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Effect of Cooling Rates on Cryopreserved Hariana Bull Spermatozoa

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ABSTRACT

This experiment was designed to study the effect cooling rate in Hariana bull spermatozoa during cryopreservation. For this purpose, ejaculates were collected from four Hariana bulls using artificial vagina at biweekly interval. The semen sample which possesses more than 70% progressive motility and above 500 million/ml spermatozoa concentration was subsequently subjected to processing for liquid nitrogen (LN_2) vapour freezing. Semen samples were extended in Tris-egg yolk-glycerol extender and split into 3 parts. These split samples were subjected to cooling from 35°C to 4°C temperature at 2.21°C/min (rapid), 0.48°C/min (moderate) and 0.25°C/min (slow cooling). Each cooled part was than subjected to LN_2 vapour freezing in a programmable biological freezer followed by plunging into LN_2. Samples were evaluated at pre-freeze and post-thaw stages for per cent live spermatozoa, per cent progressively motile spermatozoa, per cent Hypo-osmotic swelling test (HOST) positive spermatozoa and percent spermatozoa with intact acrosome by Fluorescein isothiocyanate- pisum sativum agglutinin (FITC-PSA) method. Maximum damage of cooling was observed in rapid cooling whereas damage found to be minimal in slow cooling protocols.

Keywords: Bull semen, cryopreservation, hariana, pre-freeze, post thaw

Dairy industry is the largest growing industries in India and country ranked as highest milk producer in the world. However, need genetic improvement of their main stray of milch animals i.e. cows and buffaloes. The best tool available for this improvement is the use of Artificial Insemination which requires the use of cryo-preserved semen (Andrabi and Maxwell, 2007).

Sperm cells tolerate a wide range of temperature variation during cryopreservation and subsequently at thawing. These temperature changes results in various degrees of damage to sperm cells, cellular damage of different degrees of severity is induced by distinct mechanisms at each of the cryopreservation phases, and the functional state of the frozen-thawed cells is the result of the injuries accumulated (Medeiros *et al.,* 2002).

The decrease in temperature from normothermic to 4°C reduces cellular metabolic activity and permits some expansion of the cell lifespan. However, because sperm have very limited biosynthetic activity and depend mostly on catabolic processes to function (Hammerstedt *et al.,* 1997) the remaining metabolic activity of the sperm cell leads to death because of the intrinsic aging process. Further, reports suggest that maximum damage to sperm cells happens during lowering down of sperm cells from 4°C to – 10°C (Kumar *et al*., 2003).

Keeping the importance of temperature reduction as an essential step for cryopreservation the present study was designed to access the most suitable cooling rate for better use of cryopreservation techniques.

MATERIALS AND METHODS

Experimental animals

Four Hariana bulls between 6.5–7.5 years of age and weighing around 450-500 kg reared at Semen Biology Lab (DUVASU, Mathura) were used as semen donor. After morpho-physiological evaluation and those confirming the

criteria (apparently normal appearance, > 70% progressive motility (PM) and > 500 millions/ml sperm concentration) for freezing were subjected to extension with Tris-egg yolk-glycerol (at 35°C) to fix the concentration as 80 million spermatozoa/ml. For each bull, six ejaculates (n=6) were considered for drawing out the results. The overall value represents data of 24 observations.

Cooling protocols and equilibration

The extended semen was divided in three parts (C_1, C_2 and C_3). All these three portions were subjected to three cooling regimes. Sample C_1 was cooled from 35ºC to 4ºC in 14 minutes (2.21ºC/min), C_2 in 65 minutes (0.48ºC/min) and C_3 in 122 minutes (0.25 ^{0}C/min) The cooling of sample C_1, C_2 and C_3 were termed as rapid, moderate and slow cooling. All cooled samples were kept for equilibration at 4ºC for 4 hr.

Filling and Sealing of Straws

French mini straws (0.25 ml, 135 mm length and 2 mm diameter, IMV) of different colors were used for filling of semen using automatic straw filling and sealing machine (IMV, France). All procedure of filling and sealing was done avoiding temperature variation.

