EFFECT OF INCUBATION PERIOD AT 30°C AND AT 40°C ON POST-THAW CHARACTERISTICS OF FROZEN SEMEN OF HF CROSSBRED BULLS*

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ABSTRACT

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To observe the effect of post-thaw incubation periods, frozen semen samples of six HF crossbred bulls were thawed at 37°C for 30 seconds and thawed semen was evaluated immediately at 0 min and after 15, 30, 45 and 60 min of incubation at 30°C and 4°C, respectively. There was non-significant difference in post-thaw motility between 30°C and 4°C at each incubation period except at 15 min of incubation. Non-significant difference was recorded in post-thaw viability during incubation at 15, 30, 45 and 60 min between 30°C and 4°C temperature. Higher proportion ($P< 0.05$) of HOST positive spermatozoa were recorded at 30, 45 and 60 min of incubation at 4°C than at 30°C. There was non-significant difference in proportion of spermatozoa with intact acrosome between 30°C and 4°C at all incubation periods. Post-thaw motility was recorded nearly 50 per cent at 15 min of incubation with both incubation temperatures and therefore, it may be suggested that frozen-thawed semen may be used for insemination up to 15 min by keeping frozen-thawed semen straw either at 4°C or in warm water at 30°C after thawing at 37°C for 30 seconds.

Key words: Crossbred bulls, frozen semen, post-thaw semen characteristics.

INTRODUCTION

Many AI workers carry frozen-thawed semen straw either in pocket or in thermos flask containing ice as user-friendly alternative mode of semen transport to the doorstep of dairy farmers to carry out insemination under field conditions; although such practice must be discouraged. Frozen semen with egg-yolk-base extender is universally thawed at 37°C for 30 seconds. This thawing protocol of frozen semen resulted in the highest survival of spermatozoa in terms of motility and acrosome integrity as compared to thawing at different temperature with different time (Senger *et al.*, 1976; Nur *et al.*, 2005). However, information on effect of incubation of frozen-thawed semen at lower temperature (< 37°C) on post-thaw characteristics of spermatozoa in crossbred bulls is scanty. Study on effect of duration of incubation period at different temperature on post-thaw seminal characteristics would provide an idea regarding length of time at which frozen-thawed semen may be used effectively for insemination under field conditions. Therefore, present study was undertaken to evaluate the effect of incubation period at 30°C and at 4°C on post-thaw characteristics of frozen semen of HF crossbred bulls.

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MATERIALS AND METHODS

The present study was conducted on six HF crossbred bulls aged between 4 to 6 years and maintained at Central Semen Station, Anjora, Durg (C.G.). The bulls were maintained in identical feeding and management regimes according to minimum standard protocol (MSP) of Government of India. Semen was collected twice per week from the bulls using an artificial vagina. Ejaculates having 70 per cent or more initial progressive motility and concentration of 500 x 10^6 spermatozoa/ml or more, were subjected to processing for cryopreservation, else discarded. Semen was diluted in Tris–yolk–citric acid–fructose-glycerol (7.0%) dilutor to contain 20 million spermatozoa per dose in French mini straw (0.25 ml). After equilibration of 4 hrs at 4°C, cryopreservation of semen was carried out in liquid nitrogen vapours using Programmable Bio freezer (Digit Cool 5300, IMV, France) and stored in liquid nitrogen. Two straws of frozen semen of same batch of an individual bull was thawed at 37°C for 30 seconds; thawed semen was pooled and post-thaw characteristics of frozen semen, viz. motility, viability, acrosomal integrity and HOST positive spermatozoa were observed at 0 min. Two aliquotes of each sample were prepared and each one was incubated at 30°C (water bath) and 4°C (crushed ice) for 15, 30, 45 and 60 minutes; and post-thaw semen characteristics of frozen semen, viz. motility, viability, acrosomal integrity and HOST positive spermatozoa were observed after each incubation period. Post-thaw motility was estimated under 200x magnification of phase contrast microscope objectively. Post-thaw sperm viability was determined using modified differential staining technique with Eosin-Nigrosin stain (Campbell *et al.*, 1953). The acrosomal integrity (per cent normal acrosome) in frozen-thawed semen was estimated in Giemsa stained semen smears (Watson, 1975). Hypo-osmotic swelling test (HOST) positive spermatozoa were calculated using millipore water (osmolarity 100 m.osm., Bhosrekar, 2005). Paired't' test was applied to compare different post-thaw characteristics of frozen semen among different time intervals between two incubation periods (Snedecor and Cochran, 1967) using SPSS soft-ware version 10.

RESULTS AND DISCUSSION

The post-thaw characteristics of frozen semen of six HF crossbred bulls at different incubation periods are presented in Table. Post-thaw motility, sperm viability, percentage of HOST reacted spermatozoa and percentage of spermatozoa with intact acrosome of frozen-thawed semen consistently decreased, as the incubation period increased at both the incubation temperatures. There were non-significant differences in post-thaw motility, viability and acrosomal integrity at each incubation period between 30°C and 4°C temperature except that post-thaw motility was significantly lower ($P< 0.05$) at incubation temp of 4°C than 30°C. However, significantly ($P< 0.05$) higher proportion of HOST positive spermatozoa were recorded at 30, 45 and 60 min of incubation at 4°C than at 30°C.

