“OVIDIUS” UNIVERSITY OF CONSTANTA

DOCTORAL SCHOOL OF APPLIED SCIENCES

PHD DOMAIN BIOLOGY

DOCTORAL THESIS SUMMARY

RESEARCHES CONCERNING THE STUDY OF SPERM CELLS MEMBRANES FUNCTIONS THAT ARE SUBJECTED TO DIFFERENT CRYOBIOLOGICAL TREATMENTS

Scientific Coordinator

Prof.univ.dr. **COPREAN DRAGOMIR**

PHD Student

DOBRIN NICOLAE

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CONTENTS

INTRODUCTION

Semen cryopreservation is a widely used method for artificial insemination because it facilitates dissemination of valuable genetic material even for small herds of livestock, leading to a higher quality genetic background. Frozen semen packed in different forms (straws, glass vials or pills) became the universally accepted method to store and transfer genetics for sheep and goats, a procedure that depends on the maintaining of the functional activity of sperm (viability and capability of fertilization). In the freezing process any biological activities are stopped until thawing (Jondet, 1972). Sperm cell thawing phase returns it at body temperature with the reactivation of its metabolism, so that thawing should be done with care to avoid damaging the sperm cell (Bearden et all, 2004). So far there have been numerous studies to determine the optimal temperature and duration of thawing, thereby increasing the interest in knowing the appropriate thawing speed that can give the highest percentage of viable sperm after the thawing process (Pace et al. 1981 ; Dham and Sahni, 1993). Bearden et all (2004) define the thawing process of semen stored in straws as beeing its thawing speed at a certain temperature in a certain amount of time. The interplay of various factors in the thawing procedures affect the sperm motility after thawing, such as the type of diluent, the concentration of glycerol, the semen packaging, the cooling speed, the handling of the semen during the cryopreservation procedure (Rodriguez et all, 1975) as well as the experimental conditions such as available facilities, equipment and reagents, ranging from one country to another or even from one area to another (Vishwanath and Shannon, 2000; Thibier and Wagner, 2002). Thus, methods of semen freezing and thawing should be examined according to each species and race (Hayashi and Isobe, 2005).

Even though cryogenic core damage can be morphologic and lead to cell dysfunction, the physical stress undergone by the sperm membrane during the freezing process must be considered as a limiting factor in achieving an acceptable fecundity (Pesch and Bergmann, 2006). Even if sperm motility and morphology assessments can be used as rapid methods of analysis of a sample of thawed semen, these tests do not reveal the morphological changes regarding defects of nanometer size (Crespilho et al., 2006). Different areas of the sperm cell plasma membrane plays different roles in its function and survival. The structural components of the head, intermediate part and tail respond differently to osmotic and heat shock during preservation by refrigeration or freezing

(Nagy et al. 1999). Using transmission electron microscopy (TEM) as a method for assessing the quality of semen, favors a complementary diagnosis of its precise fertilising capacity.

Cryopreservation changes the behavioral and functional ability of spermatozoa, leading to reduced motility and reduced ability to cross the cervix, as well as lowering their viability in the female reproductive tract (Salamon and Maxwell, 1995). These changes often lead to obtaining an unacceptably low level of fertility in sheep and goats inseminated with frozen semen (Gillan et al., 1999). Therefore, it is necessary to find new methods to improve the quality of the cryopreserved spermatozoa. Irradiation of the sperm cells using a Helium-Neon (He-Ne) laser is a new method through which the photo-stimulative effect of laser irradiation of different biological systems is demonstrated (Lubart and Breitbart, 2000). For example, in somatic cells the irradiation stimulates the release of the growth factor fibroblast (Yu et all, 1994) and accelerates their mitosis (Lubart et al., 1992a), regenerates the skeletal muscles and fix the bone tissue (Bibikov et al., 1994). The irradiation with the He-Ne laser may also improve the fertilizing potential of the spermatozoa by speeding up the Ca^{2+} transport through the sperm mitochondria membrane and through the plasma membrane of irradiated sperm cells (Lubart et all, 1992b, 1997; Breitbart et all, 1996; Quero Ocana et all, 1997; Cohen et all, 1998). In isolated mitochondria, He-Ne laser irradiation induces an increase in the electrochemical potential and additional ATP synthesis at mitochondria level producing more power and positive influence on sperm motility (Passarella et al., 1984). Other authors have also shown that the cell motility depends on increasing the availability of metabolic energy (Ruiz-Pesini et all, 2000; Baqués Corral et all, 2005, 2009). It was shown that laser irradiation improved the quality of frozen rabbit and turkey sperm (Iaffaldano et al., 2005; Iaffaldano et al., 2010).

The specialized literature provides little and sometimes conflicting data on how to improve the quality of ram and buck semen and especially all information are not much morphologically supported, knowing that any cell that has no cell membrane integrity is sentenced to death. In this context the research objectives of this thesis were set.

The research objectives were:

• determining the optimum thawing procedure, in order to know the proper thawing speed that can give the highest percentage of viable sperm after thawing;

• assessing the relationship between the thawing technique and the survival during incubation at 37 ° C of the thawed sperm cells;

• establishing ultrastructural changes after the freeze-thaw of ram and buck sperm at different thawing speeds (temperature, time) and the correlations between these and the cytological parameters;
• investigating the hypothesis that laser irradiation at different energy doses of radiation (3.96 and 6.12 J/cm^2) can improve ram and buck sperm and cryobiological indices after the freeze-thaw process;
• *In vivo* testing of thawed ram sperm after the quality improvement through rapid thawing and laser irradiation.

Keywords: sperm cell membrane, flow cytometry, TEM, He-Ne laser, mitochondrial function, motility, viability

The PhD thesis contains:
The general part which consists of two chapters totaling 28 pages and 4 figures.
The personal part which consists of three chapters totaling 111 pages, 336 references, 91 figures, 11 tables and four graphics.

Note: Tables and figures inserted in the summary of the doctoral thesis retain the original numbering from the thesis. The table of contents from the summary retains the structure and page number from the thesis.

Part I. STATE OF KNOWLEDGE

Chapter 1. PLASMA MEMBRANE

This chapter is divided into five subchapters which analyze the morphology and structure aspects of the plasma membrane, plasma membrane fluidity, sperm cell membrane structure, the dynamic organization of the sperm plasma membrane and sperm plasma cell membrane damage in the cryopreservation process.

Chapter 2. MODERN METHODS FOR ASSESSING THE INTEGRITY OF THE PLASMA MEMBRANE

This chapter is divided into two subchapters aimed to describe the modern methods of assessing the integrity of the plasma membrane, ie, flow cytometry and transmission electron microscopy (TEM).

PART II. PERSONAL RESEARCH

Chapter 1. MATERIALS AND METHODS

The scientific research was conducted in the Laboratory of Reproduction and Biotechnology at the Institute of Research-Development for Sheep and Goats Breeding of Palas, Constanta, and in the Laboratory of Cell Biology of the Faculty of Natural and Agricultural Sciences at the "Ovidius" University of Constanta under the guidance of Prof.univ.dr. Zamfirescu Stela and Prof.univ.dr. Coprean Dragomir.

In this chapter we described the main methods used in the experiments, divided into:

- **Optical methods of analysis**: assessment of sperm motility, structural integrity of the plasma membrane, viability (used exclusively for experiment 6) and the functional integrity of the plasma membrane;
- **Flow cytometric methods**: determination of the viability of the sperm cells by flow cytometry, the assessment of mitochondrial function by flow cytometry using rhodamine (R123);
- **Transmission electron microscopy methods**: patented morphological assessment method for ram semen;
- **Laser irradiation**: energizing the samples using a He-Ne laser;

- **Statistical analysis of experimental data**: using IBM SPSS software, version 17.

Chapter 2. RESULTS AND DISCUSSIONS

Experiment 1. Effect of thawing time and temperature variation on the quality of frozen-thawed ram semen

Objectives:

• determining the optimal thawing procedure, in order to know the proper thawing speed that can give the highest percentage of viable sperm after thawing the ram semen;

• assessing the relationship between this thawing technique and the survival, during incubation at 37 ° C, of the sperm cells after thawing.

Animals: sperm samples were collected from five adult Merinos de Palas rams with known fertility. A total of 86 ejaculate were processed. Semen was cryopreserved in 0.25 ml fine straws.

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

Results and discussions

The objective of the research was to experimentally determine the influence of some different thawing factors (time and temperature) on the cytological parameters of the sperm cell after thawing in order to establish an optimal variant that will affect as little as possible the sperm cell during this process.

In this regard, the cryopreserved semen was thawed in 5 variants and mitochondrial activity, cell viability (by flow cytometry), motility and the functional integrity of plasma membrane (HOST test) were analyzed. Also, viability was studied over a period of three hours after thawing.

It can be noticed that the best values of motility (table 2) were obtained by thawing the straws at 50° C for 30 seconds. Thawing at 39° C for 120 seconds led also to an increased motility, between the two variants there is no statistically significant differences. Increasing thaw temperature leads to lower motility. Both at 75 ° C, in the two types of

time and at 90° C there were significantly lower values compared to the first two variants of thawing ($p < 0.05$).

Table 2 Variation of sperm qualitative parameters thawed by different methods

Temperature and time of thawing	**N** (no. of samples)	**Semen characteristics**			
		Motility (%)	**Viability (%)**	**Mitochondrial activity (%)**	**HOST (%)**
Thawing at 39 ° C for 120 seconds	12	39 ± 2.08^{a}	45.61 ± 1.83^{a}	37.03 ± 1.66^{a}	38.97 ± 1.84^{a}
Thawing at 50 ° C for 30 seconds	12	45 ± 2.24^{a}	52.47 ± 2.25^{a}	40.66 ± 2.36^{a}	46.35 ± 2.55^{a}
Thawing at 75 ° C for 10 seconds	12	20 ± 1.83^{b}	27.16 ± 1.86^{b}	22.07 ± 2^{b}	19.3 ± 2.07^{b}
Thawing at 75 ° C for 5 seconds	12	30 ± 1.83^{c}	34.48 ± 1.82^{c}	28.52 ± 1.81^{b}	31.97 ± 1.41^{c}
Thawing at 90 ° C for 2 seconds	12	10 ± 1.29^{d}	15.34 ± 1.32^{d}	7.1 ± 1.37^{c}	11.36 ± 1.36^{d}

[a-d] Different letters in the same column indicate a statistically significant difference ($p<0.05$)

The percentage of viable spermatozoa (table 2) indicate that the thawing at 50 ° C for 30 seconds and at 39 ° C for 120 seconds leads to statistically significantly greater viability ($p < 0.05$) as compared to the other variants of thawing at 75 ° C and 90 ° C. Thawing at 75° C lead to significantly better results for 5 seconds compared with thawing for 10 seconds.

