the Laboratory of Cell Biology, University Ovidius, from March to June 2013.

As dilution medium a diluent of Tris base 20% (v/v) egg yolk was used. The cryoprotectant used for freezing ram semen was glycerol (5% final concentration).

The following thawing variants were tested:

- Thawing at 90 ° C for 2 seconds
- Thawing at 75 ° C for 5 seconds
- Thawing at 75 ° C for 10 seconds
- Thawing at 50 ° C for 30 seconds
- Thawing at 39 ° C for 120 seconds

Sperm samples from the experimental variants were processed and analyzed in terms of ultrastructural view using transmission electron microscopy. The samples were processed in the usual way, namely prefixing the samples in a cacodylate buffer with 2.7% glutaraldehyde, fixing in osmic acid, dehydrating in serial alcohol baths and including in epoxy resins. The fine sections were double

stained with uranyl acetate and lead acetate, after which they were examined under a Philips 320M microscope.

Evaluation of sections

Sperm cells were assessed at all levels of the cell for plasma membrane integrity. Transversal and longitudinal sections at the level of the main part and the intermediate part of the flagella and sagittal sections through the sperm head were examined. Sections were categorized as having undamaged or damaged membranes. Also, the acrosome integrity was analyzed, which has been classified as undamaged or vesicular. The method consisted of counting at least 200 spermatozoa, serially sectioned in various planes of the sperm cells for each experimental variant [6], [13]. Characterization of whole cell was made after the appearance of the membrane on photographs taken on a small scale x 3500-6000.

Results

The results regarding the integrity of the plasma membrane at the flagella and sperm cell head level and the acrosome integrity are shown in Table 1.

Table 1. The integrity of the plasma membrane and the acrosome (%, mean ± SE, n = 10) after freeze-thaw

Variant	n	Plasma membrane		Acrosome
		Head	Flagella	
Thawing at 39 ° C for 120 seconds	10	41.59 ± 2.56^{a}	44.25 ± 3.12^{a}	46.99 ± 2.39^{a}
Thawing at 50 ° C for 30 seconds	10	46.21 ± 1.79^{a}	53.12 ± 2.72^{a}	60.02 ± 4.52^{a}
Thawing at 75 ° C for 10 seconds	10	24.66 ± 1.23^{b}	28.03 ± 1.85^{b}	27.86 ± 1.42^{b}
Thawing at 75 ° C for 5 seconds	10	29.04 ± 2.19^{c}	34.16 ± 1.62^{c}	37.92 ± 3.23^{c}
Thawing at 90 ° C for 2 seconds	10	12.66 ± 0.78^{d}	15.25 ± 1.65^{d}	17.23 ± 0.83^{d}

Sperm cell ultrastructure analysis results are in agreement with those obtained by optical microscopy analysis and flow cytometry. The best results regarding the

integrity of the plasma membrane, both at the head and the flagellum level were obtained for thawing at 50 °C for 30" and the worst results for thawing at 90° C for 2". There were no statistically significant differences between thawing at 39 º C and thawing at 50 º C, but there were statistically significant differences ($p < 0.05$) compared to the rest of the thawing variants.

1. Ultrastructure of frozen-thawed spermatozoa at 39 ° C for 120"

In general, all the head level sections shows normal membranes and a rate of 41.59% of the cell plasma shows non lysed membranes at the head level (figure 1). The acrosome has a intact structure in 42% of cells and the acrosome external membrane is generally unaffected. At the flagella level, 44.25% of the cells have integral plasma membrane.

