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Effect of post-thaw incubation on semen characteristics of ram spermatozoa cryopreserved under controlled and uncontrolled rate of cooling

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Abstract

Exposure of frozen-thawed spermatozoa to a thermal resistance test reveals damages, which are not apparent immediately after thawing but are useful to assess the fertilizing ability of ram spermatozoa. Our earlier study has shown that cryopreservation of ram spermatozoa under controlled rate of cooling and freezing significantly improves the post-thaw motility and acrosomal integrity, compared to uncontrolled rate of cooling prior to controlled rate of freezing. The purpose of this study was to assess the effect of post-thaw *in vitro* incubation on motion characteristics and acrosomal integrity of ram spermatozoa cryopreserved under controlled (Group 1) and uncontrolled rate of cooling (Group 2) followed by programmable freezing. Semen samples of good initial motility obtained from adult Malpura rams were pooled, diluted to 1 x 10^9 spermatozoa per ml with Egg yolk-Test-glycerol extender and packaged in 0.25 ml straws. Straws representing Group 1 were cooled in a programmable cell freezer from 25 to 5°C at the rate of 0.15°C per minute followed by a holding time of 2 h for equilibration, while straws of Group 2 were allowed to cool slowly up to 5°C and equilibrate for 2 h in the cold cabinet. After equilibration, straws of Group 2 were also loaded in the cell freezer for freezing straws of both the treatment groups simultaneously from 5 to –125°C at the rate of 25°C per minute. Thawing of straws was done at 50°C for 10 seconds and thawed spermatozoa were subjected to a thermal resistance test at 37°C for 4 h. Samples were assessed immediately after thawing and at hourly interval for sperm motion characteristics by computer-aided semen analysis technique. Post-thaw incubated spermatozoa were also evaluated at 0, 1, 2, 3, and 4 h for acrosomal integrity after staining the dried semen smears with Giemsa stain. The % motility, % rapid moving spermatozoa, % linearity and % sperm with normal acrosome were significantly ($P < 0.05$) higher in Group 1 compared to Group 2. The effect of incubation time was also significant ($P < 0.05$) on % motility, fraction of rapid motile spermatozoa, % linearity, curvilinear velocity, average path velocity, straight line velocity, area of sperm head, lateral head displacement and % spermatozoa with normal acrosome. The % motility, % rapid motile spermatozoa, sperm velocity, lateral head displacement and % spermatozoa with normal acrosome progressively declined during 4 h of incubation but the decline in all the traits was less in Group 1 compared to Group 2. The results showed that controlled rate of cooling conferred better cryopreserving ability to ram spermatozoa for post-thaw thermoresistance test compared to uncontrolled rate of cooling prior to programmable freezing.

Keywords: acrosome, frozen ram semen, spermatozoa, sheep.

Introduction

Ram spermatozoa are susceptible to various stresses during cryopreservation (Salamon and Maxwell, 2000; Anel *et al.,* 2006). The physiological and functional changes that occur in spermatozoa include an irreversible reduction in motility, viability and acrosome integrity (Watson, 1995; Salamon and Maxwell, 2000; Medeiros *et al.,* 2002). These alterations result in low fertility following artificial insemination with frozen-thawed semen due to impaired sperm transport through the cervix and short duration of survival in the female reproductive tract (Salamon and Maxwell, 1995a, b). *In vitro* assessment of sperm longevity (thermo-resistance) by incubation at body temperature mimics somewhat the conditions within the female reproductive tract. It has been suggested that motility of freshly thawed samples is not a good indicator for the success of *in vitro* fertilization, whereas longevity after sperm incubation is a much more reliable parameter (Roth *et al.,* 1999). The duration of motility and other sperm characteristics during the post-thaw incubation is an indication of the usability of the semen (Saacke and White, 1972). Moreover, exposure of frozen-thawed spermatozoa to a thermoresistance test reveals damages, which are not apparent immediately after thawing (Aisen *et al*., 2000). The maintenance of a higher motility of sperm during incubation reflects a greater likelihood to survive in the female genital tract, undergo capacitation and fertilize the ovum (Fiser *et al*., 1991).

