African Journal of Biotechnology Vol. 10(44), pp. 8965-8969, 15 August, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.1076
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

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

Iraj Ashrafi[1]*, Hamid Kohram[2,3], Hamid Naijian[2] , Mehdi Poorhamdollah[2],Hamid Mirzakhani[4],

[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 (A.I LAB).

Accepted 6 June, 2011

Ram spermatozoa are sensitive to extreme changes in temperature during the freeze-thaw process. 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. Ejaculates were collected using the artificial vagina from four Chal rams and diluted with a Tris-based extender and packed in 0.25 ml straws. Then, the sample was processed according to the two methods; method 1: straws were 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 and then were frozen at the rate of -0.3°C/min from 5 to -10 and -25°C from -10 to -150°C and plunged into liquid nitrogen for storage; method 2: straws were transferred to a 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. A computer-assisted sperm motility analysis was used to analyze sperm motion characteristics. The controlled rate of freezing (method 1) significantly improved the pre-freezing and post-thaw total and progressive motility compared with the uncontrolled rate (method 2). In specific kinetic parameters, method 1 gave significantly higher value for straight linear velocity (VSL) and curvilinear velocity (VCL) in comparison with method 2. There were no significant differences between the two methods for average path velocity (VAP) and linearity (LIN). In conclusion, the controlled rate of cooling conferred better cryopreserving ability to ram spermatozoa compared with the uncontrolled rate of cooling prior to programmable freezing.

Key words: Controlled cooling rate, uncontrolled cooling rate, motility parameters, ram spermatozoa.

INTRODUCTION

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 improves 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 (Anel et al., 2003). Spermatozoa are not adapted to survive cryopreservation and therefore, have variable responses to cooling and rewarming depending both on individual male and species (Holt, 2000; Thurston et al., 2002). Ram spermatozoa are susceptible to various stresses during cryopreservation (Anel et al., 2006). The physiological and functional changes that occur in spermatozoa such as an irreversible reduction in motility, viability and acrosome integrity (Watson, 1995; Salamon and Maxwell, 2000; Medeiros et al., 2002) cause

*Corresponding author. E-mail: i.ashrafi@ut.ac.ir. Tel: +98-9144190815. Fax: +98-261-2246752.

changes similar to capacitation and acrosome reaction in the surviving population (Bailey et al., 2000)

Damage to sperm membranes primarily occurs during the freezing and thawing process over the temperature range -15 to -60°C and not during storage in liquid nitrogen (Mazur, 1965). In the case of ram spermatozoa, most damage occurs between -10 and -25°C; the region of ice crystallisation (Salamon and Maxwell, 2000). 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 (Watson, 1995). O'Neill (National University of Ireland, MSc thesis) observed that semen frozen rapidly (from 5 to -25°C at -5°C/min) had significantly better viability, mitochondrial activity and acrosome integrity than after a slower (-0.5°C /min) freezing rate over the same interval.

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 occur. 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 (Kumar et al., 2003).

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 (Fiser and Fairfull, 1986; Fiser et al., 1986; Pontbriand et al., 1989; Soderquist et al., 1997; Byrne et al., 2000; Gil et al., 2000; Bag et al., 2004).

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 (Kumar et al., 2003)

Computer-aided semen analysis (CASA) technique provides precise and validated objective assessment of sperm motion characteristics (Holt and Palomo, 1996; Verstegen et al., 2002; Joshi et al., 2003) and has been applied for short-term (Kasimanickam et al., 2007) and long-term preservation of ram.

MATERIALS AND METHODS

Animals and semen collection

Semen samples were collected from four mature Chal rams (3 to 4 years) maintained at the Animal Breeding Center Farm of Iran. After collection, the raw 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

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 that varied 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 (Gil et al., 2003). 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 was processed according to the following methods and evaluated pre-freezing and post -thawing.

Semen cooling and freezing methods

Method 1

Straws were 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 the same machine at a rate of -0.3°C/min from 5 to -10°C and -25°C from -10 to -150°C and then the straws were plunged into liquid nitrogen for storage. The thermocouples of the custom-built cool in the machine recorded the chamber temperature via a computer program and drove a motor to open and close the entrance of the nitrogen vapor source.

