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STUDY ON THE OPTIMAL THAWING PROCEDURE OF CRYOPRESERVED BUCK SEMINAL MATERIAL USING FLOW CYTOMETRY

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Rezumat. *Acest studiu își propune să determine procedura optimă de decongelare a spermei de țap, în scopul de a stabili rata optimă de decongelare care poate asigura cel mai mare procent de spermatozoizi viabili. Crioconservarea spermei implică mai multe etape, cum ar răcirea, congelarea și decongelarea și fiecare dintre aceste etape poate provoca daune membranei plasmatice. Sunt necesare tehnici avansate pentru a stabili exact procentul de spermatozoizi viabili. Variantele de decongelare testate au fost decongelare: la 90 ° C- 2 s, la 75 °C - 10 s, la 50° C - 30 s și la 39° C - 120 s. Au fost analizate activitatea mitocondrială, motilitatea și viabilitatea. Rezultatele acestui studiu au arătat că viabilitatea și funcția mitocondrială sunt crescute semnificativ dacă paietele fine au fost decongelate la 39° C și 50° C în comparație cu alte temperaturi de decongelare.*

Abstract. *This study aimes to determine the optimal thawing procedure of crioconserved buck sperm, in order to establish the proper thawing rate that can assure the highest percentage of viable spermatozoa. Sperm cryopreservation involves several steps such as cooling, freezing and thawing and each of these steps can cause sperm damage to the plasma membrane, which impairs normal sperm structure and function, reduces motility and the fertilizing ability of spermatozoa. Advanced techniques are needed in order to establish exactly the percentage of viable sperm cells. The thawing variants tested were thawing: at 90° C -2 s, at 75° C -10 s, at 50° C for 30 s, and at 39° C - 120 s. The mitochondrial activity, motility and viability were analyze. The results of this study showed that the viability and mitochondrial function are significantly increased when the fine straws were thawed at 39 °C and 50 °C as compared with other thawing temperatures.*

Keywords: *Saanen bucks, flow cytometry, mitochondrial function, motility, viability.*

1. Introduction

Semen cryopreservation has a very important contribution in developing breeding techniques, such as artificial insemination (AI) and IVF. Artificial insemination will reach its full potential by the efficient use of frozen semen.Cryopreservation involves several steps, such as dilution, cooling, freezing and thawing. Each of these steps can damage the plasma membrane structure and

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functions which affect normal sperm function, can reduce mobility and fertilizing ability and can induce premature nuclear decondensation [3].

In the freezing process any biological activity is stopped until thawing [12]. Thawing returns the sperm cell to physiologic temperature reactivating the metabolism, so thawing should be done with care to avoid any damage [2].Various studies were conducted to determine the optimum temperature and duration of thawing and raised the interest regarding the most adequate speed of the thawing process so that will result the highest possible percentage of viable sperm cells [7]. The rate of thawing was defined by Bearden [2] like the thawing process of semen stored in straws at a specific temperature, in a certain period of time. The interactions of various factors with the thawing procedures, like the type of extender, the glycerol concentration, the way of packing the semen, the cooling rate, the handling of the seminal material during the freezing process, affect sperm motility after thawing, as well as the experimental conditions such as the available facilities, equipment and reagents, which varies between countries and even regions [16]. Thus, the methods of semen freezing and thawing should be looked at according to each race, country and area.

This study aimed to determine the optimal thawing procedure of crioconserved buck sperm, in order to establish the proper thawing rate that can assure the highest percentage of viable spermatozoa.

2. MATERIAL AND METHODS

The activity of freezing buck 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 [17]. 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 February to May 2013.

As dilution medium a diluent of Tris base 20% (v/v) egg yolk was used. The cryoprotectant used for freezing buck semen was glycerol (7% final concentration).