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Spermatozoa of most species are conventionally cooled to approximately 5°C before freezing to develop maximum resistance to freezing stress. Cooling is the first temperature change known to alter the physical properties of cell membranes (Hammerstedt *et al.,* 1990) and thus is one of the variables potentially affecting the success of artificial insemination with frozen-thawed semen. 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, 1989; Fiser *et al.,* 1986; Pontbriand *et al.,* 1989; Soderquist *et al.,* 1997; Byrne *et al.,* 2000; Gil *et al.,* 2000; Bag *et al.,* 2004; Joshi *et al.,* 2005). 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; Mortimer and Maxwell, 2004; Holt *et al.,* 2007; Kumar *et al.,* 2007) and has been applied for short-term (Briggs *et al.,* 1996; Joshi *et al.,* 2003; Kasimanickam *et al.,* 2007) and long-term preservation of ram spermatozoa (Edward *et al.,* 1995; Moses *et al.,* 1995; Sanchez-Partida *et al.,* 1999; Bag *et al.,* 2002a, b; Joshi *et al.,* 2006). CASA and Giemsa staining techniques have shown in our earlier study that controlled rate of cooling significantly improves the post-thaw motility and acrosomal integrity of ram spermatozoa, compared to uncontrolled rate of cooling prior to programmable freezing of semen in straws (Joshi *et al.,* 2008). The aim of the present study was, therefore, to investigate the effect of post-thaw incubation on motion characteristics and acrosomal integrity of ram spermatozoa cryopreserved under controlled and uncontrolled rate of cooling followed by controlled rate of freezing.

Materials and Methods

The study was conducted at the Central Sheep and Wool Research Institute, Avikanagar which is located at 75-28°E longitude, 26-26°N latitude and at an altitude of 320 m above sea level in the semi-arid zone of subtropical India. Adult Malpura (n = 10) rams maintained in semi-intensive system under preventive and clinical veterinary care, ranging in age from 1.2 to 4.8 years, with mean body weight of 44.14 ± 1.75 kg were used as semen donors for the experiment at the onset of the autumn, when major breeding activities commence at the farm. Malpura is a hardy native sheep of semi-arid tropical India and reared for wool and mutton production. The rams were grazed for 8-10 h daily on natural vegetation interspersed with seasonal shrubs, grasses and forbs (*Achyranthes aspera*, *Commelina forskalaei*, *Eleusine aegypticae* and *Sorghum helepense*). In addition to grazing, the rams were provided 200 g concentrate (65% barley, 32% groundnut cake, 2% mineral mixture and 1% common salt) per head, daily.

On the day of freezing, ejaculates were obtained from donor rams in quick succession by artificial vagina warmed to 42-45°C with water after mounting on the restrained estrus ewe secured in the service crate. The semen samples were evaluated for volume, consistency, wave motion (0-5 scale), concentration (photometrically) and % motile spermatozoa (0-100%; Evans and Maxwell, 1987). Ejaculates having thick consistency, rapid wave motion (4 or 5 in 0-5 scale), 90% initial motility and more than 3 x 10^9 spermatozoa per ml were pooled to avoid differences among individuals and immediately diluted with a TEST-yolk-glycerol extender (Schmehl *et al.,* 1986) at 25°C to a final concentration of 1 x 10^9 spermatozoa per ml (Bag *et al.,* 2002a, b). The time lapse between the first and last ejaculation until dilution was approximately 10 min.

Diluted samples were aspirated into 0.25 ml size French plastic straws (IMV Technologies, L' Aigle, France), sealed with polyvinyl alcohol powder, submerged in water kept in a heavy wall glass tray (200 x 200 x 58 mm dimensions) at 25°C and were divided into two groups. Straws representing Group 1 were loaded vertically in the programmable cell freezer pre-cooled to 25°C (Model Kryo 360-3.3, Planer Products Ltd., Middlesex, UK). The straws representing Group 2 were transferred along with the glass tray containing 1.5 l of water to the cold cabinet pre-cooled to 5°C for slow cooling from 25 to 5°C. A thermometer (Model T 3330, range + 30 to – 200°C, SGA Scientific Inc., USA) was placed within the glass tray with its bulb dipped in water and the temperature was recorded every 15 min from 25 up to 5°C. The uncontrolled cooling of straws was achieved over a period of 135 min in the cold cabinet at the rate of approximately 0.15°C per min, which was followed by equilibration at 5°C for 2 h. Controlled-rate cooling of Group 1 straws was initiated in the cell freezer at the linear rate of 0.15°C per min from 25 to 5°C followed by a holding time of 2 h at 5°C by mimicking the overall cooling rate and equilibration period of the uncontrolled cooling group. The equilibrated straws of Group 2 were loaded into the cell freezer at 5°C by imposing a pause of 10 min after the termination of the holding time of Group 1. The straws of both treatment groups were then simultaneously frozen by continuation of the freezing program from 5 to – 125°C in the cell freezer at the rate of 25°C per min and then plunged into liquid nitrogen for storage until required.

