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Effect of controlled and uncontrolled cooling rate on motility parameters of cryopreserved ram spermatozoa

Iraj Ashrafi[1*], Hamid Kohram[2,3], Hamid Naijian[2], Majid Bahreini[4] and Hamid Mirzakhani[4]

Abstract

Background: Ram spermatozoa are sensitive to extreme changes in temperature during the freeze-thaw process. The degree of damage depends on a combined effect of various factors including freezing temperature. The aim of this study was to determine the effects of two cooling method (controlled-rate and uncontrolled-rate) on pre-freezing and post-thaw sperm motility parameters.

Results: Ejaculates were collected using the artificial vagina from four Chal rams and three replicates of the ejaculates were diluted with a Tris-based extender and packed in 0.25 ml straws. Then, sample processed according to the two methods. Method 1: straws cooled from 37 to 5°C, at a liner rate of -0.3°C/min in a controlled-rate cooling machine (custom-built) and equilibrated at 5°C for 80 min, then the straws were frozen at rate of -0.3°C/min from 5°C to -10°C and -25°C/min from -10°C to -150°C and plunged into liquid nitrogen for storage. Method 2: straws were transferred to refrigerator and maintained at 5°C for 3 h, then the straws were frozen in liquid nitrogen vapor, 4 cm above the liquid nitrogen for 15 min and plunged into liquid nitrogen. Computer-assisted sperm motility analysis was used to analyze sperm motion characteristics.

Conclusions: Controlled rate of freezing (Method 1) significantly improve the pre-freezing and post-thaw total and progressive motility compared to uncontrolled rate (Method 2). In specific kinetic parameters, Method 1 gives significantly higher value for VSL and VCL in comparison with Method 2. There are no significant differences between the two methods for VAP and LIN. In conclusion, controlled rate of cooling conferred better cryopreserving ability to ram spermatozoa compared to uncontrolled rate of cooling prior to programmable freezing.

Background

Considerable effort has been directed towards developing techniques for artificial insemination (AI) using frozen ram semen. Its potential in sheep breeding has become evident following the development of controlled reproduction procedures and more intensive management systems. The widespread application of AI depends largely on the use of frozen semen and thus on the availability of techniques that result in acceptable fertility in a selective breeding control programme. AI with frozen semen dispensed through the cervix gives quite low fertility rates in ram and use of laparoscopy with thawed semen is more costly and time-consuming but it improve the fertility rate significantly. The need to prepare a large number of doses of ram's semen each year requires the development of a rapid and effective method for freezing semen [1]. Spermatozoa are not adapted to survive cryopreservation, and therefore have variable responses to cooling and rewarming depending both on individual male and species [2,3]. Ram spermatozoa are susceptible to various stresses during cryopreservation [4]. The physiological and functional changes that occur in spermatozoa such as an irreversible reduction in motility, viability and acrosome integrity [5-7] cause changes similar to capacitation and acrosome reaction in the surviving population [8]. Damage to sperm membranes primarily occurs during the freezing and thawing process over the temperature range -15°C

* Correspondence: i.ashrafi@ut.ac.ir
[1]Young Researchers Club, Science and Research Branch, Islamic Azad University, Tehran, Iran
Full list of author information is available at the end of the article

to -60°C and not during storage in liquid nitrogen [9]. In the case of ram spermatozoa, most damage occurs between -10°C and -25°C, the region of ice crystallization [6]. The process of cell dehydration that accompanies slow freezing is potentially beneficial for cell survival, whereas rapid freezing rates are considered more likely to cause cell death [5].

Of considerable importance in the cooling regime is the cooling rate through the critical temperature range, defined as the range when ice crystal formation and consequent cell dehydration is occurring. The cooling rate determines whether the cells remain in equilibrium with their extracellular environment or become progressively super cooled with the increasing possibility of intracellular ice formation [10]. Freezing of ram spermatozoa in cell freezer has been commonly carried out from 5°C after precooling of straws up to 5°C in the cold chamber [11-17].

Computer-controlled cooling machine was built in the laboratory to achieve rapid cooling of samples in straws under controlled conditions. A protocol based on controlled-rate cooling and freezing of ram semen in straws has been reported to improve ram semen freezing technique but the post-thaw attributes of spermatozoa were evaluated by subjective assessment [10]. Computer-aided semen analysis (CASA) technique provides precise and validated objective assessment of sperm motion characteristics [18-20] and has been applied for short-term [21] and long-term preservation of ram.

The aim of this study was to determine the effects of two cooling methods (controlled-rate and uncontrolled-rate) on pre-freezing and post-thaw sperm motility parameters.

Methods

Animals and semen collection

Semen samples were collected from four mature Chal rams (3-4 years) maintained at the Animal Breeding Center Farm of IRAN. After collection, the ram semen samples were transferred within a minute to the laboratory and kept in a water bath at 37°C for examination. The rams were fed 0.91 kg of concentrate daily and good quality hay and water were supplied ad libitum. Ejaculates were collected from the rams using the artificial vagina twice a week during the breeding season (autumn to early winter).