Freezing of semen straws

Each cooling protocols was subjected to freezing (4ºC to -10ºC @ of 5ºC/min, from -10ºC to -100ºC at the rate of 40ºC/min and from -100ºC to -140ºC @ of 20ºC/min) under liquid nitrogen vapors in a programmable biological freezer followed by plunging of straws in LN_2 canister for storage. The frozen semen was thawed in thawing unit (IMV, France) maintained at 37ºC withholding time kept as 45 seconds. Semen samples were evaluated at pre-freeze and post-thaw stages for various seminal attributes viz progressive motility, viable spermatozoa (Bloom, 1950; Hancock, 1951) HOS positive spermatozoa (Correa and Zavos, 1994) and spermatozoa with intact acrosome (FITC-PSA) as per the method of Mendoza *et al.,* (1992) with little modification.

Acrosomal Integrity (FITC-PSA assay)

Sperm suspension used for preparation of smear for FITC-PSA were washed (centrifugation with 1000 rpm for one minutes) 2-3 times with Dulbecco's Phosphate-Buffered Saline (protein, calcium and magnesium free DPBS) solution. The smear prepared was air dried and dipped in absolute methanol for 15 minutes and then allowed to dry rapidly. Methanol treated smears were then incubated for 30 minutes at room temperature in dark moisture chamber with FITC labelled PSA (50 µg/ml in DPBS). The slides were then washed in distilled water (rinsed with distilled water and further dipped in distilled water for 15 minutes) to remove unbound probe. The smear was air dried and examined immediately, without mounting, in an epifluorescence microscope. At least 200 spermatozoa were counted in the prepared smear and differentiated according to the Fluorescence pattern of their acrosome as spermatozoa with intact acrosome (bright apple green fluorescence acrosome) and damaged acrosome (no fluorescence or fluorescence at equatorial segment of acrosome (Fig. 1).

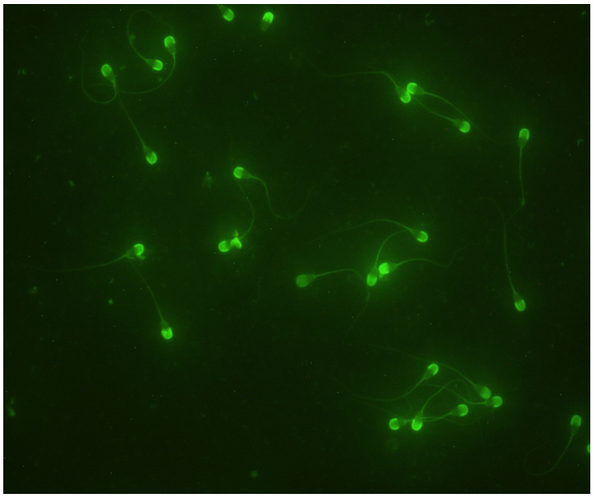

Fig. 1: Spermatozoa showing fluorescence pattern of their acrosome

Statistical analysis

The raw data were analyzed using statistical package SPSS V 23 and expressed in the form of mean and standard error of mean. Analysis of variance (ANOVA) was used as test of significance.