Frozen straws of each treatment group were randomly selected and thawed individually at 50°C for 10 sec in a water bath (Bag *et al*., 2002a, b). Thawed semen samples of each treatment group obtained from 4 straws were pooled and transferred into round bottom screw cap glass tubes (5 ml, 15 x 75 mm) and subjected to a thermoresistance test at 37°C for 4 h. The motion characteristics of spermatozoa immediately after thawing (0 h) and subsequently after 1, 2, 3, and 4 h post-thawing incubation were objectively evaluated by computer-aided semen analysis (CASA) technique using motility analyzer (Hamilton-Thorn Biosciences HTM-IVOS Animal-Version 12.1 M, Beverly, MA, USA).

In this study, all the samples were diluted approximately to the recommended range of 25 x 10^6 sperm/ml with normal saline solution at 37°C and the settings of the analyzer were kept constant prior to CASA analysis for all the observations so as to ensure reliability and repeatability of the results. Moreover, the time lapse between sample dilution and the CASA analysis was kept short, thereby enabling the spermatozoa to survive until completion of analysis (Bag *et al.*, 2004). The semen analyzer was set up as follows: Image type: Phase contrast; Frames at frame rate: 30 at 60/sec; Minimum contrast: 60; Low and high static size gates: 0.8 to 6.25; Low and high static intensity gates: 0.25 to 1.50; Low and high static elongation gates: 20 and 70; Default cell size: 5 pixels; Default cell intensity: 55; Magnification: 1.89 (Kumar *et al.,* 2007). Twenty µl of the diluted samples were placed in a prewarmed Makler counting chamber (10 µm deep, Sefi-Medical Instruments Ltd., Haifa, Israel) and 5 fields per chamber were examined at 37°C in the analyzer. The semen variables included in the analysis were: curvilinear velocity (VCL, µm/sec), average path velocity (VAP, µm/sec), straight line velocity (VSL, µm/sec), % motility, % rapid motility (VAP > 75 µm/sec), % medium motility (10 < VAP < 75 µm/sec), % slow motility (0 < VAP < 10 µm/sec), % linearity, % straightness, % elongation (ratio of minor axis/major axis x 100), area (μm^2, major axis x minor axis), beat frequency (BF, Hz) and amplitude of lateral head displacement (ALH, µm) of the spermatozoa.

Semen samples were also evaluated immediately after thawing (0 h) and subsequently after 1, 2, 3 and 4 h post-thawing incubation for acrosomal integrity using Giemsa stain (E. Merck, India), as described by Watson and Martin (1972). The stained slides were selected in unknown order by the observer to avoid subjective bias. On each slide, 100 stained spermatozoa were randomly chosen and examined with a microscope (Nikon Biophot, Nikon Corporation, Tokyo, Japan) equipped with an oil-immersion lens. The acrosomes were considered normal (intact) when the stain was clearly and evenly distributed over the spermatozoon anterior to the equatorial segment and damaged (non-intact) when the acrosome was swollen, separated or completely lost from the spermatozoon (Watson, 1995).

The study was replicated three times, under controlled and uncontrolled cooling conditions at weekly intervals using the same donor rams for semen collection. The CASA estimates and percentage of normal acrosomes were derived from six observations from the pool of each treatment group and post-thawing incubation time. The data were analyzed by analysis of variance (ANOVA) using the general linear model (GLM) repeated multivariate measures procedure of SPSS 13.0 (SPSS Inc. Headquarters, Chicago, IL, USA) after arc sin transformation of the values in percentage with three levels of week as within subject variable and effects of cooling rates and incubation period as between subject variables for each measure. Values were considered to be statistically significant when $P < 0.05$.

Results

The overall effects of cooling rate and post-thaw incubation on motion characteristics and acrosomal integrity are given in Table 1. Sperm velocities and track dimensions are depicted in Table 2. Controlled rate cooling provided better sustenance of motility and acrosomal integrity of ram spermatozoa compared to uncontrolled cooling. The percent motility, percent rapid moving spermatozoa, percent linearity and percent spermatozoa with normal acrosome were significantly ($P < 0.05$) higher in semen that was frozen under controlled rate cooling. However, no significant differences were observed between controlled and uncontrolled cooling for percent elongation of sperm head, area of sperm head, percent straightness, sperm velocities, lateral head displacement and BF of post-thaw incubated spermatozoa. The effect of incubation time was also significant ($P < 0.05$) on percent motility, percent rapid motile spermatozoa, percent linearity, sperm velocities, sperm head area, ALH and percent spermatozoa with normal acrosome. There was a progressive decline in the percent motility, percent rapid motile spermatozoa, VCL, VAP, VSL, ALH and percent spermatozoa with normal acrosome over the period of post-thaw incubation. The decline was less in samples processed to cryopreservation under controlled rate cooling compared to uncontrolled rate cooling. However, the interactions of cooling rates and incubation period with respect to all the sperm characteristics were not statistically significant.