Cryobiological procedures

General aspects

Some general procedures were established for the two methods. The volume of ejaculates was measured in a conical tube graduated at 0.1 ml intervals. The sperm concentration was determined by means of a haemocytometer and sperm motility was estimated using phase contrast microscopy (400 ×). Only ejaculates containing a semen volume varying between 1 and 2 ml, spermatozoa with >80% forward progressive motility and concentrations higher than 2.5×10^9 spermatozoa/ml were mixed in a pool balancing the sperm contribution of each male to eliminate individual differences [22]. Tris-citrate modified solution (Tris 27.1 g/l, citric acid 14.0 g/l, fructose 10.0 g/l, egg yolk 10% (v/v) glycerol 5% (v/v): 300 mOsM, pH 6.8, was used as the base extender (freezing extender). Each mixed ejaculate was split into two equal aliquots and diluted at 37°C with the base extender to a final concentration of approximately 4×10^8 spermatozoa per milliliter, in one step, in a 10 ml-glass centrifuge tube. Diluted samples were aspirated into 0.25 ml (medium-sized) French straws and sealed with polyvinyl alcohol powder. The sample processed according to the following methods and evaluated pre-freezing and post -thawing.

Semen cooling and freezing methods

Method 1: straws cooled from 37 to 5°C, at a liner rate of -0.3°C/min in a controlled-rate cooling machine (custom-built), and equilibrated at 5°C for 80 min. After equilibration, the straws were frozen in same machine at rate of -0.3°C/min from 5°C to -10°C and -25°C/min from -10°C to -150°C, then the straws were plunged into liquid nitrogen for storage. The Thermocouples of the custom-built cool in machine record the chamber temperature via a computer program and drives a motor to open and close the entrance of the nitrogen vapor source.

Method 2: straw transferred to refrigerator and maintained at 5°C for 3 h, then the straws were frozen in liquid nitrogen vapor, 4 cm above the liquid nitrogen, for 15 min and plunged into liquid nitrogen [23].

Semen thawing

Frozen straws were thawed individually at 37°C for 20 s in a water bath for evaluation immediately after thawing [23].

Evaluation of percentage of motile cells, recovery rate and kinetic parameters

A computer-assisted sperm motility analysis (CASA, Version 12 IVOS, Hamilton-Thorne Biosciences, Beverly, MA, USA) was used to analyze sperm motion characteristics. CASA was set up as follows: phase contrast; frame rate - 60 Hz; minimum contrast - 70; low and high static size gates - 0.6 to 4.32; low and high intensity gates - 0.20 to 1.92; low and high elongation gates 7 to 91; default cell size - 10 pixels; default cell intensity - 80. Semen was diluted (5 μl semen +95 μl extender) in a Tris-based extender (without egg yolk) and evaluated immediately after dilution. A 4 μl sample of diluted semen was put onto a pre-warmed chamber slide (Leja

4, Leja Products, Luzernestraat B.V., Holland) and sperm motility characteristics were determined with a 10 × objective at 37°C. Percentages of total motility and progressive motility were recorded. For both parameters the recovery rate of motility after thawing with respect to before freezing was calculated (post-thaw motility/pre-freeze motility). For thawed semen, VSL (straight linear velocity, μm/s), VCL (curvilinear velocity, μm/s), VAP (average path velocity, μm/s) and LIN = VSL/VCL × 100 (linearity, %) were noted as specific parameters of sperm motility. For each evaluation, 10 microscopic fields were analyzed to include at least 300 cells.

Analysis

The results were analyzed by Analysis of Variance with semen samples as replicates, using the following model: $Y = \mu+\alpha i+eij$

Results

As shown in Tables 1 controlled rate of freezing (method 1) significantly improve the pre-freezing and post-thaw total and progressive motility of ram spermatozoa compared to uncontrolled rate of freezing (method 2). The changes observed in motility are reflected in the recovery rate which indicates that method 1, preserve sperm motility better, showing significant difference with respect to the method 2. In general, it may be noted that the total motility is preserved better than progressive motility with recovery rates of 0.89 and 0.74, respectively.

In specific kinetic parameters, method 1 gives significantly higher value for VSL and VCL in comparison with method 2. There are no significant differences between the two methods for VAP and LIN (additional file 1).

Discussion

The cryopreservation cycle for semen samples includes the entire process from sperm preparation and dilution through to the post-thawing maintenance of functional capacity. At each of these stages, spermatozoa need to maintain a range of functional attributes that ensure their fertilizing capacity [5]. Maintenance of sperm function during freezing and thawing depends upon several interrelated factors that include cooling rate, equilibration period and freezing method [4,8,13,24] but their adverse effects are manifested on thawing. The degree of cryo-damage also depends on several factors [5,25] which limit the survival of spermatozoa during incubation. Under the best experimental conditions about half of the population of motile sperm survives the freeze-thaw process [5,24,26]. In the present study it was observed that controlled rate of cooling and freezing resulted in significantly higher sperm total and progressive motility, compared to uncontrolled rate of cooling and freezing. The overall good post-thaw may be attributed to the efficacy of controlled rate freezing protocol and the criteria of processing only those ejaculates for cryopreservation which have thick consistency, >80% initial motility, $>2.5 \times 10^9$ spermatozoa per ml.