Similar results were obtained for the functional integrity of membranes (HOST test). The best results have been obtained for the temperatures of 50° C and 39° C, the values being significantly higher ($p < 0.05$) compared to the other variants.

Bi-parametric graphs (cell size / density) (figures 12, 13, 14, 15 and 16) show the presence of four subpopulations:

- subpopulation 1, marks dead spermatozoa, colored in red stained with Propidium Iodide;
- Subpopulation 2, marks viable sperm, colored in green stained with SYBR-14;

- Subpopulation 3, colored in blue, includes other particles and was not considered because there is no sperm population;

- Subpopulation 4, spermatozoa showing a double staining with both fluorochromes, appear purple and are considered "dying".

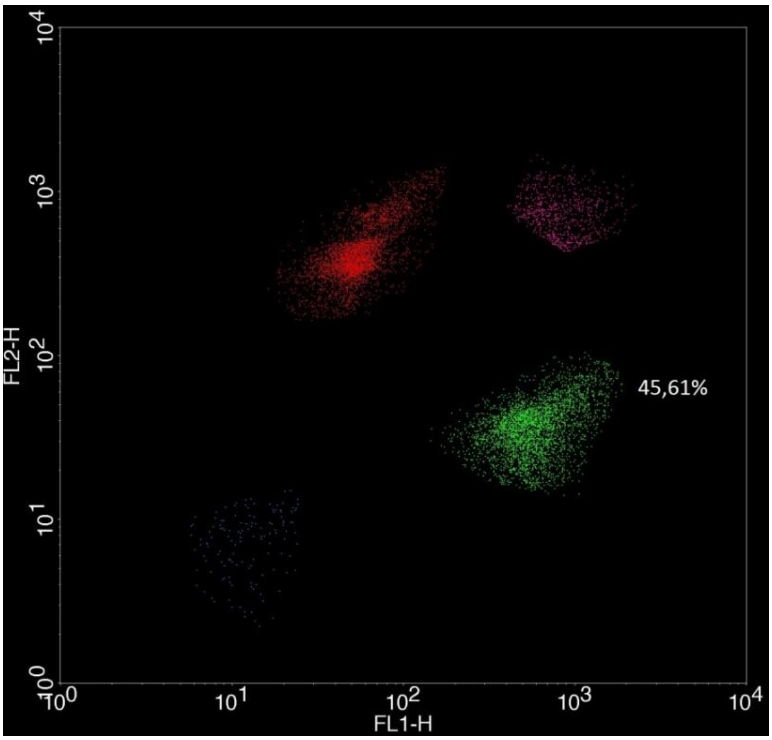


Figura 12 (originală). The comparative analysis by dot-plot graph technique for the simultaneous identification of cells, where each cell is represented by a point and a cloud by cells with appropriated characteristics, for the thawed sperm at 39^0 C for 120 seconds

In the case of mitochondrial activity study compared cytometric sperm assessments of the 5 experimental variations were analyzed. Bi-parametric graphs (figures 17, 18, 19, 20 and 21) shows the presence of four subpopulations: subpopulation of dead spermatozoa stained with propidium iodide; subpopulation 2, stained with Rhodamine 123 and representing the sperm with normal mitochondrial activity; subpopulation 3 with double staining by both fluorochromes; subpopulation 4, which includes other particles, which is not taken into account when interpreting the results.

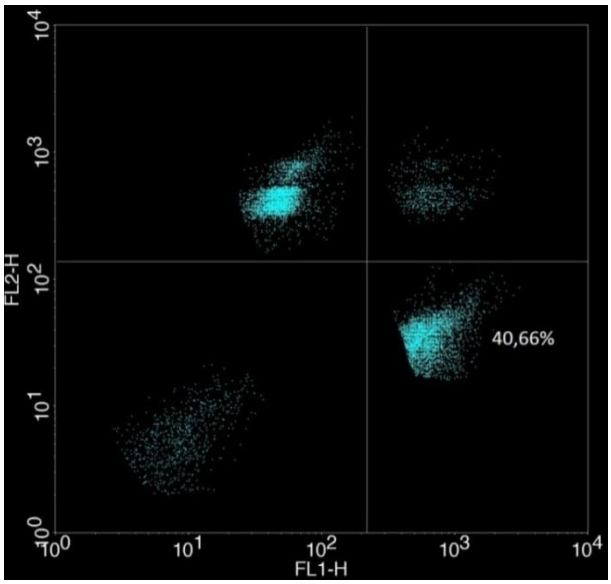


Figura 18 (originală). Comparative analysis by quadrant technique for simultaneous identification of cell with normal mitochondrial activity (lower right quadrant), death cells (square top left) and dying (double positive, upper right quadrant) and non-sperm population (left quadrant below) for the sperm thawed at 50^0 C for 30 seconds.

Regarding the time variation of semen viability incubated on water bath at 37 ° C there is a steady decline of about 6 -7 percent during the three hours of incubation for the thawing temperatures of 39° C and 50 C (Table 3). For other variants decrease is more pronounced, up to 10 percent per hour.

Table 3. Variation in time of semen viability incubated on a water bath at 37 ° C (%)

Temperature and time of thawing	**N** (no. of samples)	**The viability of the semen incubated at 37° C on a water bath (%)**		
		1 hour	2 hours	3 hours
Thawing at 39 ° C for 120 seconds	12	40.94 ± 1.8^a	33.04 ± 1.85^a	27.02 ± 1.94^a
Thawing at 50 ° C for 30 seconds	12	46.45 ± 2.65^a	41.73 ± 2.84^a	34.54 ± 2.91^a
Thawing at 75 ° C for 10 seconds	12	22.02 ± 1.94^b	11.95 ± 1.87^b	5.28 ± 1.78^b
Thawing at 75 ° C for 5 seconds	12	26.22 ± 1.37^c	17.14 ± 1.36^c	9.32 ± 1.3^c
Thawing at 90 ° C for 2 seconds	12	9.3 ± 1.39^d	2.07 ± 0.8^d	0^d

[a-d] Different letters in the same column indicate a statistically significant difference ($p<0.05$)

The results of this study showed that the best survival rate is provided by thawing at 39° C for 120 seconds and at 50° C for 30 seconds for a period of 3 hours of incubation on a water bath at 37 ° C

Conclusion

Using flow cytometry techniques lead to more accurate results due to the large number of cells analyzed.

Thawing the fine straws with ram semen at temperatures of 50° C or 39° C leads to significantly higher values of mitochondrial activity, viability, motility and plasma membrane functionality compared to other variants of thawing.

Experiment 2. Effect of thawing time and temperature on cryopreserved ram sperm cell ultrastructure

The objective of the research was to establish the ultrastructural changes after freeze-thaw of ram sperm at different thawing conditions (temperature, time) and correlations between these conditions and the cytological parameters.

Samples of frozen-thawed semen from the experimental variants were processed and ultrastructural analyzed using transmission electron microscopy. The samples were processed, prefixation in 2.7% glutaraldehyde cacodylate buffer, fixation in osmic acid, dehydration in a series of alcool baths and inclusion in epoxy resins. Finally, the fine sections were double stained with uranyl acetate and lead acetate, after which they were examined in a Philips 320M microscope.

Sections evaluation

Sperm cells were assessed at all levels for cell plasma membrane integrity. Cross and longitudinal sections were examined of the main and intermediate part of the flagella and sagittal sections through the head of the spermatozoa.

Results and discussions

The results of our research regarding the integrity of the plasma membrane at flagella and cell head level and the acrosome integrity are shown in table 4.

Table 4. Integrity of acrosome and the plasma membrane (%, mean±se, n=10), after thawing

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

[a-d] Different letters in the same column indicate a statistically significant difference ($p<0.05$)

1. Ram spermatozoa ultrastructure, frozen-thawed at 39º C for 120 seconds

Generally all sections at the head level presents normal membrane and a percentage of 41.59% of the integer cells present in the plasma membrane (Exhibit 1).

Exhibit 1

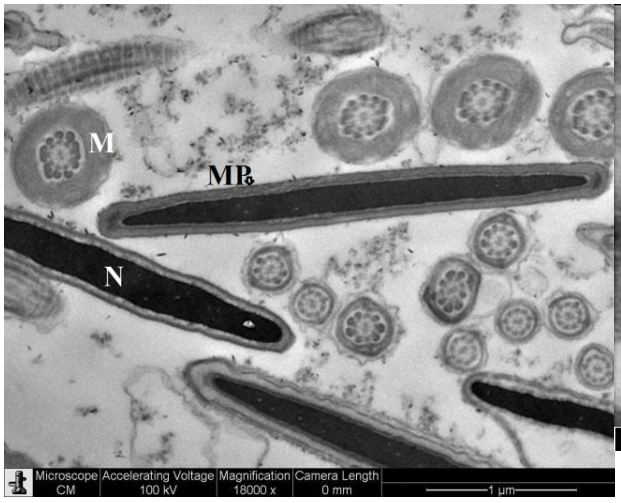


Figure 22 (original). Sagittal section of the head (x18000), acrosome comprises a matrix with a low density aspect, where the "perforator" is observed

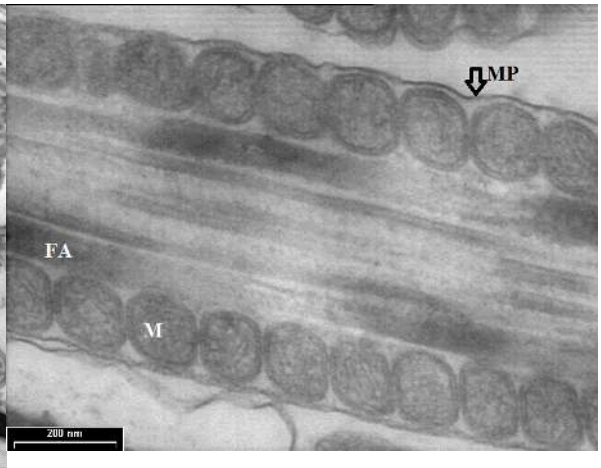


Figure 25 (original). Longitudinal section through the intermediate piece (x70000).