Table 1. Influence of cooling rate prior to programmable freezing on motion characteristics and acrosomal integrity of post-thaw incubated ram spermatozoa.

	n	% Motility	% Rapid	% Linear	% Straightness	% Elongation	% Normal acrosome
Group (G)							
G1	30	51.65 (60.5)^{a}	40.17 (40.6)^{a}	44.06 (47.3)^{a}	56.59 (68.6)	45.30 (49.5)	50.42 (58.4)^{a}
G2	30	50.72 (58.9)^{b}	38.59 (37.9)^{b}	43.35 (46.1)^{b}	56.19 (67.9)	45.37 (49.6)	49.45 (56.7)^{b}
S.E.M.		0.29	0.22	0.21	0.28	0.26	0.16
Significance		P < 0.05	P < 0.05	P < 0.05	n.s.	n.s.	P < 0.05
Incubation hours (H)							
0 h	12	59.61 (73.4)^{a}	43.83 (46.9)^{a}	44.57 (48.2)^{a}	55.84 (67.4)	45.09 (49.1)	52.25 (61.5)^{a}
1 h	12	54.68 (65.5)^{b}	40.86 (41.8)^{b}	43.96 (47.1)^{ab}	56.69 (68.8)	45.01 (49.0)	50.58 (58.7)^{b}
2 h	12	51.35 (60.0)^{c}	39.84 (40.0)^{c}	43.15 (45.7)^{b}	57.01 (69.4)	45.85 (50.5)	49.46 (56.7)^{c}
3 h	12	47.32 (53.0)^{d}	37.32 (35.7)^{d}	43.50 (46.4)^{b}	56.44 (68.4)	45.55 (50.9)	49.04 (56.0)^{cd}
4 h	12	42.96 (45.4)^{e}	35.05 (32.0)^{e}	43.33 (46.1)^{b}	55.99 (67.7)	45.16 (49.3)	48.34 (54.8)^{d}
S.E.M.		0.46	0.34	0.34	0.44	0.41	0.26
Significance		P < 0.05	P < 0.05	P < 0.05	n.s.	n.s.	P < 0.05
H x G interaction							
0h, G1	6	60.22 (74.3)	44.23 (47.7)	45.10 (49.2)	56.23 (68.1)	44.54 (48.2)	52.74 (62.3)
0h, G2	6	59.00 (72.5)	43.43 (46.3)	44.04 (47.3)	55.45 (66.8)	45.64 (50.1)	51.75 (60.7)
1h, G1	6	55.10 (66.3)	41.55 (43.0)	44.65 (48.4)	56.93 (69.2)	45.12 (49.2)	50.81 (59.1)
1h, G2	6	54.25 (64.9)	40.17 (40.6)	43.28 (46.0)	56.45 (68.4)	44.90 (48.8)	50.35 (58.3)
2h, G1	6	51.37 (60.0)	40.57 (41.3)	43.50 (46.4)	56.98 (69.3)	46.34 (51.3)	50.09 (57.8)
2h, G2	6	51.33 (60.0)	39.11 (38.8)	42.80 (45.2)	57.04 (69.4)	45.35 (49.6)	48.84 (55.7)
3h, G1	6	47.72 (53.7)	38.32 (37.3)	43.79 (46.9)	57.06 (69.4)	45.38 (49.7)	49.55 (56.9)
3h, G2	6	46.93 (52.4)	36.33 (34.1)	43.21 (45.9)	55.82 (67.4)	45.72 (50.2)	48.54 (55.2)
4h, G1	6	43.85 (47.0)	36.18 (33.8)	43.25 (46.0)	55.78 (67.4)	45.10 (49.2)	48.90 (55.8)
4h, G2	6	42.08 (43.9)	33.92 (30.1)	43.41 (46.2)	56.20 (68.0)	45.22 (49.4)	47.78 (53.8)
S.E.M.		0.66	0.48	0.47	0.62	0.58	0.36
Significance		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Overall	60	51.19 (59.7)	39.38 (39.2)	43.70 (46.7)	56.39 (68.3)	45.33 (49.6)	49.93 (57.5)
S.E.M.		0.21	0.15	0.15	0.20	0.18	0.12

Values are the means of the arc sin transformed values in percentage, whereas values in parentheses are actual means of data.
The values within the same column followed by dissimilar letters are significantly different (P < 0.05).

