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ORIGINAL PAPER

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

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Abstract

The study aimed to determine the optimal thawing procedure, in order to establish the proper thawing rate that can assure the highest percentage of viable spermatozoa after thawing and the assessment of the relationship between this technique of thawing and sperm survival during incubation at 37° C. Sperm samples were collected from five adult Merinos de Palas rams with known fertility. Sperm samples from each animal were analyzed separately in order to take into account the variability in the individual. 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 showed that the motility, viability and functional and structural integrity of the plasma membrane is significantly increased when the fine straws were thawed at 39° C and 50° C as compared with other thawing temperatures.

Keywords: Merinos de Palas rams, flow cytometry, mitochondrial function, motility, viability

Introduction

The straw containing frozen semen has become the universally accepted unit for storage and genetic transfer in sheep, procedure that depends on maintaining the functional activity of the spermatozoa (viability and fertilizing capacity). In the freezing process any biological activity is stopped until thawing (Jondet [16]). Thawing returns the sperm cell to physiologic temperature reactivating the metabolism, so thawing should be done with care to avoid any damage (Bearden [3]).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 (Pace [21], Dhami [9]). The rate of thawing was defined by Bearden [3] 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 (Rodriguez [22]) as well as the experimental conditions such as the available facilities, equipment and reagents, which varies between countries and even regions (Vishwanath [28], Thibier [26]).

Thus, the methods of semen freezing and thawing should be looked at according to each race, country and area (Hayashi [13]).

This study aims to determine the optimal thawing procedure in order to know the proper thawing rate that can give the highest percentage of viable spermatozoa after thawing and evaluating the relationship between the thawing technique and the survival of sperm after thawing during incubation at 37° C.

Materials and methods

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 (Zamfirescu [29]). Experiments were conducted in the normal breeding season, during December 2012 - February 2013. 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).

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 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

Methods for assessing the cryobiological indices after thawing

1. 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 (Ronot [23]).

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.

2. 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 (Garner [10]).

3. Assessment of sperm motility

Manual evaluation in wet preparation technique (Zamfirescu [29]) was used for assessing sperm motility using a Novex optical microscope with hot plate (x100 magnification).

4. Functional integrity of the plasma membrane

The hypo-osmotic (HOST) test is used to assess the functional integrity of the sperm membrane, and is based on the semi-permeability of intact cell membranes, that, in the hypo-osmotic conditions, leads to swelling the sperm cell flagella, these being an indication that the transport of water through the membrane is normally carried out.

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.

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

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

The temperature and time of thawing	Semen characteristics				The viability of the semen incubated at 39° C in water bath (%)		
	Motility (%)	Viability (%)	Mitochondrial activity (%)	HOST (%)	1 hour	2 hours	3 hours
Thawing at 39 ° C for 120 seconds	39 ± 2.08[a]	45.61 ± 1.83[a]	37.03 ± 1.66[a]	38.97 ± 1.84[a]	40.94 ± 1.8[a]	33.04 ± 1.85[a]	27.02 ± 1.94[a]

Thawing at 50 ° C for 30 seconds	45 ± 2.24[a]	52.47 ± 2.25[a]	40.66 ± 2.36[a]	46.35 ± 2.55[a]	46.45 ± 2.65[a]	41.73 ± 2.84[a]	34.54 ± 2.91[a]
Thawing at 75 ° C for 10 seconds	20 ± 1.83[b]	27.16 ± 1.86[b]	22.07 ± 2[b]	19.3 ± 2.07[b]	22.02 ± 1.94[b]	11.95 ± 1.87[b]	5.28 ± 1.78[b]
Thawing at 75 ° C for 5 seconds	30 ± 1.83[c]	34.48 ± 1.82[c]	28.52 ± 1.81[b]	31.97 ± 1.41[c]	26.22 ± 1.37[c]	17.14 ± 1.36[c]	9.32 ± 1.3[c]
Thawing at 90 ° C for 2 seconds	10 ± 1.29[d]	15.34 ± 1.32[d]	7.1 ± 1.37[c]	11.36 ± 1.36[d]	9.3 ± 1.39[d]	2.07 ± 0.8[d]	0[d]

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

Motility: In table 1 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).

Viability: 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.

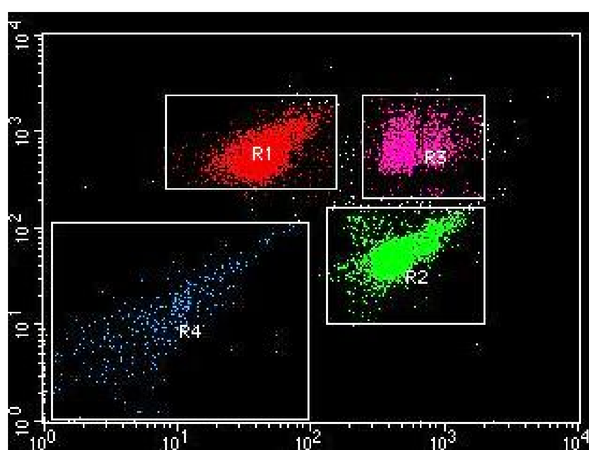

Figure 1 The comparative analysis through the quadrants technique of identifying simultaneously viable cells (green dots), dead (red dots) and dying (double positive - purple dots) and non-sperm population (blue dots) from frozen-thawed sperm at different temperatures. Abscissa: intensity of cells stained 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 the functional integrity of membranes (HOST test) and 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.

The viability of the semen incubated at 39 ° C (%) on water bath show a steady decrease of about 6 -7 percent during the three hours of incubation for the variants of thawing at 39° C and 50° C. For the other variants decrease is more pronounced, up to 10 percent per hour.

International researches has shown that traditionally the fine straws with ram 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 (Deka [8]).

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) (Tuli [27]).

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 (Senger [25], Ileri [15], Correa [6], Almquist [2]).

Salisbury [24] reported that semen thawing should be fast because the slow thawing (at 5° C) allows the re-crystallisation of ice within the cell, leading to membrane rupture (Senger [25]), while Ahmad [1], Ozkoca [20] and Nur [19] reported that, after thawing, the sperm motility as well as acrosome integrity and the survival rate of sperm cells was significantly higher when the straws were thawed at 70° C for 5 seconds compared to thawing at 37° C for 30 seconds, and at 50° C for 15 seconds.

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 [28] showed that rapid thawing of semen prevented physiological damage suffered during thawing process. Holt [14] 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 [7]. Gordon [11] 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. Mitchell [17] reported that semen is thawed frequently at temperatures between 33° C and 37° C, for a period of 30 to 40 seconds. Pace [21] and Nur [18] 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). Bravo [4] have emphasized that straws should be thawed on a water bath at 37° C for 30-40 seconds and at 40° C for 8 seconds. Chaiprasat [5] 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

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

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

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