2. Ram spermatozoa ultrastructure, frozen-thawed at 50º C for 30 seconds

It is noted that most of the damage occurs throughout the membrane at the head level, as we found in the first experimental variant (thawing at 39° C for 120 seconds), although the differences from damage flagella are quite small or moderate. Fenestrated membranes appear in 46% of the sperm thawed at 50° C (Exhibit 2 and 3).

At the intermediate and main piece the damage occurred in 49% of cells. Intact acrosome structure is found in 60% of cells (Figure 28).

Exhibit 2 and Exhibit 3

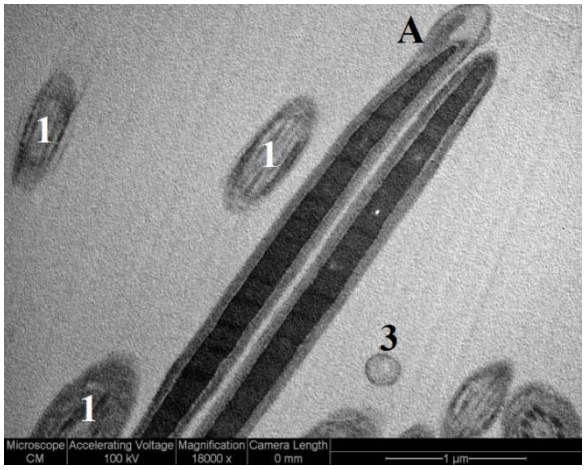


Figure 28 (original). Ram sperm cells, frozen - thawed at 50 °C (x18000). Intermediate piece (1), main piece (2) and terminal piece (3).

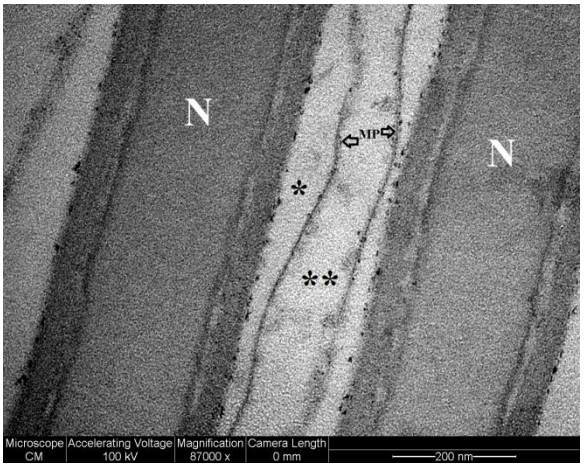


Figure 30 (original). Ultrastructure of bloated intact membranes (x87000). Nucleus (N), Terminal piece (PT), Tip tail (VC), Plasma membrane (MP), Inter-membrane space (*), Extracellular space (**).

3. Ram spermatozoa ultrastructure, frozen-thawed at 75º C for 5 and 10 seconds

Thawing at 75º C, both for the period of 5 and 10 seconds lead to results significantly lower than thawing at 39ºC respectively at 50° C. Better results were obtained when thawing for 5 seconds (Exhibit 4).

At the flagella level, the plasma membrane has a serrated appearance, is broken and partially or completely detached (Exhibit 4).

Exhibit 4

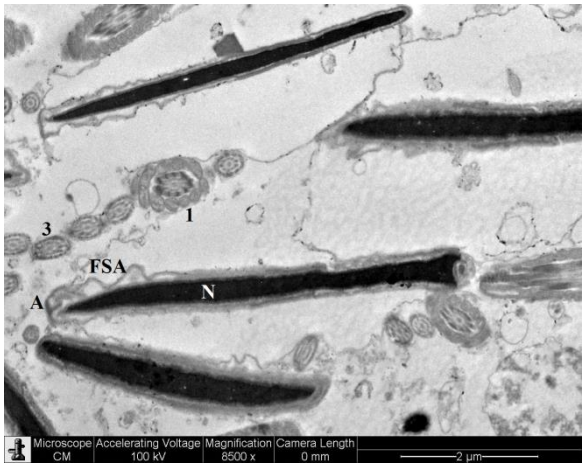


Figure 33 (original). Ram sperm cells, thawed at 75 ° C for 5 seconds (x8500). Intermediate piece (1) and terminal piece (3).

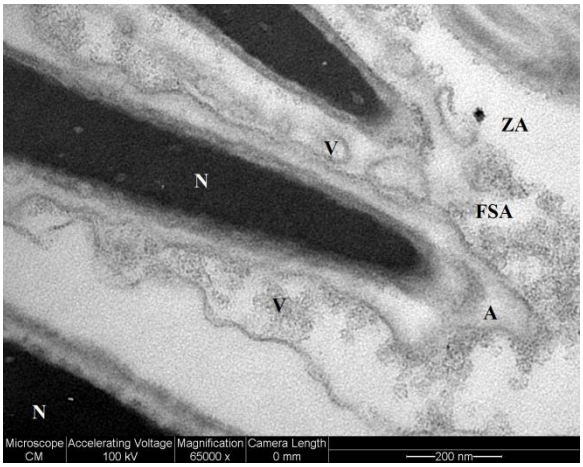


Figure 36 (original). Longitudinal section through the head of ram sperm cell in the acrosome area (x65000). Vesicles (V), white areas (ZA).

4. Ram spermatozoa ultrastructure, frozen-thawed at 90º C for 2 seconds

In the case of thawing at 90 ° C, statistically significantly lower results were obtained than the rest of variants. Only 12.66% of the cells have integer plasma membrane at the head level and 15.25% at the flagella level (Exhibit 5, figures 37-42). Acrosome is structurally intact only in 17.23% of cells.

Exhibit 5

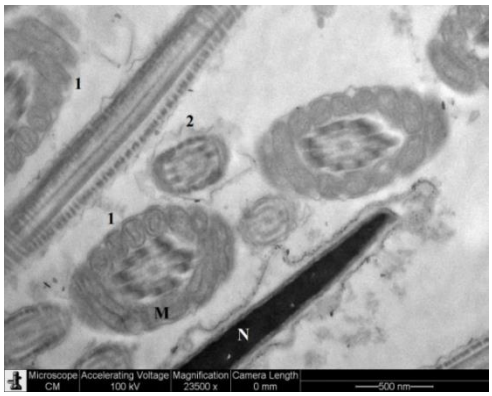


Figure 37 (original). Ram sperm cells thawed at 90° C for 2 seconds (x23500). Intermediate piece (1) and main piece (2).

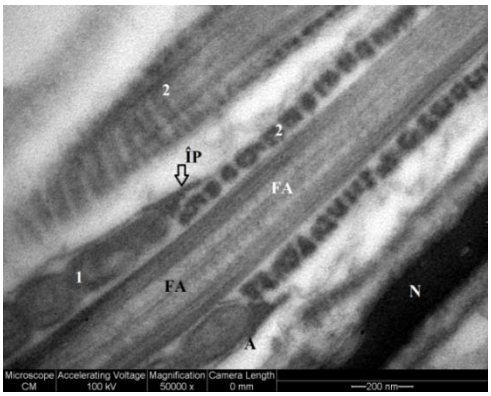


Figure 42 (original). Section through the flagellum at Jensen's ring level (x50000). Intermediate piece (1) and main piece (2). Protein coating (ip), axial filament (FA).

Conclusion

Freezing-thawing leads to alterations in plasma membrane which include tearing, especially at the head level, and membrane detachment or vacuolization at the head and flagella level. Photomicrographs analysis shows that the acrosomes were only partially affected by the freeze-thaw 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 flagellum.

The results regarding the degree of damage on the plasma membrane after freezing-thawing in various conditions show that the best rates are thawing at 39º C for 120 seconds and thawing at 50 ° C for 30 seconds.

Experiment 3. Effect of thawing time and temperature variation on the quality of frozen-thawed buck semen

The objective of this study is determining the optimal thawing procedure, in order to know the proper thawing speed that can give the highest percentage of viable sperm after thawing the buck semen and assessing the relationship between this thawing technique and the survival, during incubation at 37 ° C, of the sperm cells after thawing.

Animals: sperm samples were collected from five adult Saanen bucks with known fertility. A total of 78 ejaculate were processed. Semen was cryopreserved in 0.25 ml fine straws.

The following thawing variants were tested:

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

Results

The objective of the research was to experimentally determine the influence of some different thawing factors (time and temperature) on the cytological parameters of the sperm cell after thawing in order to establish an optimal variant that will affect as little as possible the sperm cell during this process.

Table 5. Variaţia parametrilor calitativi ai materialului seminal decongelat prin diferite metode

Temperature and time of thawing	N (no. of samples)	Semen characteristics			
		Motility (%)	Viability (%)	Mitochondrial activity (%)	HOST (%)
Thawing at 39 ° C for 120 seconds	12	37 ± 1.12[a]	43.29 ± 1.23[a]	35.16 ± 1.66[a]	36.25 ± 1.47[a]
Thawing at 50 ° C for 30 seconds	12	41 ± 2.18[a]	50.69 ± 2.38[a]	40.02 ± 2.17[a]	42.61 ± 3.17[a]
Thawing at 75 ° C for 10 seconds	12	19 ± 1.33[b]	23.33± 1.32[b]	21.29 ± 2,31[b]	21.36 ± 2.22[b]
Thawing at 75 ° C for 5 seconds	12	28 ± 1.46[c]	31.55 ± 1.53[c]	26.34 ± 1.57[b]	30.23 ± 1.79[c]
Thawing at 90 ° C for 2 seconds	12	12 ± 1.67[d]	14.75± 1.26[d]	8.22 ± 1.29[c]	11.21 ± 1.28[d]

[a-d] Different letters in the same column indicate a statistically significant difference ($p<0.05$)

In this regard, the cryopreserved semen was thawed in 5 variants and mitochondrial activity, cell viability (by flow cytometry), motility and the functional integrity of plasma membrane (HOST test) were analyzed. Also, viability was studied over a period of three hours after thawing.

Motility: table 5 presents the values of sperm motility. It is noted that the best values were obtained by thawing the straws at 50° C for 30 seconds. Thawing at 39° C for 120 seconds led also to an increased motility, between the two variants there is no statistically significant differences. Increasing temperature leads to lower thaw motility. Both at 75° C, for 5 and 10 seconds, and at 90° C there were significantly lower values compared to the first two variants of thawing ($p <0.05$).