Table 1: Various seminal attributes in the fresh ejaculates of Hariana bull

Seminal Attributes	Bull No 623	Bull No 580	Bull No 533	Bull No 531	Overall
pH	6.50 ± 0.09	6.60 ± 0.07	6.50 ± 0.09	6.50 ± 0.10	6.52 ± 0.04
	(6.20 – 6.80)	(6.40 – 6.80)	(6.20 - 6.80)	(6.20 – 6.80)	(6.20 – 6.80)
Volume	6.40^{a} ± 0.26	6.33^{a} ± 0.26	4.58^{b} ± 0.56	7.03^{a} ± 0.60	6.09 ± 0.28
(ml)	(5.80 – 7.20)	(5.60 ± 7.20)	(3.50 – 7.20)	(4.80 – 9.40)	(3.50 – 9.40)
Sperm concentration	1356.67 ± 122.14	1341.33 ± 117.42	1110.50 ±233.66	1057.50 ± 76.45	1216.50 ± 74.99
(millions/ml)	(987.00 – 1822.00)	(960.00 - 1700.00)	(787.00 –2252.00)	(816 – 1224)	(787 – 2252)
Mass motility	4.33 ± 0.17	4.08 ± 0.15	4.00 ± 0.18	4.17 ± 0.17	4.15 ± 0.08
(0 - +5 scale)	(4.00 – 5.00)	(3.50 – 4.50)	(3.50 – 4.50	(3.50 – 4.50)	(3.5 – 5)
Viable spermatozoa	96.50 ± 0.56	96.00 ± 0.86	93.67 ± 1.60	93.17 ± 1.14	94.83 ± 0.60
(Percentage)	(95.00 – 98.00)	(93.00 – 98.00)	(88.00 – 98.00)	(90.00 – 97.00)	(88 – 98)
Progressively motile spermatozoa	92.50 ± 1.12	92.50 ± 1.12	88.33 ± 2.47	89.17 ± 1.54	0.87
(Percentage)	(90.00 – 95.00)	(90.00 – 95.00)	(80.00 – 95.00)	(85.00 – 95.00)	(80– 95)

Means bearing different superscripts in row differ significantly ($p<0.05$).

Table 2: Effect of cooling on different seminal attributes at pre-freeze and post-thaw stages

Parameter (%)	Stages of evaluation	Cooling protocol		
		Rapid cooling	Moderate cooling	Slow cooling
Progressive motility	Pre freeze	46.67^{c} ± 1.72	56.46^{b} ± 1.92	65.42^{a} ± 2.08
	Post thaw	36.04^{c} ± 1.47	44.58^{b} ± 1.59	58.38^{a} ± 1.79
Viability	Pre freeze	53.17^{c} ± 1.78	64.08^{b} ± 1.83	73.58^{a} ± 2.00
	Post thaw	43.50^{c} ± 1.49	54.13^{b} ± 1.58	62.50^{a} ± 1.61
HOS Positive	Pre freeze	49.58^{c} ± 1.53	61.21^{b} ± 1.69	68.83^{a} ± 1.79
	Post thaw	39.63^{c} ± 1.48	50.38^{b} ± 1.76	59.25^{a} ± 1.68
Acrosomal integrity	Pre freeze	52.75^{c} ± 1.82	64.67^{b} ± 1.88	70.17^{a} ± 1.75
	Post thaw	43.00 ± 1.43	55.00 ± 1.73	61.00 ± 171

Means bearing different superscripts in rows differ significantly ($p<0.05$).

RESULTS AND DISCUSSION

The present study was conducted to evaluate the effect of different cooling [rapid (2.22ºC/min), moderate (0.48ºC/min), and slow (0.25ºC/min)] rates on various seminal attribute. In the fresh ejaculates, a significant ($p<0.01$) difference between the bulls was observed for seminal volume whereas the other attributes remain non-significant (Table 1). The individual seminal volume may vary and is attributed to the difference in age, size, frequency of collection, level of teasing, nutrition, season etc. (Hafez, 2000).

Hence, in subsequent comparison overall mean values (n=24) were considered. The fresh semen extended in Tris-egg yolk-glycerol and subjected to vapor freezing. At pre-freeze stage, PM, Viability, HOS positive spermatozoa and spermatozoa with intact acrosome differed significantly ($p<0.05$) and this trend follow at post-thaw stage for all the parameters. The maximum post-thaw results for PM, Viability, HOS positive spermatozoa and spermatozoa with intact acrosome were observed with slow cooling while minimum results for these parameters were with rapid cooling (Table 2).

Cooling, freezing, and thawing exert physical as well as chemical stresses on the sperm membrane (Chatterjee *et al*., 2001). When sperms in suspension are cooled, frozen and then thawed a number of critical physical changes occur which affects motility, morphology, sperm chromatin alterations, membrane permeability changes, membrane destabilization and generation of reactive oxygen species (ROS).