Table 2. Influence of cooling rate prior to programmable freezing on sperm velocity and track characteristics of post-thaw incubated ram spermatozoa

	n	VCL (μm/s)	VAP (μm/s)	VSL (μm/s)	ALH (μm)	BCF (Hz)	AREA (μm^2)
Group (G)							
G1	30	113.79	74.92	52.66	7.29	37.9	7.84
G2	30	112.61	74.32	51.87	7.46	37.5	7.70
S.E.M.		0.99	0.45	0.43	0.07	0.37	0.10
Significance		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Incubation hours (H)							
0 h	12	119.67^{a}	77.00^{a}	55.97^{a}	7.77^{a}	37.25	8.35^{a}
1 h	12	117.37^{a}	76.35^{a}	53.55^{b}	7.60^{ac}	37.57	7.87^{b}
2 h	12	111.56^{b}	74.34^{cd}	51.17^{c}	7.36^{bc}	38.43	7.73^{bc}
3 h	12	110.03^{b}	73.62^{bd}	51.13^{c}	7.16^{bd}	37.54	7.45^{bc}
4 h	12	107.38^{b}	71.79^{b}	49.48^{c}	6.99^{d}	37.74	7.43^{c}
S.E.M.		1.57	0.70	0.67	0.11	0.59	0.15
Significance		$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	n.s.	$P < 0.05$
H x G interaction							
0h, G1	6	119.08	75.50	55.52	7.52	38.04	8.39
0h, G2	6	120.25	78.49	56.42	8.02	36.46	8.31
1h, G1	6	117.68	76.71	54.21	7.47	37.02	7.84
1h, G2	6	117.05	75.99	52.89	7.73	38.12	7.90
2h, G1	6	111.90	74.69	51.83	7.37	38.19	7.89
2h, G2	6	111.22	74.00	50.51	7.34	38.67	7.57
3h, G1	6	110.47	74.64	51.41	7.10	37.87	7.53
3h, G2	6	109.59	72.59	50.86	7.23	37.21	7.37
4h, G1	6	109.83	73.08	50.32	6.99	38.43	7.52
4h, G2	6	104.93	70.51	48.64	6.98	37.06	7.34
S.E.M.		2.22	1.00	0.95	0.15	0.83	0.22
Significance		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Overall	60	113.20	74.62	52.26	7.38	37.71	7.77
S.E.M.		0.70	0.32	0.30	0.05	0.26	0.07

The mean values with different letters within columns differ significantly ($P < 0.05$).

Discussion

Maintenance of sperm function during freezing and thawing depends upon several interrelated factors that includes cooling rate, equilibration period and freezing method (Fiser and Fairfull, 1986, 1989; Pontbriand *et al*., 1989; Maxwell and Salamon, 1993; Salamon and Maxwell, 1995a; 2000; Bailey *et al*., 2000; Curry, 2000; Anel *et al*., 2006) but their adverse effects are manifested on thawing (Holt *et al*., 1992; Holt and North, 1994). The degree of cryo-damage also depends on several factors (Salamon and Maxwell, 1995b, 2000; Watson, 1995, 2000; Naqvi *et al*., 2001), which limit the survival of spermatozoa during incubation (Aisen *et al*., 2000; Bag *et al*., 2002a). 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 the present study it was observed that controlled rate of cooling resulted in significantly higher sperm motility and acrosomal integrity up to 4 h of incubation, compared to uncontrolled rate of cooling. The overall good post-thaw recovery obtained following long-term preservation of ram spermatozoa and maintenance of more than 50% motile sperm after incubation in this study may be attributed to (i) the efficacy of controlled rate freezing protocol and (ii) the criteria of processing only those ejaculates for cryopreservation which have thick consistency, rapid wave motion, 90% initial motility, >3 x 10^9 spermatozoa per ml.

The main factors that can provide erroneous CASA results are settings of the semen analyzer on the basis of sperm dimensions, sperm concentration and image digitization. The role of all these factors is very important for meeting the operational standards and comparability of automated semen analyzers (Davis and Siemers, 1995; Verstegen *et al*., 2002; Mortimer and Maxwell, 2004). CASA technique is accurate for objective measurements of sperm kinematics if care is taken when preparing the semen samples and proper setting of the instrument. The results of CASA are inaccurate if the sperm count for analysis is below 20 X 10^6 sperm/ml or above 50 x 10^6 sperm/ml (Davis and Katz, 1992; 1993). 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 (Holt and Palomo, 1996; Joshi *et al*., 2003; Kumar *et al*., 2007).