Viability: The percentage of viable spermatozoa (table 5) indicates that thawing at 50° C for 30 seconds and at 39° C for 120 seconds leads to significantly greater viability ($p <0.05$) as compared to the case of thawing at 75 ° C and 90 ° C.

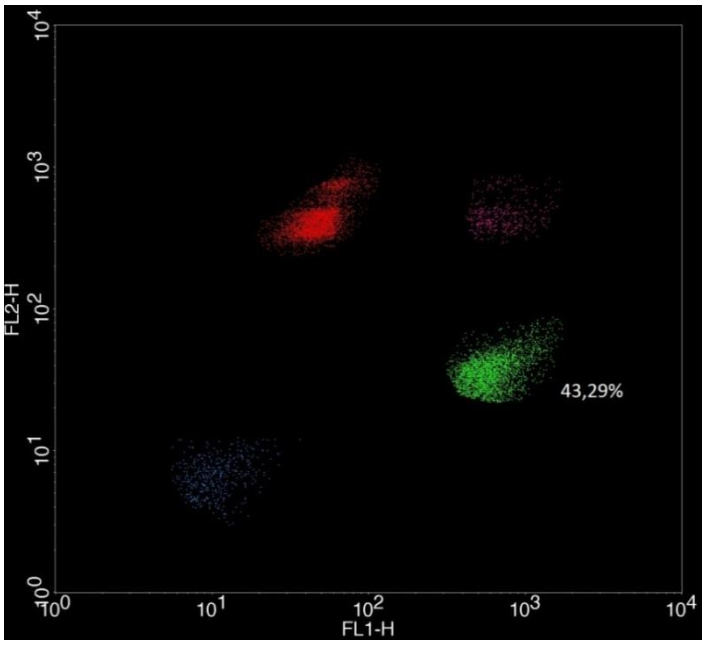


Figure 43 (original). Comparative analysis by dot-plot graphic technique for simultaneously identification of viable cells (stained green), death (colored red) and dying (double positive, colored purple) and non-sperm population (colored blue) for spermatozoa thawed at 39^0 C for 120 seconds

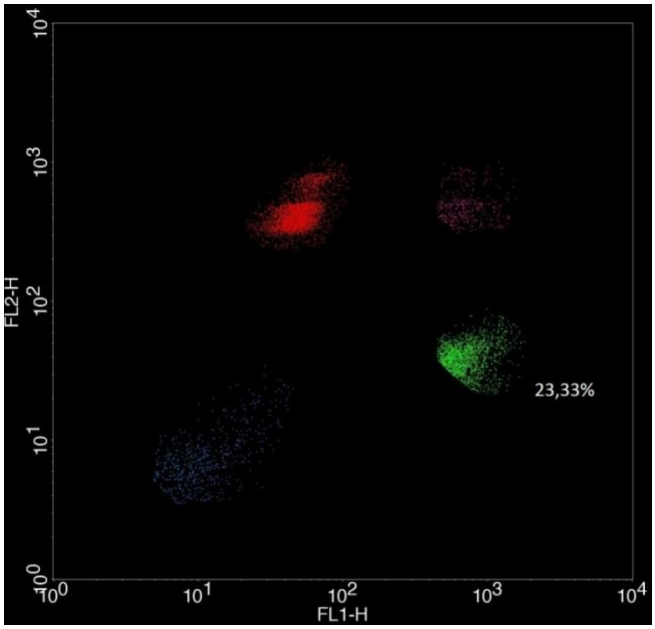


Figure 45 (original). Comparative analysis by dot-plot graphic technique for frozen-thawed spermatozoa at 75^0 C for 10 seconds

Similar results were obtained for the functional integrity of the membranes (table 5) and for the assessment of mitochondrial activity test (table 5). The best results have been obtained for the temperatures of 50° C or 39° C, the values being significantly higher ($p < 0.05$) compared to the other variants.

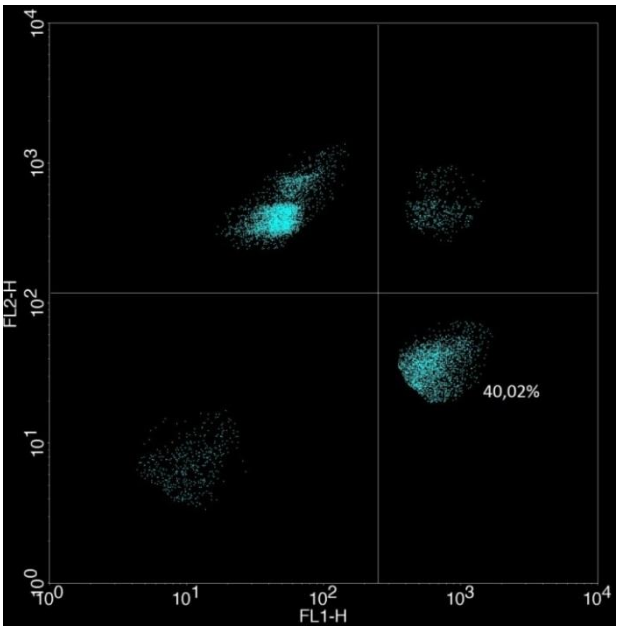


Figure 49 (original). Comparative analysis by quadrant technique for simultaneous identification of cell with normal mitochondrial activity (lower right quadrant), death cells (square top left) and dying (double positive, upper right quadrant) and non-sperm population (left quadrant below) for the sperm thawed at 50^0 C for 30 seconds.

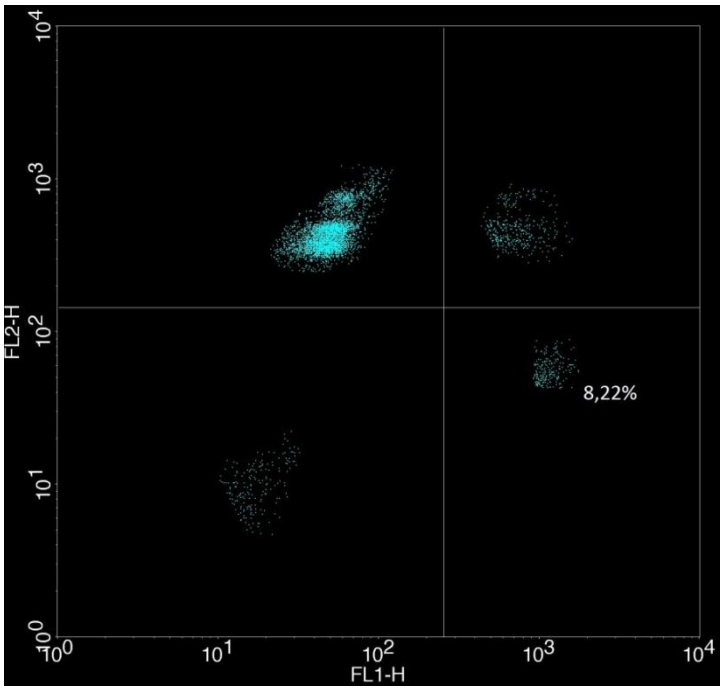


Figure 52 (original). Comparative analysis by quadrant technique for the sperm thawed at at 90^0 C for 2 seconds

The viability of the semen incubated at 37° C (%) on a water bath recorded a steady decline of around 7-9 percent during the three hours of incubation for the thawing temperatures of 39° C and 50° C. For other variants decrease is more pronounced, up to 20-30 percent per hour.

Tabel 6. Variation in time of semen viability incubated on a water bath at 37 ° C (%)

Temperature and time of thawing	The viability of the semen incubated at 37° C on a water bath (%)		
	1 hour	2 hours	3 hours
Thawing at 39 ° C for 120 seconds	40.69 ± 1.79[a]	37.84 ± 2.31[a]	34.81 ± 1.72[a]
Thawing at 50 ° C for 30 seconds	47.64 ± 2.37[a]	43.82 ± 2.95[a]	39.44 ± 2.19[a]
Thawing at 75 ° C for 10 seconds	21 ± 1.73[b]	16.8 ± 1.67[b]	10.08 ± 1.59[b]
Thawing at 75 ° C for 5 seconds	25.24 ± 1.28[c]	15.15 ± 1.43[c]	7.58 ± 1.41[c]
Thawing at 90 ° C for 2 seconds	10.32 ± 1.48[d]	3.61 ± 0.54[d]	0,36 ±0,17[d]

[a-d] Different letters in the same column indicate a statistically significant difference ($p<0.05$)

Discusions

The results of this study showed that the structural and functional integrity of the plasma membrane is significantly increased when the straws were thawed at 39° C and 50° C as compared with other thawing temperature. The better survival rate is obtained by thawing at 39° C for 120 seconds and at 50° C for 30 seconds for a period of 3 hours of incubation on a water bath at 37 ° C.

Conclusion

Using flow cytometry techniques lead to more accurate results due to the large number of cells analyzed.

Thawing the fine straws with buck semen at temperatures of 50° C or 39° C leads to significantly higher values of mitochondrial activity, viability, motility and plasma membrane functionality compared to other variants of thawing.

Experiment 4. Effect of thawing time and temperature on cryopreserved buck sperm cell ultrastructure

The objective of the research was to establish the ultrastructural changes after freeze-thaw of buck sperm at different thawing conditions (temperature, time) and correlations between these conditions and the cytological parameters.

Fine straws with buck sperm were thawed using the 5 variants described, the sperm was mixed and the resulting sample was processed for electronic microscopic examination using the same method that was applied for ram sperm. Electron-photomicrographs were obtained with a Philips 320 M electron microscope.

Results and discussions

Morphostructural integrity of thawed buck sperm cells showed an ultrastructural profile better than the ram, but the major structural changes are present at the acrosome level and the membranary system surrounding it, then at the entire head level, the flagellum being affected the least.

The least structural changes were found in sperm thawed at 50° C for 30 seconds, where at the entire head level, acrosome and flagellum, the percentage of normal structure is 52.13, 49.41 and respectively 58.29%. Conventional thawing at 39° C for 120 seconds displayed cells with ultrastructural profile which had the same trend but normal structures percentage values are lower by about 7, 6 and 8 percent. Percentage values of normal sperm cell structures thawed with the 3,4 and 5 variants are very small, being in the range of 15 to 27% at the head level, 11-20% at the acrosome level and 24-31% at the flagella level.