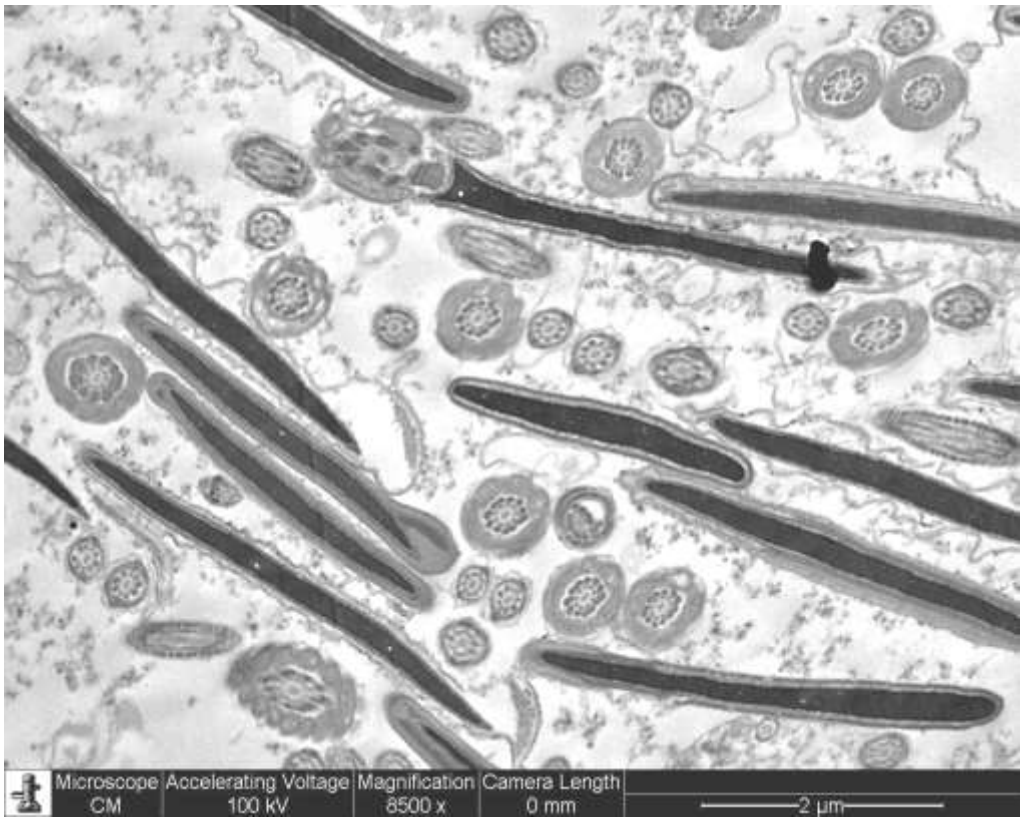

Fig. 1 (original). Sagittal section at the head level (x8500), acrosome contains an acrosome matrix with an electron-dense layout, in which the apical ridge is observed

2. Ultrastructure of frozen-thawed spermatozoa at 50 ° C for 30"

It is found that most of the membrane damage occurs at the head level, similar to the other experimental variants, although differences between the damage of the flagella are very small. Interrupted membranes occur in 46% of ram spermatozoa thawed at 50 ° C. At the intermediate piece and the main piece level damage occurs in 49% of cells (figure 2). The acrosome has a intact structure in 60% of the cells.

The increased motility observed implies the existence of a cytoskeleton and a motor system of the microtubule and intact mitochondria.

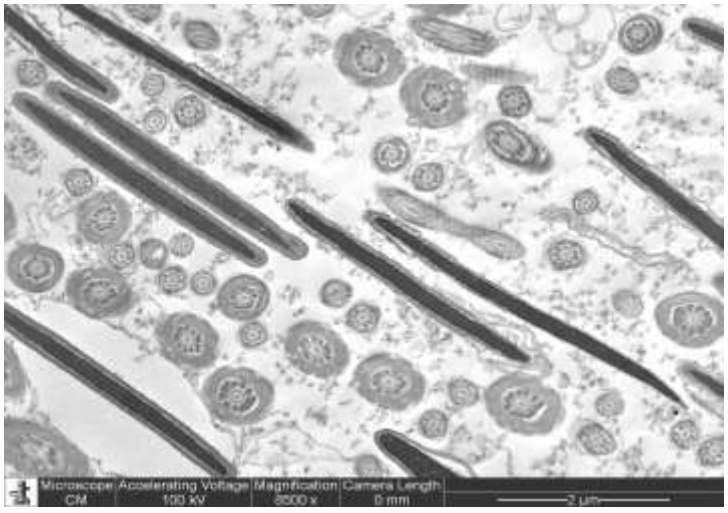

Fig. 1 (original). Ram sperm cells thawed at 50 O C (x8500)

3. Ultrastructure of frozen-thawed spermatozoa at 75 ° C for 5" and 10"

Thawing at 75 ° C, both for the period of 5 seconds and 10 seconds lead to significantly lower results compared to thawing at 39º C and 50º C. Better results were obtained thawing for 5 seconds.

At the flagella level the plasma membrane has a serrated look, is partially detached or completely broken (figure 3).