Maintenance of higher sperm motility and acrosomal integrity under controlled rate of cooling, compared to uncontrolled rate of cooling suggests its beneficial effect on sperm plasma membrane, which is one of the primary sites for sperm injury sustained during cooling (De Leeuw *et al*., 1990). The mechanism of plasma membrane damage is not completely understood, but there is increasing evidence that membranes are compromised due to reordering of membrane lipids during cooling, thus disturbing the lipid-lipid and lipid-protein associations required for normal membrane function (Bailey *et al*., 2000). The results obtained in the present study revealed that the percent motility and fractions of rapidly motile spermatozoa were significantly decreased during post-thaw incubation. Although there were statistical differences, no biological implications on reproduction efficiency are expected because semen characteristics means between treatments are too closed. Similar results have been reported for ram sperm frozen in mini straws during post-thawing incubation following the CASA analysis (Gil *et al.,* 2000; Bag *et al*., 2002a, Joshi *et al*., 2005). The significant decline in the percentage of motile and rapidly motile sperm during post-thawing incubation may be due to the inability of frozen-thawed spermatozoa to generate enough ATP through mitochondrial respiration as a consequence of mitochondrial ageing (Cummins *et al*., 1994; Viswanath and Shanon, 1997) or the toxic effect of membrane-bound aromatic amino acid oxidase enzyme released by the dead sperm (Shanon and Curson, 1972).

Freezing and thawing causes damage to acrosomal membrane of spermatozoa (Watson, 1975; Watson and Martin, 1975; Pontbriand *et al*., 1989; Aisen *et al*., 2000; Bag *et al*., 2002a; 2004). Evaluation of acrosomal integrity of frozen-thawed ram spermatozoa is useful for improvement of ram semen preservation protocols (Anel *et al*., 2006). Prolonged incubation of sperm also causes deterioration and changes in the acrosome integrity of ram spermatozoa (Pontbriand *et al.*, 1989). Similar changes in the acrosomal integrity of ram spermatozoa were observed in the present study after post-thaw incubation, which was in agreement with our earlier findings (Bag *et al.,* 2004; Joshi *et al.,* 2005). 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.

The measurement of sperm velocity has been considered as an indirect indicator of mitochondrial function in spermatozoa (Graham *et al*., 1984). 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 the present study, the mean VCL, VAP and VSL of post-thaw incubated spermatozoa were higher in samples cooled at a controlled rate, compared to samples cooled at an uncontrolled-rate, but the effect was not significant thereby implying that the magnitude of mitochondrial damage was almost similar under both the cooling treatments. However, after 4 h of incubation significantly higher VCL was observed in samples cooled under a controlled rate suggesting subtle protective effect of controlled rate cooling.

Ram spermatozoa can tolerate a wide range of freezing rates (Entwistel and Martin, 1972; Watson and Martin, 1974; Colas, 1975; Fiser and Fairfull, 1986, 1989;

Pontbriand *et al*., 1989; Kumar *et al*., 2003). In this study, the overall cooling rate of straws achieved under uncontrolled conditions was approximately at the rate of 0.15ºC per min from 25 to 5°C, which was close to the approximate cooling rate of 0.14ºC per min reported by Morrier *et al.* (2002) 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 0.4ºC per min from 25ºC for 15 min, and continuing at the rate of 0.2ºC 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. In our earlier studies we have observed higher post-thaw survival of sperm frozen at –125°C, compared to –25 or –75°C (Bag *et al*., 2002a, b). Similarly, the higher survival of post-thaw incubated sperm under controlled rate of cooling achieved in this study might be attributed to lesser structural damage to frozen-thawed spermatozoa due to uniform cooling at the rate of 0.15°C from 25 to 5°C followed by freezing at the linear rate of 25°C per min from 5 to –125°C.

In conclusion, the results indicated that controlled rate of cooling had significant effect on spermatozoa survivability and acrosomal integrity during post-thaw incubation, 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.

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References

Aisen EG, Alvarez HL, Venturino A, Garde JJ. 2000. Effect of trehalose and EDTA on cryoprotective action of ram semen diluents. *Theriogenology*, 53:1053-1061.

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*, 41:30-42.

Bag S, Joshi A, Naqvi SMK, Rawat PS, Mittal JP. 2002a. Effect of freezing temperature, at which straws were plunged into liquid nitrogen, on the post-thaw motility and acrosomal status of ram spermatozoa. *Anim Reprod Sci*, 72:175-183.

Bag S, Joshi A, Rawat PS, Mittal JP. 2002b. Effect of initial freezing temperature on the semen characteristics of frozen-thawed ram spermatozoa in a semi-arid tropical environment. *Small Rumin Res*, 43:23-29.