Present results demonstrated that there was gradual reduction in post-thaw motility, viability, proportion of HOST positive spermatozoa and proportion of spermatozoa with intact acrosome as the post-thaw incubation period was increased at both incubation temperatures. Present finding is in agreement with the observation reported by Dhami *et al.* (1994), Dhami and Sahni (1995), Pramanik (1996) and Pathak (2008); however, post-thaw incubation temp was 37°C in these studies. Perusal of literature showed lack of information on post-thaw motility at incubation temp of 30°C. Present observation of HOST positive sperm percentage at 0 min at 37°C is higher than reported by Shrivastava and Kumar (2006) who reported 21.88 ± 0.44 and 21.00 ± 0.57 per cent HOST positive sperm in frozen thawed semen of HF and HF x Hariana cross bull, respectively. Similarly, Mandal *et al.* (2006) could observe 33.96 ± 1.78 per cent spermatozoa showing swelling response in hypo-osmotic solution in frozen thawed semen from Frieswal bulls. Higher percentage of HOST positive spermatozoa in present study could be due to i) use of water as hypo-osmotic solution with osmolarity of 100, whereas previous workers used hypo-osmotic solution with osmolarity of 150 and ii) variations in duration of exposure of spermatozoa to hypo-osmotic solution. The present finding of higher percentage of HOST positive spermatozoa approximates with early report (Pathak,

2008), in which hypo-osmotic solution with osmolarity of 100 was used at incubation temp of 37°C.

It is interesting to note that proportion of live spermatozoa was recorded lesser than proportion of post-thawed motile spermatozoa in the present study. This discrepancy between these two characteristics might be due to staining of live spermatozoa owing to alteration in characteristics of plasma membrane, which are common consequences of semen cryopreservation. The concentration of eosin was reduced to one-fourth of original composition of stain for live and dead count of spermatozoa and staining period was reduced to 10 seconds to overcome the problem of staining of live spermatozoa in frozen-thawed semen. Despite these modifications in staining procedure, proportion of post-thawed viable spermatozoa was recorded lesser than proportion of post-thawed motile spermatozoa in the present study. Hence, it may be suggested that alternative method should be developed for differential staining of frozen-thawed semen for study of viability of spermatozoa.

There is scarcity of liquid nitrogen containers in developing countries like India. Inseminators use to carry frozen-thawed semen straw either in pocket (DeJarnette *et al.*, 2000) or in thermos flask containing ice as user-friendly alternative mode of semen transport to insemination site. Generally, insemination is accomplished after a time interval ranging from 15 to 60 min under field conditions with this mode of semen transport. Present experiment was conducted to assess the post-thaw quality of frozen semen in relation to post-thaw length of time at which semen may be effectively used for insemination. The visual estimation of percentage of progressively motile spermatozoa is the most common method of semen analysis conducted in the laboratory but it is not consistently highly correlated with fertility (Graham and Moce, 2005). In addition to progressive motility, plasma membrane integrity and acrosomal integrity are the tests of choice to assess post-thaw quality of frozen semen.

Frozen semen samples having 40 per cent and above post-thaw motility are considered suitable for the use of artificial insemination (Mandal *et al.*, 2010); however, some scientists reported that frozen semen sample having 30 per cent post-thaw motility with more than 65 per cent normal spermatozoa with respect of acrosome, mid piece and tail in combination is regarded as good quality semen (Bhuiyan, 1998). Singh and Pant (1999) reported 54.2, 50.8 and 44.2 per cent conception rate when post-thawed semen was inseminated within 15 min, within 1 hr and within 2 hrs of thawing, respectively. They concluded that frozen thawed semen straws can be used for AI by keeping them either wrapped in cotton or in a thermos flask containing water at 37°C, without marked loss in fertility provided the animals were inseminated within one hour post-thaw. As a matter of fact that it is the number (10-12 million per dose) of motile spermatozoa at the time of AI that matters for obtaining conception. Presently, frozen semen straws contain 20 million spermatozoa as per minimum standard protocol of Government of India and with this concentartion of sparmatozoa, 50 per cent and above post-thaw motility is considered suitable for the use of artificial insemination. Post-thaw motility was recorded nearly 50 per cent at 15 min of incubation, both at 30°C and 4°C temperature in the present study with viability > 44 per cent, HOST posltive spermatozoa > 59 per cent and acrosomal integrity > 48 per cent. Therefore, it may be suggested that frozen-thawed semen may be safely used for artificial insemination up to 15 min by keeping frozen-thawed semen straw either at 4°C or at warm water at 30°C after thawing of frozen semen at 37°C for 30 seconds.

Table. Effect of incubation period on post-thaw semen characteristics in Holstein-Friesian crossbred bull

Incubation Time (Min)	Post-thaw motility		Viability		HOST		Acrosomal Integrity	
	30°C	4°C	30°C	4°C	30°C	4°C	30°C	4°C
0	58.33±1.77	58.33±1.77	52.0±2.60	52.0±2.60	65.05±2.01	68.38±1.57	60.11±2.59	60.11±2.59
15	52.61±1.56	48.61±1.93	45.38±2.84	44.66± 2.01	59.72±1.98	62.61±1.43	53.38 ±2.68	48.83±2.53
30	49.16±1.60*	44.16±2.00	40.61±2.7	39.83±2.04	54.61±1.90	58.88±1.64*	47.88±2.62	44.88±2.76
45	39.66±1.42	40.11±2.07	35.38±2.73	35.27±2.13	48.94±1.62	54.61±1.67*	44.66±2.44	40.83±2.70
60	37.44 ±2.13	36.44±2.07	30.27±2.66	29.16 ±1.72	43.61±1.70	48.77±1.47*	39.61±2.08	37.61±2.75

Data with superscript * differed significantly (P < 0.05)

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