Tabel 7. Integrity of acrosome and the plasma membrane (%, mean±se, n=10), after thawing

Variants	n	Plasma membrane		Acrosome
		Head	Head	
Thawing at 39 ° C for 120 seconds	10	45.27 ± 2.83[a]	50.37 ± 3.23[a]	43.52 ± 2.23[a]
Thawing at 50 ° C for 30 seconds	10	52.13 ± 1.76[a]	58.29 ± 2.63[a]	49.41 ± 4.52[a]
Thawing at 75 ° C for 10 seconds	10	27.72 ± 1.35[b]	31.23 ± 1.97[b]	20.15 ± 1.17[b]
Thawing at 75 ° C for 5 seconds	10	23.05 ± 1.95[c]	35.12 ± 1.74[c]	18.13 ± 2.93[c]
Thawing at 90 ° C for 2 seconds	10	15.27 ± 0.59[d]	24.19 ± 1.47[d]	11.27 ± 0.67[d]

[a-d] Different letters in the same column indicate a statistically significant difference ($p<0.05$)

In the photomicrographs shown on exhibit 6-7 ultrastructural defects of buck sperm cell are observed, consisting of bloating more or less exaggerated of the external plasma membrane with fenestration of various sizes (figures 53, 54, 59, 60 , 63, 64), damage of the external plasma membrane of the acrosome, loss of acrosomal content (Figure 63), the presence of vesicles of different sizes around the head (Figure 61), "fluffy" membrane structures due to displacement of phospholipids or of dismantling of the transmembrane proteins (figures 55, 56, 57).

Exhibit 6

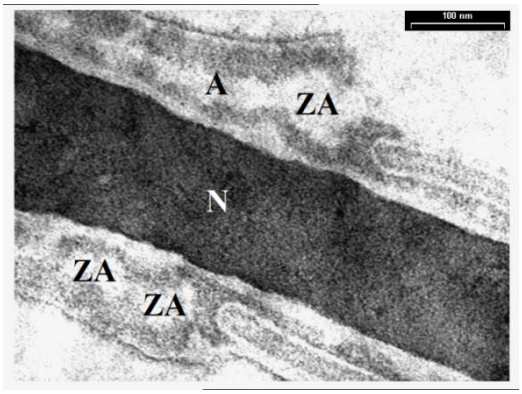


Figure 55 (original). Longitudinal section through the head in the equatorial zone (x50000). The distal portion of the acrosome destroyed, white areas (ZA).

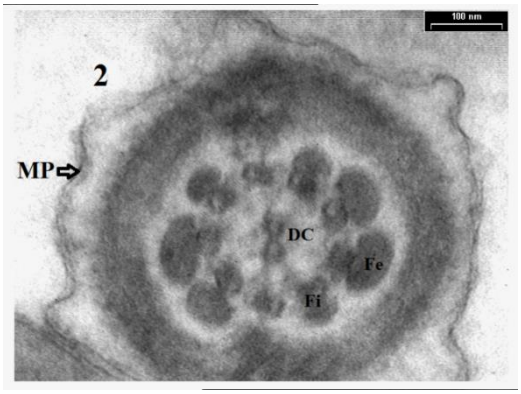


Figure 58 (original). Cross-section through main piece (x230000). MP and "fluffy" protein coating, main piece (2), central doublets (DC), external filaments (Fe), internal filament (Fi).

Exhibit 7

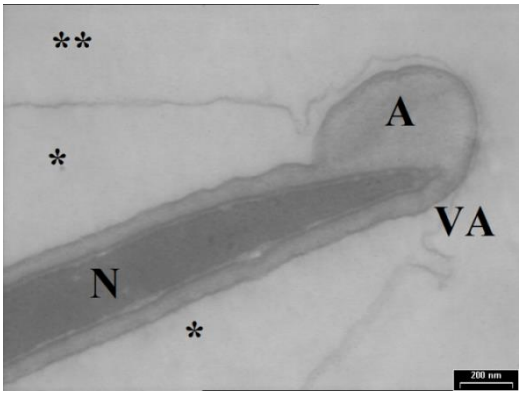


Figure 59 (original). Longitudinal section through the head (x50000). Excessively bloated MP detached from the acrosome, few fenestrations. Extracellular space (**), intracellular space (*), apical tip (VA).

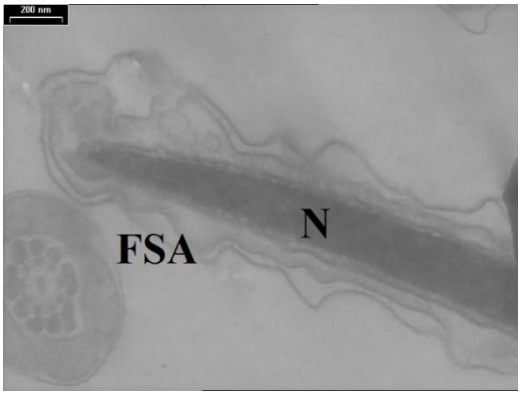


Figure 61 (original). Longitudinal section through the destroyed acrosome (x50000). Bloated MP without fenestration.

Ultrastructural changes of each thawing variant are shown in exhibits 8-12. General changes shown in exhibits 6-7 are present in all types of thawed buck sperm cell in the five

variants, but the frequency of serious injuries that cause cell death, are more common in the 3, 4 and 5 thawing variants.

Exhibit 8

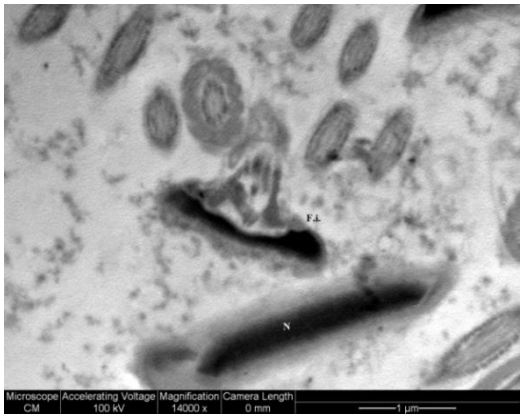


Figure 66 (original). Skew longitudinal section through the head of the sperm at the implantation fossa level of the flagella (Fi) (x14000).

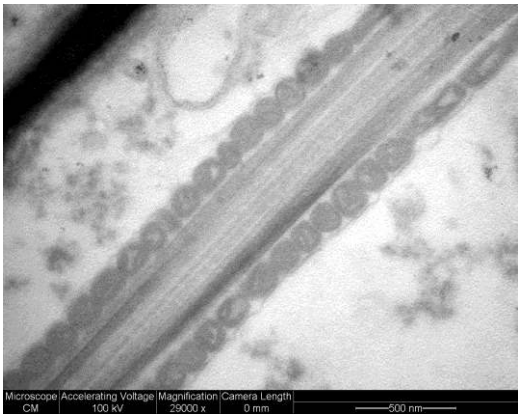


Figure 69 (original). Longitudinal section through the intermediate piece (x 29,000). Tightly adherent MP to the mitochondria (M).

Exhibit 9

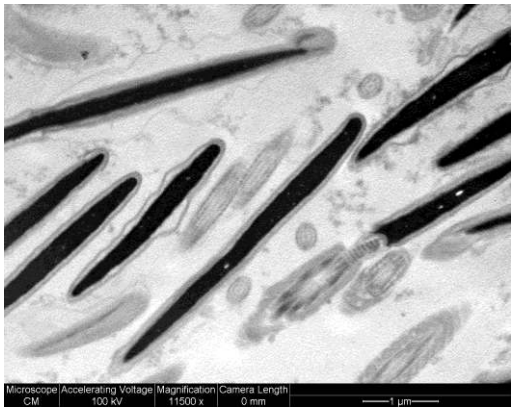


Figure 70 (original). Semen thawed at 50 ° C for 30 seconds (x 11500). Sectioned sperm cells in various fields through the head and flagella. Most cells are normal.

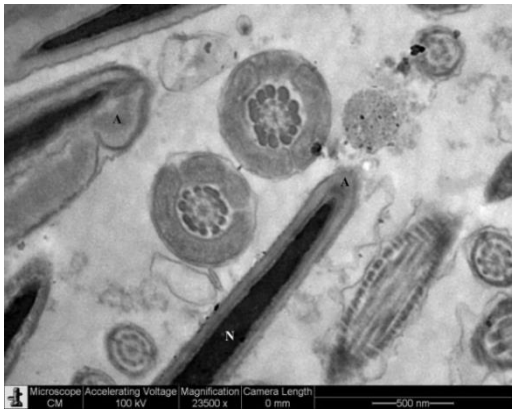


Figure 73 (original). Sperm sectioned in various planes with small changes (x23500).

Exhibit 10

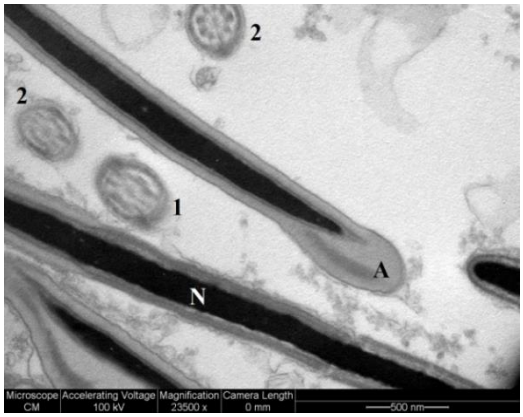


Figure 75 (original). Thawed sperm at 75º C for 10 seconds (x 23500). The large sizes images in this section displays disorganized MP more or less detached from the N. Intermediate piece (1) and main piece (2).

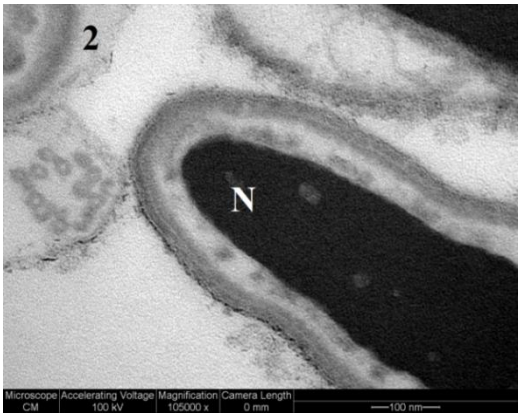


Figure 77 (original). Dotted areas with electrondense aspect coming out of the nucleus through the nuclear membrane pores (x105000). The images are not found in other methods of thawing. The main part (2).