Bag S, Joshi A, Naqvi SMK, 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.

Briggs RM, Upreti GC, Smith JF, Duganzich DM. 1996. The effect of inorganic ions on ram sperm motility using computer-assisted sperm analysis (CASA). *Proc NZ Soc Anim Prod*, 56:353-354.

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 freezing ability of deep frozen ram semen. *J Reprod Fertil*, 42:277-285.

Cummins JM, Jequier AM, Kan R. 1994. Molecular biology of the human male fertility: links with ageing, mitochondrial genetics and oxidative stress. *Mol Reprod Dev*, 37:345-362.

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

Davis RO, Katz DF. 1992. Standardization and comparability of CASA instruments. *J Androl*, 13:81-85.

Davis RO, Katz DF. 1993. Operational standards for CASA instruments. *J Androl*, 14:385-394.

Davis RO, Siemers RJ. 1995. Derivation and reliability of kinematic measures of sperm motion. *Reprod Fertil Dev*, 7:857-869.

De Leeuw FE, Colenbrander B, Verkleij AJ. 1990. The role membrane damage plays in cold shock and freezing injury. *Reprod Domest Anim Suppl*, 1:95-104.

Edward AY, Windsor DP, Purvis IW, Sanchez-Partida LG, Maxwell WMC. 1995. Distribution of variance associated with measurement of post-thaw function in ram sperm. *Reprod Fertil Dev*, 7:129-134.

Entwistel KW, Martin ICA. 1972. Effects of composition of diluent, method of addition of glycerol, freezing rate and storage temperature on the revival of ram spermatozoa after deep freezing. *Aust J Biol Sci*, 25:379-386.

Evans G, Maxwell WMC. 1987. Handling and examination of semen. *In*: Salamon S (Ed.). *Artificial Insemination of Sheep and Goats*. Sydney: Butterworths. pp. 93-106.

Fiser PS, Fairfull RW. 1986. Combined effects of glycerol concentration, cooling velocity and osmolality of skim milk diluents on cryopreservation of ram semen. *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.

Fiser PS, Fairfull RW. 1989. The effect of glycerol-related osmotic changes on post-thaw motility and acrosomal integrity of ram spermatozoa. *Cryobiology*, 26:64-69.

Fiser PS, Hansen C, Underhill H, Marcus GJ. 1991. New thermal stress test to assess the viability of cryopreserved boar sperm. *Cryobiology*, 28:454-459.

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 WMC, Evans G. 2004. Preservation and evaluation of semen for artificial insemination. *Reprod Fertil Dev*, 16:447-454.

Graham EF, Schmehl MKL, Deyo RCM. 1984. Cryopreservation and fertility of fish, poultry and mammalian spermatozoa. *In*: Proceedings of the 10th NAAB Technical Conference on Artificial Insemination and Reproduction 1984, Milwaukee, WI. Columbia, MO: National Association of Animal Breeders. pp. 4-24.

Hammerstedt RH, Graham JK, Nolan JP. 1990. Cryopreservation of mammalian sperm: what we ask them to survive. *J Androl*, 11:73-88.

Holt WV, Head MF, North RD. 1992. Freeze-induced membrane damage in ram spermatozoa is manifested after thawing: observation with experimental cryomicroscopy. *Biol Reprod*, 46:1086-1094.

Holt WV, North RD. 1994. Effects of temperature and restoration of osmotic equilibrium during thawing on the induction of plasma membrane damage in cryopreserved ram spermatozoa. *Biol Reprod*, 51:414-424.

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.

Holt WV, O'Brien J, Abaigar T. 2007. Applications and interpretation of computer-assisted sperm analysis and sperm sorting methods in assisted breeding and comparative research. *Reprod Fertil Dev*, 19:709-718.

Joshi A, Naqvi SMK, 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 Hlth Prod*, 35:249-257.

Joshi A, Bag S, Naqvi SMK, Sharma RC, Mittal JP. 2005. Effect of post-thawing incubation on sperm motility and acrosomal integrity of cryopreserved Garole ram semen. *Small Rumin Res*, 56:231-238.

Joshi A, Mathur AK, Naqvi SMK, Mittal JP. 2006. Influence of osmolality of complete semen extender on motion characteristics of frozen-thawed ram spermatozoa. *Asian-Aust J Anim Sci*, 19:1716-1721.

Joshi A, Kumar D, Naqvi SMK, Maurya VP. 2008. Effect of controlled-rate cooling and freezing on motion characteristics and acrosomal integrity of cryopreserved ram spermatozoa. *Cell Preserv Technol*, 6:277-284.