The decrease in motility following cooling is indirectly a reflection of mitochondrial damage of spermatozoa as they could not derive their ATP need by oxidative phsophorylation (Ruiz-Pesini *et al.*, 2001; Januskauskas and Zillinskas, 2002) resulting in a poor motility. Our result is in agreement with the finding of other reports (Martin, 1965; Gilbert and Almquist, 1978; Fiser and Fairfull, 1986; Evans and Maxwell, 1987; Mathur *et al.,* 1991; Dhami and Sahani, 1993, 1994; Talevi *et al.,* 1994; Parks, 1997) where a cooling down of spermatozoa from 30^{o}C to 5^{o}C in a rapid manner (taking less time to reach 5^{o}C) has resulted in a significantly low pre-freeze motility compared to slow cooling.

The percentage of viable spermatozoa which is also an indicator of sperm intactness as well as had a correlation with fertility did not provide the functional intactness of the membrane. HOS test is a further indicator of potential sperm function and has been related to the fertilizing ability of spermatozoa (Barratt *et al.,* 1989, Avery *et.al*, 1990). Moreover, intactness is essential for the various physiological process of fertilization (Henkel *et al*., 1993, Lechniak *et al*., 2002). In the present study viability and HOS positive spermatozoa were maximum with slow cooling. Our findings are in agreement with the findings of Keel and Webster (1990).

Another test that can be a close indicator of fertilizing potential of sperm was intactness of acrosome. A cryopreserved semen must have sufficient spermatozoa with a full range of functions (the ability to survive, reach the oviduct, interact with the oviductal epithelium, attach to and penetrate the zona pellucida, and interact with the oocyte) to achieve satisfactory fertility.

A true acrosome reaction, which precedes fertilization, occurs only in live, intact spermatozoa. Several techniques have been proposed to differentiate acrosome intact from acrosome-reacted spermatozoa (Talbot and Chacon, 1981). Merely to measure the total acrosomal integrity is not useful since a non-viable cell will deteriorate rapidly and is not relevant to further consideration. Thus test determining viable and acrosomally intact spermatozoa is a better indicator for fertilizing ability. In the present study, we employed FITC – PSA which differentiate the intact acrosome with reacted one (Jankovicova *et al*., 2006; Jankovicova *et al.,* 2008) and hence is a more accurate index for intact acrosome. Our result for effect of cooling revealed that slow cooling inflict less cold shock compare to rapid and moderate cooling at pre -freeze stage and such finding has been reported by other workers too (Bryne *et al.,* 2000; Nur *et al.,* 2011). Thus our study demonstrates that for cryopreservation of Hariana bull semen, slow cooling from 35^{o}C to 4^{o}C in 122 minutes, followed by equilibration of 4 hr duration and subsequent freezing of semen from 4^{o}C to -10^{o}C @ of 5^{o}C/min, from -10^{o}C to -100^{o}C @ of 40^{o}C/min and from -100^{o}C to -140^{o}C @ of 20^{o}C/min is a better choice compare with the other cooling and freezing rate.

CONCLUSION

Cooling rates have profound effect on various seminal attributes including viability, motility, and acrosomal integrity of spermatozoa. In this context slow rate of cooling followed by four hours of equilibration and finally freezing (4^{o}C to -10^{o}C @ of 5^{o}C/min, from -10^{o}C to -100^{o}C @ of 40^{o}C/min and from -100^{o}C to -140^{o}C at the rate of 20^{o}C/min, followed by plunging in LN_2) resulted as a better protocol for cryopreservation of Hariana bull semen. Maximum damage to spermatozoa occurs during the cooling process (35^{o}C to 4^{o}C) and the slow cooling rate was found better. Acrosomal integrity by FITC- PSA and subjective assessment compare to routine tests (viability and progressive motility) and can predict fertility status more closely than the routine tests.

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