Exhibit 11

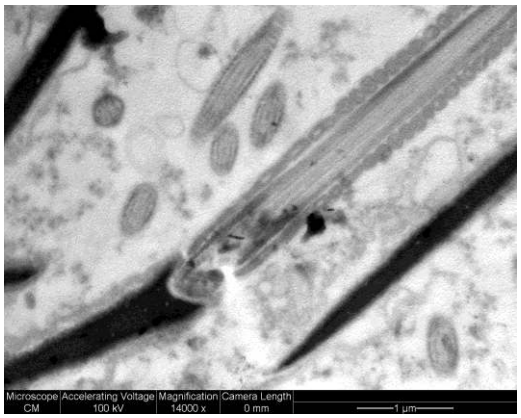


Figure 80 (original). Thawed sperm at 75º C for 5 seconds (x 14000). Cells without MP and loss of cellular matrix.

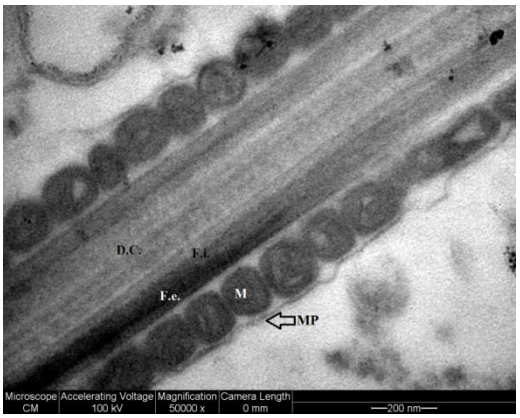


Figure 81 (original). Sperm thawed at 75 ° C for 5 seconds (x 50000). Mitochondria destroyed on one side and on the opposite MP is fenestrated and the mitochondrial matrix destroyed. Central doublets (DC), internal filaments (F.i.), external filament (F.E.)

Exhibit 12

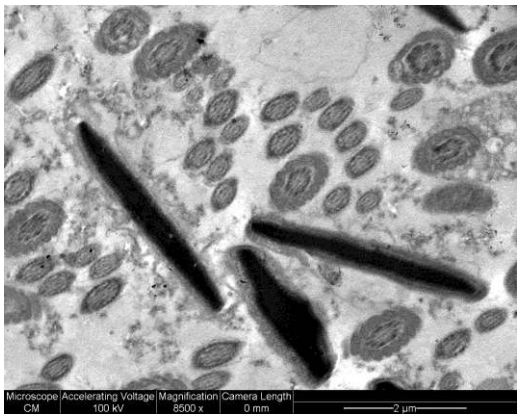


Figure 82 (original). Thawed sperm at 90° C for 2 seconds (x8500). Sperm cells without plasma membrane and disorganized ultrastructural aspect.

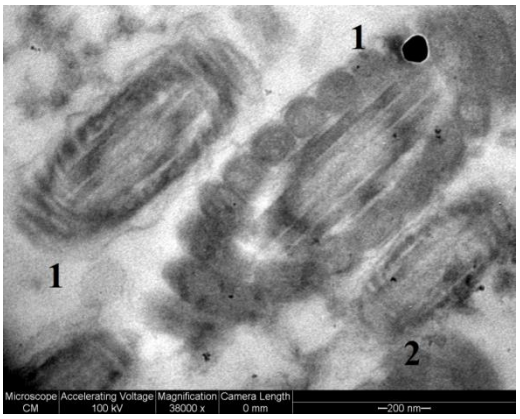


Figure 83 (original). Thawed sperm at 90° C for 2 seconds (x 38000). Skew sections through main and terminal piece of the flagella completely destroyed.
Intermediate piece (1) and main piece (2).

Conclusion

Thawing buck sperm at 39^0 C for 120 seconds (V1- slow conventional method), at 50˚C for 30 seconds (V2- fast method) at 75° C for 10˚and 5 seconds and 90˚C for 2 seconds (V3, V4, V5 – ultrafast method) was assessed by electron microscopy examination to determine the ultrastructural profile of the 5 experimental variants. The main conclusions that were drawn from the research are: the differences were not statistically significant between V1 and V2 thawing; ultrastructural profile of the ultrafast thawed specimens in V3, V4 and V5 presents normal membrane in a range from 11.27% - 31.23% for all cellular compartments, with statistically significant differences from variants V1 and V2; the principal structural changes have had different frequencies in the 5 variants, the most common defects have been bloating more or less exaggerated of the external plasma membrane with fenestration of different sizes, damage of the external acrosome plasma membrane, loss of acrosomal content; the frequency of serious injuries, which cause cell death, are more common in the V3, V4 and V5 ultrafast thawing variants.

Experiment 5. Study on the effects of exposure to different doses of energy generated by a He-Ne laseron the qualityof frozen-thawed ram and buck semen

The aim of the research was to determine whether and how the two doses of laser irradiation energy (3.96 and 6.12 J/cm2) can improve the quality characteristics of ram and buck sperm after the freeze-thaw process.

Cryopreserved semen samples were thawed on a water bath at 39° C and were subjected to two different doses of irradiation energy.

Results

The objective of the research was to determine whether and how the two doses of laser irradiation energy (3.96 and 6.12 J/cm2) can improve the quality characteristics of ram and buck sperm after the freeze-thaw process.

For that, the straws were thawed on a water bath at 39^{o} C for 120 seconds. The semen was divided into three samples: one was the control sample and the other two were irradiated with a He-Ne laser using two different doses of energy (3.96 și 6.12 J/cm^2) and the mitochondrial activity (by flow cytometry), cell viability (by flow cytometry), motility and function of plasma membrane integrity (HOST test) were analyzed. The results are presented in tables 8 and 9.

Table 8. Variation of quality parameters of thawed ram semen irradiated with He-Ne laser

Samples	Semen characteristics			
	Motility (%)	Viability (%)	Mitochondrial activity (%)	HOST (%)
Control	39.2 ± 1.96^{a}	45.09 ± 2.46^{a}	36.47 ± 1.32^{a}	38.67 ± 1.46^{a}
Sample 2 (3.96 J/cm^2)	38.5 ± 1.59^{a}	43.86 ± 1.68^{b}	39.96 ± 2.08^{a}	39.02 ± 1.91^{a}
Sample 3 (6.12 J/cm^2)	44.23 ± 2.38^{a}	50.73 ± 2.77^{a}	43.76 ± 1.94^{b}	45.12 ± 2^{a}

$^{a-b}$ Within column different superscript letters represents statistical significant differences at ($p<0.05$)

Tabel 9. Variation of quality parameters of thawed buck semen irradiated with He-Ne laser

Samples	Semen characteristics			
	Motility(%)	Viability(%)	Mitochondrial activity(%)	HOST (%)
Control	37.95 ± 1.79[a]	44.01 ± 2.29[a]	35.59 ± 1.02[a]	37.74 ± 1.4[a]
Sample 2 (3.96 J/cm^2)	33.05 ± 1.21[a]	37.84 ± 1.51[b]	34.76 ± 1.7[a]	33.66 ± 1.63[a]
Sample 3 (6.12 J/cm^2)	40.86 ± 2.03[a]	46.85 ± 2.39[a]	40.42 ± 2.21[b]	41.68 ± 1.86[a]

[a-b] Within column different superscript letters represents statistical significant differences at ($p<0.05$)

Motility: It is observed that the best values were obtained by He-Ne laser irradiation at a dose of 6.12 J/cm^2. The exposure at lower doses of energy lead to a reduced motility compared to the other irradiated samples and control. Statistically insignificant differences are between motility values ($p <0.05$).

Viability: The percentage of viable spermatozoa (tables 8 and 9) is statistically significantly higher ($p <0.05$) for the control sample and the sample irradiated with a dose of energy of 6.12 J/cm^{2}compared to the sample irradiated with the dose of 3.96 J/cm^2.

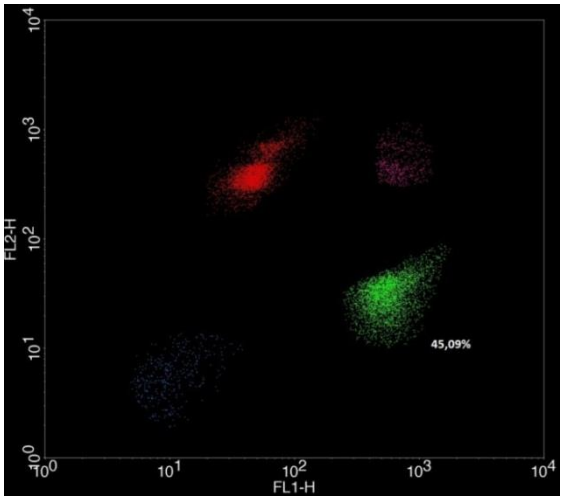


Figure 84 (original). Comparative analysis through the dot-plot graph technique for simultaneous identification of viable cells (stained green), death (colored red) and dying (double positive, colored purple) and non-sperm population (colored blue) for control sample of ram sperm

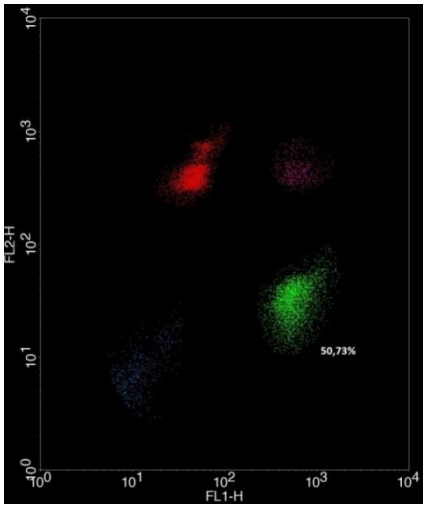


Figura 86 (originală). Comparative analysis through the dot-plot graph technique for sample 3 of ram sperm (irradiated with 6.12 J/cm^2)