Method 2

Straw was transferred to a 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 (Bucak et al., 2007).

Semen thawing

Frozen straws were thawed individually at 37°C for 20 s in a water bath for evaluation immediately after thawing (Bucak et al., 2007).

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 of 60 Hz; minimum contrast of 70; low and high static size gates of 0.6 to 4.32; low and high intensity gates of 0.20 to 1.92; low and high elongation gates of 7 to 91; default cell size of 10 pixels; default cell intensity of 80. Semen was diluted (5 µl semen +95 µl extender) in a Tris-based extender (without egg yolk) and evaluated immediately after dilution. 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

Table 1. Percentage of motile cells (total and progressive motility) pre-freezing and post-thawing and recovery rate, evaluated by CASA for spermatozoa frozen using two different methods (means ±S.E.M.).

Method	Total motility (%)			Progressive motility (%)		
	Pre-freezing	Post-thawing	Recovery rate	Pre-freezing	Post-thawing	Recovery rate
Method 1	83.2±8.2[a]	74.6±7.8[a]	0.89	73.1±13.5[a]	53.9±10.5[a]	0.74
Method 2	79.1±8.0[b]	61.2±11.1[b]	0.77	64.8±12.5[b]	46.1±6.7[b]	0.71

NS, Non-significant; a,b different superscripts within columns are significantly different.

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 analysed by analysis of variance with semen samples as replicates, using the following model:

$Y= \mu+\alpha i+eij$

RESULTS AND DISCUSSION

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

In specific kinetic parameters (Tables 3 and 4), method 1 gave significantly higher value for VSL and VCL in comparison with method 2. There were no significant differences between the two methods for VAP and LIN.

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 (Watson, 1995)

Maintenance of sperm function during freezing and thawing depends upon several interrelated factors that include cooling rate, equilibration period and freezing method (Pontbriand et al., 1989; Bailey et al., 2000; Curry, 2000; Anel et al., 2006), but their adverse effects are manifested on thawing. The degree of cryo-damage also depends on several factors (Watson, 1995; 2000) 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 (Watson, 1995; Sanchez-Partida et al., 1999; Curry, 2000). In this study, it was observed that controlled rate of cooling resulted in significantly higher sperm total and progressive motility compared with uncontrolled rate of cooling. 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 had thick consistency >80% and initial motility >2.5 × 10^9 spermatozoa per ml.

The recovery rate indicated that motility is better preserved with method 1. The fact that progressive motility was more affected by the freezing process than individual motility implied 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 (Anel et al., 2003)

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 and measure the mean sperm velocity and related sperm track dimensions (Joshi et al., 2003). The measurement of sperm velocity has been considered as an indirect indicator of mitochondrial function in spermatozoa. During cryopreservation, spermatozoal mitochondria undergo damages (Gillan et al., 2004; Peris et al., 2004) resulting in the decrease of respiratory rate of frozen-thawed ram spermatozoa (Windsor, 1997). In this study, the mean VCL and VSL of post-thaw spermatozoa were significantly higher in samples cooled at a controlled rate (method 1), compared with samples cooled at an uncontrolled-rate (method 2); VAP of post-thaw was higher in sample cooled at method 1, but the effect was not significant thereby, implying that the magnitude of mitochondrial damage was almost similar under both cooling treatments.

Ram spermatozoa can tolerate a wide range of freezing rates (Colas, 1975; Pontbriand et al., 1989; Kumar et al., 2003). In this study, the overall cooling rate of straws

Table 2. Analysis of variance showing the effect of freezing methods on percentage of motile cells (total and progressive motility).

Source of variation	df	TMPrf MS	F	TMPot MS	F	PMPrf MS	F	PMPotMS	F
Method	1	25.215	14.409*	269.340	34.137**	103.335	19.315*	91.260	19.417*
Error	4	1.750		7.890		5.350		4.700	
CV %	-	1.63		4.14		3.35		4.34	

TMPrf, total motility pre-freezing; TMPot, total motility post-thawing; PMPrf, progressive motility pre-freezing; PMPot, progressive motility post-thawing. *P < 0.05;**P < 0.01.