The recovery rate indicates that motility is better preserved with Method 1. The fact that progressive motility is more affected by the freezing process than individual motility implies that these parameters measure different aspects of cell physiology and in particular, that the physiological basis for the progressive motility parameter is more sensitive to cryobiological damage [1]. The controlled-rate cooling protocol, besides providing complete automation in the cryopreservation process, might also protect spermatozoa against some adverse effect caused by minor fluctuation in temperature imposed by the transfer of cooled straws from cold cabinet to cell freezer as done in the uncontrolled cooling rate ram semen freezing protocol.

Apart from identifying motile and static spermatozoa CASA can also categorize spermatozoa on the basis of velocity of each motile sperm, measure the mean sperm velocity and related sperm track dimensions [20]. The measurement of sperm velocity has been considered as an indirect indicator of mitochondrial function in spermatozoa. During cryopreservation spermatozoal mitochondria undergo damages [27,28] resulting in the decrease of respiratory rate of frozen-thawed ram spermatozoa [29]. In the present study, the mean VCL and VSL of post-thaw spermatozoa were significantly higher in samples cooled at a controlled rate (method 1), compared to samples cooled at an uncontrolled-rate (method 2), VAP of post-thaw was higher in sample

Table 1 Kinetic parameters (CASA) of pre-freezing and post-thawing ram sperm for two freezing methods

	Pre-freezing		Post-thawin						RR. for TM	RR. for PM
	TM (%)	PM (%)	TM (%)	PM (%)	VSL (μm/s)	VAP (μm/s)	VCL (μm/s)	LIN (%)		
Method 1	83.2 ± 8.2[a]	73.1 ± 13.5[a]	74.6 ± 7.8[a]	53.9 ± 10.5[a]	96.1 ± 44.4[a]	107.3 ± 40.5	158.6 ± 51.8[a]	60.59 ± 19	0.89	0.74
Method 2	79.1 ± 8.0[b]	64.8 ± 12.5[b]	61.2 ± 11.1[b]	46.1 ± 6.7[b]	78.4 ± 18.4[b]	91.1 ± 20.3	133.0 ± 28.3[b]	58.9 ± 9	0.77	0.71

[a,b] Different superscripts within columns are significantly differ.

VSL (straight linear velocity, μm/s), VCL (curvilinear velocity, μm/s), VAP (average path velocity, μm/s), LIN = VSL/VCL × 100 (linearity, %), RR (recovery rate), TM (total motility) and PM (progressives motility).

cooled at method 1 but the effect was not significant thereby implying that the magnitude of mitochondrial damage was almost similar under both the cooling treatments.

Ram spermatozoa can tolerate a wide range of freezing rates [10,13,30]. In this study, the overall cooling rate of straws achieved under uncontrolled conditions was approximately at the rate of 0.15°C/min from 25 to 5°C, which was close to the approximate cooling rate of 0.14° C/min on cooling straws from 30 to 5°C in the cold chamber. However, under uncontrolled conditions, cooling over the period of 135 min was not at a linear rate, commencing at the rate of 0.4°C/min from 25°C for 15 min, and continuing at the rate of 0.2°C/for 15 min, 0.13°C/min for 60 min and thereafter progressed at the rate of 0.06°C/min for 45 min up to 5°C. Kumar et al. (2003) observed optimal cryosurvival of ram spermatozoa when cooled at the rate of 0.2°C/min from 22 to 5° C over a period of 90 min followed by freezing at the rate of 30°C/min from 5 to -50°C and concluded that careful control of the cooling and freezing rates are essential for maximal recovery of viable and functional cells.

Conclusions

The results indicated that controlled rate of cooling had significant effect on percentage of motile cells (total and progressive motility) pre-freezing and post-thawing and kinetic parameters of post-thawing ram spermatozoa, compared to uncontrolled rate of cooling prior to programmable freezing. Further research efforts are needed to comparatively assess the fertilizing ability of ram semen frozen by controlled and uncontrolled cooling rate cryopreservation protocols.

Additional material

Additional file 1: Tubular data.

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Author details
[1]Young Researchers Club, Science and Research Branch, Islamic Azad University, Tehran, Iran. [2]Department of Animal Science, Faculty College of Agriculture and Natural Resources, University of Tehran, Karaj, Iran. [3]Department of Clinical Sciences, Faculty of Veterinary Medicine, Shahid Chamran University, Ahvaz, Iran. [4]Animal Breeding Center of Iran.

Authors' contributions
IA carried out the design of the study and performed the cryopreservation process and analyze the sample. HK participated in its design and coordination and helped to draft the manuscript. HN helped to sample collection and cryopreservation process. MB helped to the cryopreservation process. HM make the programmable cooling machine. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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