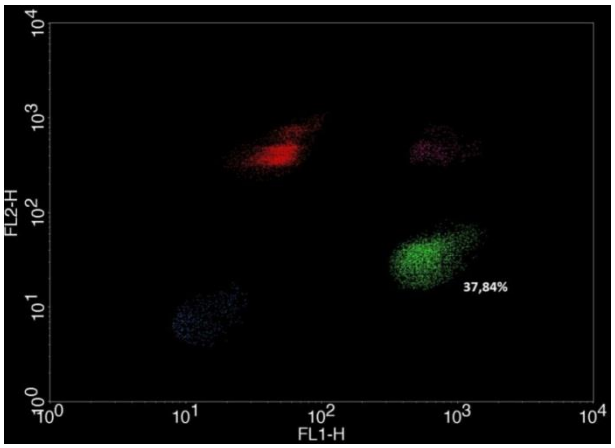


Figure 88 (original). Comparative analysis through the dot-plot graph technique for simultaneous identification of viable cells (stained green), death (colored red) and dying (double positive, colored purple) and non-sperm population (colored blue) for sample 2 of buck sperm (irradiated with 3.96 J/cm^2)

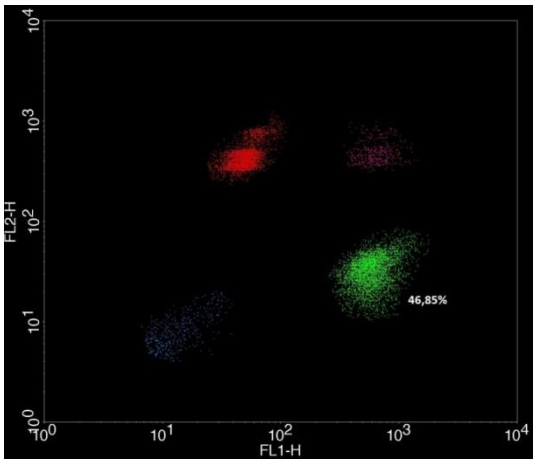


Figura 89 (originală). Comparative analysis through the dot-plot graph technique for sample 3 of buck sperm (irradiated with 6.12 J/cm^2)

Similar results were obtained for the functional integrity of the membranes (tables 8 and 9) and for the test for the assessment of mitochondrial activity (tables 8 and 9). The best results were obtained for sample 3 (6.12 J/cm^2), the values being significantly higher ($p < 0.05$) compared to the other variants in the case of mitochondrial activity.

For studying the mitochondrial activity compared cytometric evaluations of ram and goat sperm were analyzed from the experimental samples.

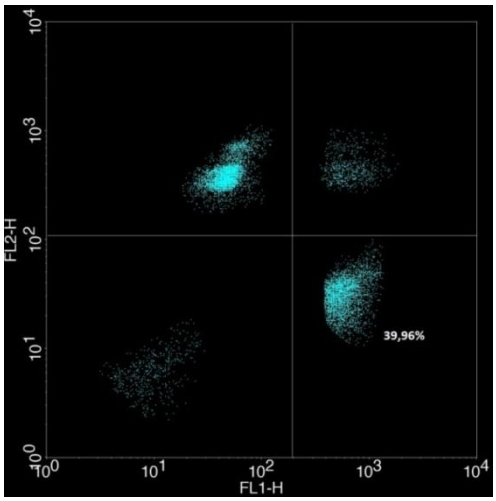


Figure 91 (original). Comparative analysis by quadrant technique for simultaneous identification of cell with normal mitochondrial activity (lower right quadrant), death cells (square top left) and dying (double positive, upper right quadrant) and non-sperm population (left quadrant below) for sample 2 of ram sperm (irradiated with 3.96 J/cm^2).

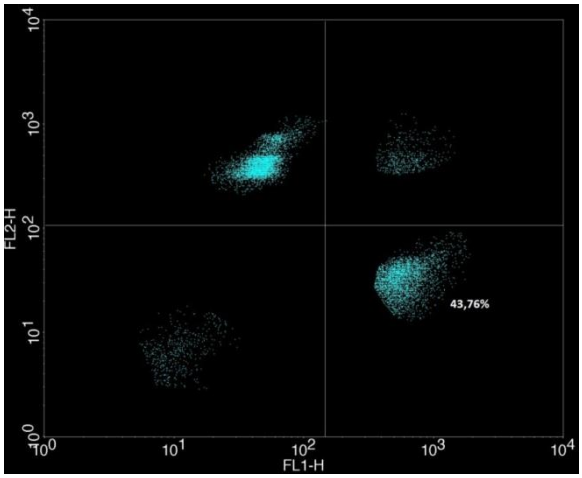


Figure 92 (original). Comparative analysis by quadrant technique for sample 3 of ram sperm (irradiated with 6.12 J/cm^2)

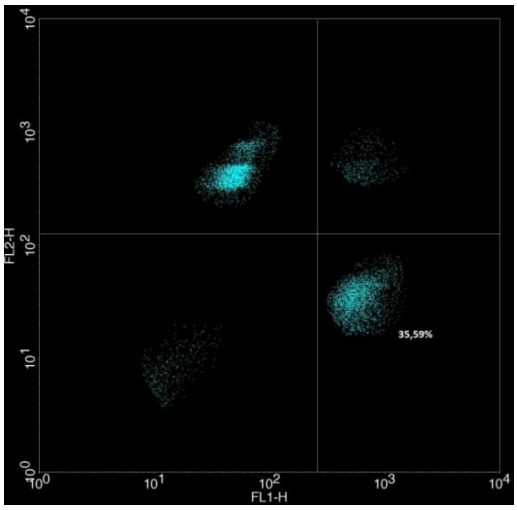


Figure 93 (original). Comparative analysis by quadrant technique for simultaneous identification of cell with normal mitochondrial activity (lower right quadrant), death cells (square top left) and dying (double positive, upper right quadrant) and non-sperm population (left quadrant below) for control sample of buck sperm.

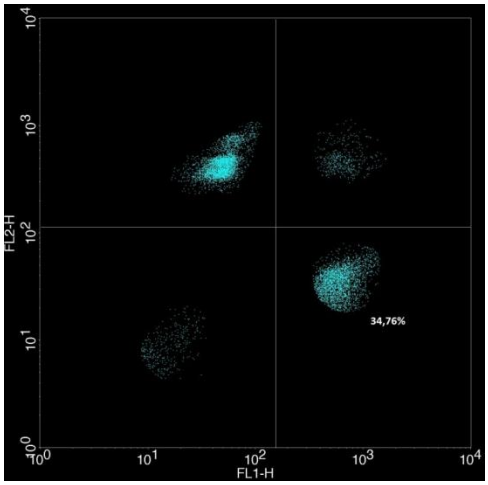


Figure 94 (original). Comparative analysis by quadrant technique for sample 2 of buck sperm (irradiated with 3.19 J/cm^2)

Discussion

The results of this study showed that irradiation with a He-Ne laser of the thawed ram and buck sperm leads to an increase of the motility, viability and functional membrane integrity of sperm in the case of the dose of 6.12 J/cm^2. At a lower dose of energy result was found to be ineffective in comparison to other irradiated samples and control.

Conclusion

Using flow cytometry techniques leads to more accurate results due to the large number of cells analyzed.

The action of an He-Ne laser on thawed ram or buck semen lead to an improvement in motility, viability, mitochondrial function and functional integrity of the membrane for the dose of 6.12 J/cm^2, unlike the dose of 3.96 J/cm^2 which recorded decreases in the semen qualitative parameters relative to the control sample.

Experiment 6. *In vivo* testing of frozen sperm subjected to thawing experiments at different temperatures and irradiated

In vivo testing was performed in normal breeding season through artificial insemination of 58 sheep from Merinos of Palas line, the meat line. Frozen semen was collected from the Merinos rams line, the meat line, reared in the biobase of I.C.D.C.O.C. Palas Constanta. Thawing was done in two ways:

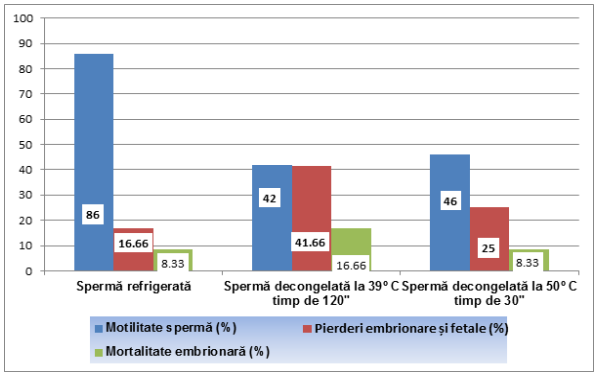
- using the slow conventional thawing method in warm water at 39º C for 120 seconds and the fast method at 50° C for 30 seconds;
- using the He-Ne laser energizing method with two energy levels of 3.96 J/cm^2 and 6.12 J/cm^2.

Assessment of the results was performed by calculating the indices of reproduction, namely: the percentage of goats non returning in oestrus (NR%), fertility (F%), prolificacy (P%) embryo mortality (ME%) and abortions (A%).

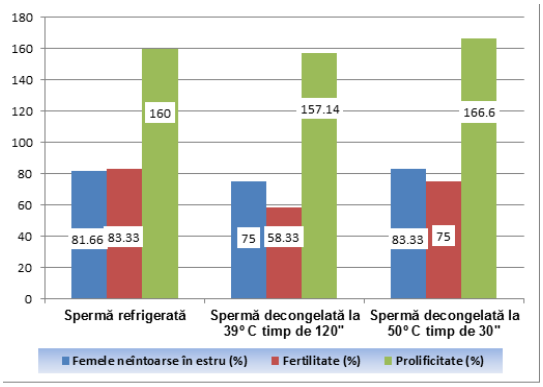
a) Breeding indices obtained after the *in vivo* testing with frozen-thawed sperm at different temperatures.

Comparing the main breeding indices obtained by the thawed sperm using the two thawing methods, thawing fast at 50° C for 30 seconds achieved a 11% greater fertility rate and a 6% greater fecundity. A better fertility was obtained in the case of a longer survival of sperm cells in the sheep genital tract, which requires lower morphostructural changes.

Graph 1. Refrigerated and frozen-thawed sperm motility, thawed through the slow and fast method.