Animals: sperm samples were collected from five adult Saanen buck 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 60 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 10 seconds
Thawing at 50 ° C for 30 seconds
Thawing at 39°C for 120 seconds

Assessment of mitochondrial function by flow cytometry with Rhodamine (R123)

A Beckton-Dickinson FACS Calibur flow cytometer was used for quantitative analysis of fluorescent labeled spermatozoa, the inputs were registered and processed using a Apple computer and the specialized software CellQuest Pro. The lipophilic fluorochrome Rhodamine 123 has a positive charge at physiological pH which favors its concentration in the mitochondria under the influence of potential difference generated by the respiratory function. This fluorochrome is typically used in the assessment of mitochondrial activity, but may also be used to determine dead cells in the population, since these accumulate in small quantity Rhodamine 123 [10, 14]. The red fluorescence emitted by the dead cells stained using propidium iodide is captured by the FL2 detector and the green fluorescence emitted by cells with functional mitochondria stained with Rhodamine 123 was captured by the FL1 detector. Interpretation of results was done through dot-plot graphs statistics FL1/FL2, where each cell read is represented as a point and each population is represented as a cloud of points.

Determination of viability of sperm cells by flow cytometry

To determine the percentage of viable sperm cells a Live-Dead Sperm viability kit (Invitrogen) was used that allows flow cytometric analysis of viability, but can also be used to determine viability by fluorescence microscopy technique. The method used is the double staining, in which, in order to determine the viability of sperm cells 2 fluorochromes are used that stain the nucleic acids. SYBR-14, which staines the spermatozoa with intact membranes and the propidium iodide, which stains cells with damaged membranes were used. The method was used to determine the viability in most species of mammals [8].

Assessment of sperm motility

Manual evaluation in wet preparation technique [17] was used for assessing sperm motility using a Novex optical microscope with hot plate (x100 magnification).

3. STATISTICAL ANALYSIS OF EXPERIMENTAL DATA

IBM SPSS, version 17.01 was used for descriptive statistics. The results are expressed as mean ± standard error. To determine the normal distribution of the

results and therefore the choice of using parametric or nonparametric tests for significant differences of means we used the Kolmogorov-Smirnov test and for added security, because the number of samples was small, the Shapiro-Wilk test. To determine significant differences, the means were analyzed using paired Student T-test.

4. RESULTS AND DISCUSSIONS

The objective of the research was to determine experimentally the influence of some thawing rates (time and temperature) on different cytological parameters of sperm cells after thawing in order to establish an optimal variant which would cause the least cell damage throughout this process.

In this sense the cryopreserved semen was thawed in 4 variants and mitochondrial activity, cell viability (by flow cytometry) and motility were analyzed.

Table 1. Variation of quality parameters of thawed semen by different methods

	Semen characteristics		
The temperature and time of thawing	*Motility (%)*	*Viability (%)*	*Mitochondrial activity (%)*
Thawing at 39 ° C for 120 seconds	$40 \pm 1.36^{\mathbf{a}}$	48.12 ± 1.56^{a}	41.05 ± 1.96^{a}
Thawing at 50 ° C for 30 seconds	43.2 ± 2.36^{a}	52.32 ± 2.65^{a}	41.56 ± 3.19^{a}
Thawing at 75 ° C for 10 seconds	22.3 ± 3.26^{b}	25.76 ± 1.89^{b}	24.35 ± 4.52^{b}
Thawing at 90 ° C for 2 seconds	13.44 ± 2.13^{d}	17.22 ± 3.18^{d}	9 ± 2.41^{c}

Within colum different small letters significant at ($p<0.05$)

The results of semen characteristics are presented in table 1. It is observed that the best values for sperm motility were obtained by thawing straws at 50° C for 30 seconds. Thawing at 39° C for 120 seconds has also led to an increased motility, between the two versions there were no significant statistical differences. Increasing the temperature leads to lower thawing motility. Both at 75° C, in the two variants of time, and at 90° C there were significantly lower values for sperm motility compared to the first two variants of thawing ($p <0,05$).