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°C. *Anim Reprod Sci*, 101:60-73.

Kumar D, Joshi A, Naqvi SMK, Kumar S, Mishra AK, Maurya VP, Arora AL, Mittal JP, Singh VK. 2007. Sperm motion characteristics of Garole X Malpura sheep evolved in a semiarid tropical environment through introgression of FecB gene. *Anim Reprod Sci*, 100:51-60.

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.

Maxwell WMC, Salaman S. 1993. Liquid storage of ram semen: a review. *Reprod Fertil Dev*, 14:83-89.

Medeiros CMO, Forell F, Oliveira ATD, Rodriguez JL. 2002. Current status of sperm cryopreservation: why isn't it better? *Theriogenology*, 57:327-344.

Morrier A, Castonguay F, Bailey JL. 2002. Glycerol addition and conservation of fresh and cryopreserved ram spermatozoa. *Can J Anim Sci*, 82:347-356.

Mortimer ST, Maxwell WMC. 2004. Effect of medium on the kinematics of frozen-thawed ram spermatozoa. *Reproduction*, 127:285-291.

Moses DF, de las Heras, MA, Valcarel A, Perez L, Baldassarre H. 1995. Use of computerized motility analyser for the evaluation of frozen-thawed ram spermatozoa. *Andrologia*, 27:25-29.

Naqvi SMK, Joshi A, Das GK, Mittal JP. 2001. Development and application of ovine reproductive technologies: an Indian experience. *Small Rumin Res*, 39:199-208.

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.

Roth T, Bush LM, Wildt DE, Weiss RB. 1999. Scimitar-Horned Oryx (*Oryx dammah*) spermatozoa are functionally competent in a heterologus bovine in vitro fertilization system after cryopreservation on dry ice, in a dry shipper, or over liquid nitrogen vapour. *Biol Reprod*, 60:493-498.

Saacke RG, White JM. 1972. Semen quality tests and their relationship to fertility. *In*: Proceedings of the 4th NAAB Technical Conference on Artificial Insemination and Reproduction, 1972, Madison, WI. Columbia, MO: National Association of Animal Breeders. pp. 22-27.

Salamon S, Maxwell WMC. 1995a. Frozen storage of ram semen. I. Processing, freezing, thawing and fertility after cervical insemination. *Anim Reprod Sci*, 37:185-249.

Salamon S, Maxwell WMC. 1995b. Frozen storage of ram semen. II. Causes of low fertility after cervical insemination and methods of improvement. *Anim Reprod Sci*, 38:1-36.

Salamon S, Maxwell WMC. 2000. Storage of ram

semen. *Anim Reprod Sci*, 62:77-111.

Sanchez-Partida LG, Windsor DP, Eppleston J, Setchell BP, Maxwell WMC. 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.

Schmehl MK, Anderson SP, Vazques IA, Graham EF. 1986. The effect of dialysis of extended ram semen prior to freezing on post-thaw survival and fertility. *Cryobiology*, 23:406-414.

Shannon P, Curson B. 1972. Toxic effect and action of dead sperm on diluted bovine semen. *J Dairy Sci*, 55:614-620.

Soderquist L, Madrid-Bury N, Rodriguez-Martinez H. 1997. Assessment of ram sperm membrane integrity following different thawing procedures. *Theriogenology*, 48:1115-1125.

Verstegen J, Iguer-Ouada M, Onclin K. 2002. Computer assisted semen analyzers in andrology research and veterinary practice. *Theriogenology*, 57:149-179.

Viswanath R, Shannon P. 1997. Do sperm cells age? A review of the physiological change in sperm during storage at ambient temperature. *Reprod Fertil Dev*, 9:321-331.

Watson PF, Martin ICA. 1972. A comparison of changes in the acrosomes of deep-frozen ram and bull spermatozoa. *J Reprod Fertil*, 28:99-101.

Watson PF, Martin ICA. 1974. Regions of the freezing curve causing changes in structure and viability of ram sperm. *Nature*, 251:315-316.

Watson PF. 1975. Use of a giemsa stain to detect changes in acrosomes of frozen ram spermatozoa. *Vet Rec*, 97:12-15.

Watson PF, Martin ICA. 1975. Effects of egg yolk, glycerol and the freezing rate on the viability and acrosomal structure of frozen ram spermatozoa. *Aust J Biol Sci*, 28:153-159.

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,* 60/61:481-492.

Windsor DP. 1997. Mitochondrial function and ram sperm fertility. *Reprod Fertil Dev*, 9:279-284.