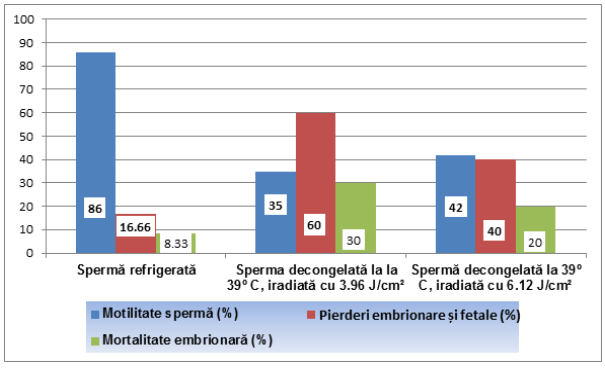
Graph 2. Conception rate in sheep inseminated with refrigerated semen and frozen-thawed sperm by slow and fast thawing method.



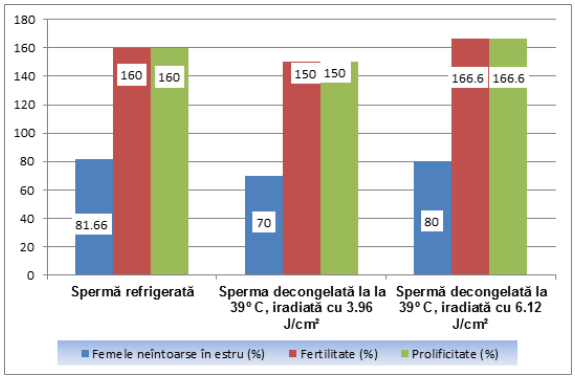
b) Breeding indices obtained by *in vivo* testing using frozen sperm – thawed using a He-Ne laser (Table 11, graphs 3 and 4).

The laser energizing method is a difficult method to put in practice, but *in vivo* results were higher when using a higher energy. There is no information in the literature regarding *in vivo* testing of semen thawed by this method, so that the results can be regarded as reference data for other authors.

Graph 3. Refrigerated and frozen-thawed energized sperm motility.



Graph 4. Conception rate in sheep inseminated with refrigerated semen and frozen-thawed energized sperm.



Conclusion

The main results after the artificial insemination with sperm thawed by the two methods are:

- Comparing the results obtained *in vivo* for the thawed sperm varying the temperature and time, it can be concluded that the fast thawing of sperm at 50° C for 30 seconds has achieved a 11% higher rate of fertility and a 6% higher prolificacy than the conventional slow thawing;
- *In vivo* testing of laser thawed sperm using two energy levels (3.96 J/cm^2 and 6.12 J/ cm^2) was carried out on two experimental groups and one control (refrigerated semen). Very good breeding indices were obtained for the thawed sperm using higher energy, namely 60% (6/8) fertility and 166.66% (n = 10) prolificacy;
- Comparing conception rates of thawed sperm using the fast method and the laser energizing method with a dose of 6.12 J/cm^2, very good results were obtained for the fast thawing method. This method is easy to use in farm conditions, is fast ensuring a higher progression and survival of the sperm cells than the rest of the variants used in this experiment;
- The control group of ewes inseminated with refrigerated semen registered greater reproduction indices than those inseminated with conventional or fast thawed semen, but the advantages of frozen-thawed sperm, regardless of the technologies used in freezing or thawing, results from the availability throughout the year, fom the fact that it is not limited to conditions, season, time and/or space.

Chapter 3. GENERAL CONCLUSIONS

Using flow cytometry techniques lead to more accurate results due to the large number of cells analyzed.

The analysis of ram and buck sperm cells packed in fine straws, thawed at temperatures of 50° C or 39° C leads to significantly higher values for the mitochondrial activity, viability, motility and functionality of the plasma membrane compared to other thawing variants.

The method is recommended for determining the quality of frozen sperm in sperm banks holding centers, because the mitochondrial activity can not be determined by other methods. The survival time of the cell after thawing dependents on mitochondrial activity.

Freezing-thawing leads to alterations in plasma membrane which include tearing, especially at the head level, and membrane detachment or vacuolization at the head and flagella level. Photomicrographs analysis shows that the acrosomes were only partially affected by the freeze-thaw 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 flagellum.

The results regarding the degree of damage on the plasma membrane after freezing-thawing in various conditions show that higher percentage of normal cells is obtained by thawing at 39º C for 120 seconds and at 50 ° C for 30 seconds.

Thawing buck sperm at 39º C for 120 seconds, at 50 ° C for 30 seconds, at 75 ° C for 5 and 10 seconds and at 90° C for 2 seconds was assessed by electron microscope examination in order to determine the ultrastructural profile of buck sperm cells using 5 experimental variants. The conclusions that were drawn from the research are:

- Motility and viability of frozen buck semen thawed samples were 8-10% higher than the frozen ram sperm, which confirms that the suitability at freezing of buck semen is higher.
- After thawing at 50° C for 30 seconds, the ultrastructural profile of buck sperm samples displayed normal ultrastructure with integral plasma membrane: 52.13 ± 1.76% at the head level, 58.29 ± 2.63% at cells with an normal acrosome level and 49.41 ± 4.52% at flagellum level.
- There were no statistically significant differences between the samples thawed at 39º C for 120 seconds and those fast thawed at 50° C for 30 seconds and integral plasma membranes at the head, acrosome and flagella level were normal for 45.27%, 50.37% and, respectively, 43.52% of the cells.
- Ultrastructural profile of the ultrafast thawed specimens at 75º C and 90º C presents normal membrane in a range from 11.27% - 31.23% for all cellular compartments, with statistically significant differences from thawing at 39º C for 120 seconds and at 50° C for 30 seconds variants;
- The principal structural changes have had different frequencies in the 5 variants, the most common defects have been bloating more or less exaggerated of the external plasma membrane with fenestration of different sizes, damage of the external acrosome plasma membrane, loss of acrosomal content;
- Fast and ultra fast thawed cells show a higher frequency of vesicles of different sizes around the head and "fluffy" membrane structures due to phospholipids displacement or transmembrane proteins dismantling and excessively bloated plasma membrane. These are the original observations, which are not found in the literature.
- In the ultra fast thawed sperm cells were observed in a higher frequency

submembranary areas without electron-optics density, which implies a transfer of water and electrolytes from the extracellular area, by membrane transport, because the membranes are not fenestrated.

- The presence of ice crystals is demonstrated by the presence of "white" areas, without structure, water being removed during the processing for examination by electron microscope of specimens, with moderate frequency in both the extracellular environment and submembranary at the spermatozoa head level.
- At the flagella level, sectioned into different plans, changes in the plasma membrane is limited to mild swelling with or without fenestration, sometimes with partial or total separation.
- The frequency of serious injuries, which cause cell death, are more common in the ultra fast thawing variants.

The action of an He-Ne laser on thawed ram or buck semen lead to an improvement in motility, viability, mitochondrial function and functional integrity of the membrane for the dose of 6.12 J/cm^2, unlike the dose of 3.96 J/cm^2 which recorded decreases in the semen qualitative parameters relative to the control sample.

The thawing of fine straws containing ram and buck semen method using a He-Ne laser for energizing the samples is a national premiere, this method was experimented for the first time in Italy.

The assessment of *in vivo* results for the thawed ram sperm by the conventional method (39º C for 120 seconds) and the fast method (50° C for 30 seconds) was performed by calculating the main reproduction indices, namely: the percentage of goats non returning in oestrus (NR%), fertility (F%), prolificacy (P%), embryon mortality (ME%) and abortion (A%). The results obtained after the artificial insemination with thawed sperm by the two methods are:

- After two estrous cycles from the insemination with thawed sperm at 39º C for 120 seconds, the percentage of conception (NR%) was 75% and that of fertility (F%) calculated, after the normal parturition of the females, was 58.33% . The loss of pregnancy (fetal and abortions) were 41.66%.
- After two estrous cycles from the insemination with thawed sperm at 50º C for 30 seconds, the percentage of conception (NR%) was 83.33% and that of fertility (F%) was 75% and the loss of pregnancy (embryo mortality and abortions) were 25%.

- Prolificacy (P%) between the two experimental groups was 157.7%, respectively, of 166.66%.
- From the total loss of gestation, embryonic mortality was 25% and respectively 16.66%, which confirms that they occurred due to frozen-thawed sperm with reduced mitochondrial activity and viability. Abortions were placed in the normal range, did not have an infectious etiology and were caused by environmental factors or technological factors.
- Comparing the results obtained *in vivo* for the thawed sperm using the two methods, it can be concluded that the fast thawing of sperm at 50° C for 30 seconds has achieved a 11% higher rate of fertility and a 6% higher prolificacy than the conventional slow thawing.
- *In vivo* testing of laser thawed sperm using two energy levels (3.96 J/cm^2 and 6.12 J/ cm^2) was carried out on two experimental groups and one control (refrigerated semen). Very good breeding indices were obtained for the thawed sperm using higher energy, namely 60% fertility and 166.66% prolificacy.
- The control group of ewes inseminated with refrigerated semen registered greater reproduction indices than those inseminated with conventional or fast thawed semen, but the advantages of frozen-thawed sperm, regardless of the technologies used in freezing or thawing, results from the availability throughout the year, fom the fact that it is not limited to conditions, season, time and/or space.
- In general, the obtained reproduction indices were comparable with other research done, given that the objective of the experiment is very little studied in the speciality literature and is not verified *in vivo*, so that the obtained original data represents scientific informations of reference for this objective which was studied *in vitro* but not *in vivo*.
- Using the flowcytometric technique for studying the viability and mitochondrial activity is a novelty at national level.

MAIN PERSONAL CONTRIBUTIONS

- The thawing of fine straws containing ram and buck semen method using a He-Ne laser for energizing the samples is a national premiere, this method was experimented for the first time in Italy.
- The morphology assesment method for ram sperm was patented:
  Invention patent number 127850/29.04.2016 title: "Method of morphological assessment of ram semen" - Authors: Zamfirescu Stela, Nicolae Dobrin
- Using the flowcytometric technique for studying the viability and mitochondrial activity is a novelty at national level.
- All the electron microscopy and flowcytometry photos are original.
- Fast and ultra fast thawed cells show a higher frequency of vesicles of different sizes around the head and "fluffy" membrane structures due to phospholipids displacement or transmembrane proteins dismantling and excessively bloated plasma membrane. **These are the original observations, which are not found in the literature.**
- In general, the obtained reproduction indices were comparable with other research done, given that the objective of the experiment is very little studied in the speciality literature and is **not verified *in vivo***, so that **the obtained original data represents scientific informations of reference for this objective which was studied *in vitro* but not *in vivo*.**

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