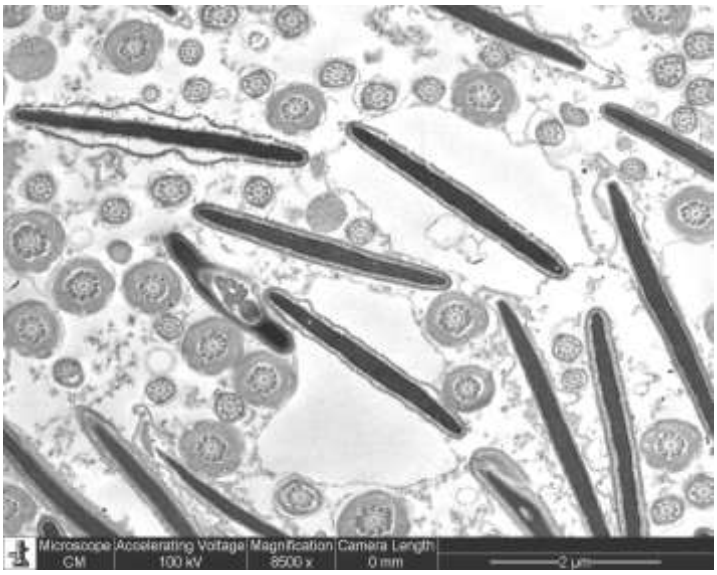

Fig. 3 (original). Ram sperm cells thawed at 75 O C for 5" (x8500)

4. Ultrastructure of frozen-thawed spermatozoa at 90° C for 2"

In the case of thawing at 90º C statistically significantly lower results were obtained compared to the rest of variants. Only 12.66% of the cells shows integral plasma membranes at the head level and 15.25% at the flagella level (figure 4). The acrosome has intact structure only in 17.23% of cells.

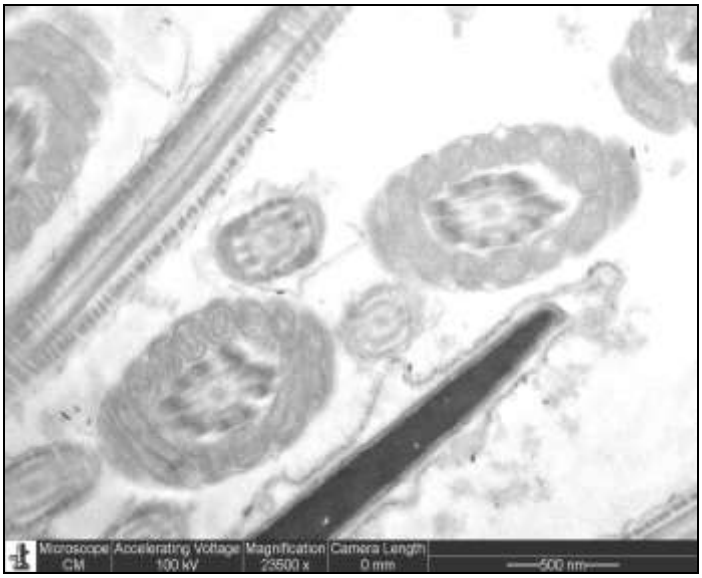

Fig. 4 (original). Ram sperm cells thawed at 90 O C for 2" (x23500)

DISCUSIONS

During the process of cryopreservation sperm cells suffers ultrastructural changes (plasma membrane, mitochondrial, acrosome), biochemical and functional [10]. Damage can occur at any stage of the process, but to a greater extent during cooling at 0° C and thawing and less during storage at -180 ° C. Structures and spermatozoa organelles respond differently in different phases to the osmotic or environment temperature changes. Ultrastructural damage are accompanied by biochemical changes or cell loss of vital content.

Plasma membrane integrity and mitochondrial function are the main attributes of a sperm cell to fertilize an egg. Damage to the plasma membrane and mitochondrial function may lead to membrane destabilization and impaired mitochondrial energy metabolism and cell viability [9], [11]. After freeze-thawing the alteration of mitochondrial filament was observed, also in a smaller proportion at the axonema, the filament and the fibril flagellation level.

The main target of the damage caused by cryopreservation is the spermatozoa plasma membrane. Due to variations in temperature and osmolarity, such as freeze-thaw induced alternations of the cell volume of water generating considerable mechanical stress on the cell membrane [5]. Since only sperm with intact membranes may be subject to capacitation and acrosome reaction, it is important to know the type and location of changes in order to optimize the technology of freezing [2].

In mammals, sperm cell membrane has a specific lipid content, distinct from that of other cells. It contains high levels of phospholipid, sterols, saturated and unsaturated fatty acids, plasminogen and sphingomyelins . This structure is responsible for the specific flow, flexibility and the ability of sperm cell operation. Polyunsaturated fatty acids have an important role in ensuring fluidity and in regulation of spermatogenesis [1]. Sperm plasma membrane has a heterogeneous structure in five specific areas: acrosome, equatorial segment, basal segment, intermediate and terminal piece. The differences between these regions are related to different physiological functions. Before and after ejaculation the plasma membrane suffers from some changes in the integration of lipid, modification of the degree of saturation of fatty acids and the loss of cholesterol from its composition resulting in a marked decrease in the cholesterol / phospholipid ratio. The various regions of the membranes differ in respect to this report.