Table 3. Kinetic parameters (CASA) of post-thawing ram sperm for two freezing methods (means ± S.E.M.).

Method	VSL (µm/s)	VCL (µm/s)	VAP (µm/s)	LIN (%)
Method 1	96.1±44.4^{a}	158.6±51.8^{a}	107.3±40.5^{NS}	60.59±19^{NS}
Method 2	78.4±18.4^{b}	133.0±28.3^{b}	91.1±20.3^{NS}	58.9±9^{NS}

NS, Non-significant; a,b, different superscripts within columns are significantly differ.

Table 4. Analysis of variance showing effect of freezing methods on kinetic parameters (CASA of post-thawing ram sperm).

Source of variation	df	VSL MS	F	VCL MS	F	VAP MS	F	LIN MS	F
Method	1	496.935	17.113*	975.375	14.119*	393.660	0.609	1.5	0.035
Error	4	27.460		69.080		79.529		43	
CV %	-	6.01		5.70		8.99		11.02	

*P < 0.05;**P < 0.01.

achieved under uncontrolled conditions was approximately at the rate of 0.15°C per min from 25 to 5°C, which was close to the approximate cooling rate of 0.14°C per min on cooling straws from 30 to 5°C in the cold chamber. However, under the uncontrolled conditions, cooling over the period of 135 min was not at a linear rate, commencing at the rate of 0.4°C per min from 25°C for 15 min and continuing at the rate of 0.2°C per min for 15 min, 0.13°C per min for 60 min and thereafter, progressed at the rate of 0.06°C per 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 per min from 22 to 5°C over a period of 90 min followed by freezing at the rate of 30°C per 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.

Conclusion

The results indicated that the controlled rate of cooling had significant effect on percentage of motile cells (total and progressive motility) pre-freezing, post-thawing and kinetic parameters of post-thawing ram spermatozoa, compared with the 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.

ACKNOWLEDGEMENT

The authors are indebted to Dr. Majid Bahreini for providing the technical assistance to conduct the experiments.

REFERENCES

Anel L, Alvarez M, Martinez-Pastor F, Garcia-Macias V, Anel E, de Paz P (2006). Improvement strategies in ovine artificial insemination. Reprod. Domest. Anim. 41(l 2): 30-42.

Anel L, De Paz P, Alvarez M, Chamorro CA, Boixo JC, Manso A, Gonzalez M, Kaabi M, Anel E (2003). Field and *in vitro* assay of three methods for freezing ram semen. Theriogenology, 60: 1293-1308.

Bag S, Joshi A, Naqvi SM, Mittal JP (2004). Effect of post-thaw incubation on sperm kinematics and acrosomal integrity of ram spermatozoa cryopreserved in medium-sized French straws. Theriogenology, 62: 415-424.

Bailey JL, Bilodeau JF, Cormier N (2000). Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. J. Androl. 21: 1-7.

Bucak MN, Atessahin A, Varisli O, Yuce A, Tekin N, Akcay A (2007). The influence of trehalose, taurine, cysteamine and hyaluronan on ram semen Microscopic and oxidative stress parameters after freeze-thawing process. Theriogenology, 67: 1060-1067.

Byrne GP, Lonergan P, Wade M, Duffy P, Donovan A, Hanrahan JP, Boland MP (2000). Effect of freezing rate of ram spermatozoa on subsequent fertility *in vivo* and *in vitro*. Anim. Reprod. Sci. 62: 265-275.

Colas G (1975). Effect of initial freezing temperature, addition of glycerol and dilution on the survival and fertilizing ability of deep-frozen ram semen. J. Reprod. Fertil. 42: 277-285.

Curry MR (2000). Cryopreservation of semen from domestic livestock. Rev. Reprod. 5: 46-52.