The percentage of viable spermatozoa (table 1) indicates that thawing at 39° C for 120 seconds and at 50° C for 30 seconds results in a significant higher viability ($p <0,05$) compared with the variants of thawing at 75° C and 90° C (Figure 1).

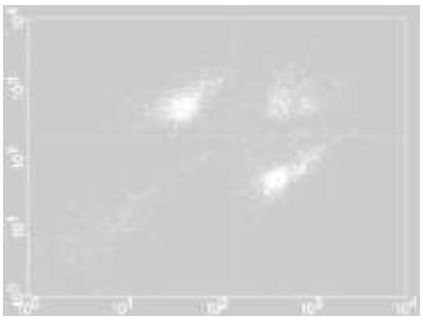

Fig. 1 The comparative analysis through the quadrants technique of identifying simultaneously viable cells (green dots), dead (red dots) and dying (double positive - purple dots) and non-sperm population (blue dots) from frozen-thawed sperm at different temperatures. Abscissa: intensity of cells stained with PI red fluorescence (FL2) in logarithmic scale. On the ordinate: green fluorescence intensity of cells stained with SYBR-14 (FL1), in logarithmic scale.

Similar results were obtained for assessing the mitochondria activity test. The best results were obtained for the temperatures of 50° C and 39° C, values being significantly higher ($p < 0,05$) as compared to other variants (Figure 2).

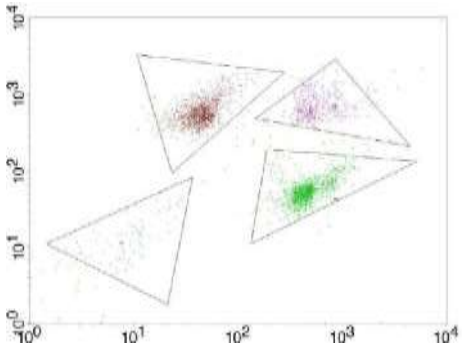

Fig. 2 The comparative analysis through the quadrants technique of identifying simultaneously cells with active mitochondria (green dots), dead (red dots) and dying (double positive - purple dots) and non-sperm population (blue dots) from frozen-thawed sperm.

International researches has shown that traditionally the fine straws with buck semen are thawed on a water bath at 38° C for 12-30 seconds. The method leads to good results (36.1% motility) compared to the method of slow thawing (18.9% motility) in which the fine straws are thawed on a water bath at 5° C for 2 minutes [6]. Other studies have shown that increasing the temperature of thawing at 70° C for only 7 seconds leads to a significant improvement of motility (36.9%) and plasma membrane integrity (39.8%) compared with thawing at 37° C for 2 minutes (31.5% motility and 33.7% integrity of the plasma membrane) or at 40° C for 20 seconds (32.4% motility and 33.5% integrity of the plasma membrane) [15].

The results of our research are similar to those in the literature, which demonstrates that the thawing time and temperature must be carefully monitored and timed [1, 4]. The results of this research 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 temperatures. Vishwanath [16] showed that rapid thawing of semen prevented physiological damage suffered during thawing process. Holt [11] observed that rapid thawing can prevent crystallization of water molecules and damage to cell membranes while the slow thawing favors spermatozoa abnormalities, this result being in accordance with the results communicated by Curry [5].

Gordon [9] noted that the optimal thawing rate that maintains a high percentage of sperm motility and viability during thawing is 30-37° C for 30 seconds. Nur [13] showed that thawing the straws of bull semen at 37° C has led to a higher fertility than those thawed at low temperatures (10° C or ice water). Chaiprasat [3] found the high and the low rate of progressive motility of spermatozoa thawed at 37° C for 30 seconds and, respectively, thawed at 20° C for 30 seconds.

Conclusions

Conclusion (1).

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

Conclusion (2).

Thawing the fine straws of buck semen at temperatures of 39° C, respectively 50°C results in a significant higher values for mitochondrial activity, viability and motility compared to other variants of thawing.

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