Microscopic examination of ram sperm labeled with an membrane integrity indicator demonstrated that exposure to low temperature followed by

heating has a different effect on plasma membrane, especially at the head and intermediate piece level [6]. Ultrastructural analysis results show that the head plasma membranes are more affected than the flagella. While several cytoskeletal proteins were identified, their role in maintaining the integrity of the plasma membrane remains unclear [4]. Also, research shows that the acrosome is less affected, although the sperm plasma membrane that surrounds the sperm cell head presents major detachment, vacuolation and even interruptions. Similar results were obtained in studies [8] showing that the sperm plasma membrane surrounding the head is considerably more labile than the one at flagellum level and the outer acrosome membrane is more vulnerable than the internal one .

CONCLUSIONS

Freezing-thawing leads to alterations in the plasma membrane that include rupture, especially at the head level, and membrane detachment and vacuolation at the head and flagella level. Analysis of photomicrographs shows that the acrosome were only partially affected by the freezing-thawing process. Although many cells shows a bloated acrosome, the internal acrosome membrane is intact. Plasma membrane has several degenerative changes at the head level compared to the flagella level.

The results regarding the degree of damage to the plasma membrane after freezing-thawing at different rates show that the best rates are thawing at 39 ° C for 120" and thawing at 50° C for 30".

References

1. Alvarez JG, Storey BT, Evidence that membrane stress contributes more than lipid peroxidation to sublethal cryodamage in cryopreserved human sperm: glycerol and other polyols as sole cryoprotectant, *Journal of Andrology* 14(3):199-209, (1993)
2. Baril G, Chemineau P, Vallet JC, Manuel de formation pour l'insemination artificielle chez les ovins et les caprines, *FAO Animal Production and Health Paper*, (1993)
3. Crespilho, A.M., Landim-Alvarenga, F.C., Papa, F.O. Infertilidade associada a defeito microtubular dos espermatozóides de jumento (Equus asinus) avaliados por microscopia eletrônica de transmissão. Ciência Rural, v.36, p.1507-1510, (2006).

4. Gadella BM, Flesch FM, van Golde LM, Colenbrander B, Dynamics in the membrane organization of the mammalian sperm cell and functionality in fertilization, *The veterinary quarterly,* 21(4):142-146, (1999)

5. Hammerstedt RH, Graham JK, Nolan JP, Cryopreservation of mammalian sperm: what we ask them to survive, *Journal of Andrology*, 11(1):73–88, (1990)

6. Holt WV, North RD, Effects of Temperature and Restoration of Osmotic Equilibrium during Thawing on the Induction of Plasma Membrane Damage in Cryopreserved Ram Spermatozoa, *Biology Of Reproduction* , 51: 414-424 (1994)

7. Nagy, S.; Házas, G.; Bali-Papp, A.; Ivancsics, J.; Sas, J.R.F.; Kovács, A.; Foote, R.H. Evaluation of sperm tail membrana by light microscopy. Theriogenology, v.52, p.1153-1159, (1999).

8. Pesch S, Hoffmann B, Cryopreservation of Spermatozoa in Veterinary Medicine, *Journal für Reproduktionsmedizin und Endokrinologie*, 4 (2):101-105, (2007)

9. Zamfirescu S, Ciupina V, Nadolu D, Utilizarea testului hipoosmotic pentru evaluarea integrităţii funcţionale a membranei spermatozoizilor de berbec după congelare- decongelare. *Buletinul SNBC,* 29: 248, (2001)

10. Zamfirescu S, Caloianu M, 2000, Changes in structure of plasmalema in ram sperm cells upon cryotreatment, *Vol. Abstracte Primul Congres de Biologie Celulară şi Moleculară*, Iaşi, pag. 35, (2000)

11. Zamfirescu S, Nadolu D, Ciupină V, Coprean D, The biochemical and electronomicroscopic research of ram semen befor and after freezing various extenders, *National Congres of Biotehnology (Biotehnology present and outlook in the thirt millennium), Abstract of paper: pag.*23, (2003a)

12. Zamfirescu S, Şonea A, Biotehnologii de reproducţie la rumegatoarele mici, *Ed Ex Ponto, Constanţa,* (2004)

13. Zamfirescu S, Anghel A, Researches regarding the ultrastructural modifications of sperm cells before and after freezing in different media, *Lucrări ştiinţifice, Seria Zootehnie*, 53 (15):67-74,(2010)