Fiser PS, Fairfull RW (1986). Combined effects of glycerol

concentration, cooling velocity, and osmolality of skim milk diluents on cryopreservation of ram spermatozoa. Theriogenology, 25: 473-484.
Fiser PS, Fairfull RW, Marcus GJ (1986). The effect of thawing velocity on survival and acrosomal integrity of ram spermatozoa frozen at optimal and suboptimal rates in straws. Cryobiology, 23: 141-149.
Gil J, Lundeheim N, Soderquist L, Rodriiuez-Martinez H (2003). Influence of extender, temperature, and addition of glycerol on post-thaw sperm parameters in ram semen. Theriogenology, 59: 1241-1255.
Gil J, Soderquist L, Rodriguez-Martinez H (2000). Influence of centrifugation and different extenders on post-thaw sperm quality of ram semen. Theriogenology, 54: 93-108.
Gillan L, Maxwell WM, Evans G (2004). Preservation and evaluation of semen for artificial insemination. Reprod. Fertil. Dev. 16: 447-454.
Holt WV (2000). Fundamental aspects of sperm cryobiology: the importance of species and individual differences. Theriogenology, 53: 47-58.
Holt WV, Palomo MJ (1996). Optimization of a continuous real-time computerized semen analysis system for ram sperm motility assessment, and evaluation of four methods of semen preparation. Reprod. Fertil. Dev. 8: 219-230.
Joshi A, Naqvi SM, Bag S, Dang AK, Sharma RC, Rawat PS, Mittal JP (2003). Sperm motion characteristics of Garole rams raised for a prolonged period in a semi-arid tropical environment. Trop. Anim. Health Prod. 35: 249-257.
Kasimanickam R, Kasimanickam V, Pelzer KD, Dascanio JJ (2007). Effect of breed and sperm concentration on the changes in structural, functional and motility parameters of ram-lamb spermatozoa during storage at 4 degrees C. Anim. Reprod. Sci. 101: 60-73.
Kumar S, Millar JD, Watson PF (2003). The effect of cooling rate on the survival of cryopreserved bull, ram, and boar spermatozoa: a comparison of two controlled-rate cooling machines. Cryobiology, 46: 246-253.
Mazur P (1965). Causes of Injury in Frozen and Thawed Cells. Fed. Proc. 24:175-182.
Medeiros CM, Forell F, Oliveira AT, Rodrigues JL (2002). Current status of sperm cryopreservation: why isn't it better?. Theriogenology. 57: 327-344.
Peris SI, Morrier A, Dufour M, Bailey JL (2004). Cryopreservation of ram semen facilitates sperm DNA damage: relationship between sperm andrological parameters and the sperm chromatin structure assay. J. Androl. 25: 224-233.
Pontbriand D, Howard JG, Schiewe MC, Stuart LD, Wildt DE (1989). Effect of cryoprotective diluent and method of freeze-thawing on survival and acrosomal integrity of ram spermatozoa. Cryobiology, 26: 341-354.
Salamon S, Maxwell WM (2000). Storage of ram semen. Anim. Reprod. Sci. 62: 77-111.
Sanchez-Partida LG, Windsor DP, Eppleston J, Setchell BP, Maxwell WM (1999). Fertility and its relationship to motility characteristics of spermatozoa in ewes after cervical, transcervical, and intrauterine insemination with frozen-thawed ram semen. J. Androl. 20: 280-288.
Soderquist L, Madrid-Bury N, Rodriguez-Martinez H (1997). Assessment of ram sperm membrane integrity following different thawing procedures. Theriogenology, 48: 1115-1125.
Thurston LM, Siggins K, Mileham AJ, Watson PF, Holt WV (2002). Identification of amplified restriction fragment length polymorphism markers linked to genes controlling boar sperm viability following cryopreservation. Biol. Reprod. 66: 545-554.
Verstegen J, Iguer-Ouada M, Onclin K (2002). Computer assisted semen analyzers in andrology research and veterinary practice. Theriogenology, 57: 149-179.
Watson PF (1995). Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. Reprod. Fertil. Dev. 7: 871-891.
Watson PF (2000). The causes of reduced fertility with cryopreserved semen. Anim. Reprod. Sci. 61: 481-492.
Windsor DP (1997). Mitochondrial function and ram sperm fertility. Reprod. Fertil. Dev. 9: 279-284.