EFFECT OF LONG-TERM STORAGE ON POST-THAW CHARACTERISTICS OF FROZEN SEMEN OF HF CROSSBRED BULLS*

NOOPUR SHRIVATAVA[1], M.K. AWASTHI[2], A. K. NAIR[3], R.P. TIWARI[4], M.R. POYAM[5] AND G.K. MISHRA[6]

VETERINARY GYNAECOLOGY & OBSTETRICS DEPARTMENT

COLLEGE OF VETERINARY SCIENCE & A.H., ANJORA, DURG (C.G.)

Received : 02.06.15

ABSTRACT

Frozen semen sample of three HF crossbred bulls stored for 4, 8, 12, 16, 20 and 24 months were used to study the effect of long-term storage on post-thaw characteristics of frozen semen. Three frozen semen straws of same batch from individual bull were thawed at 37°C for 30 seconds; pooled and post-thaw motility, viability, acrosomal integrity and proportion of spermatozoa with intact functional membrane were recorded for different storage period. Post-thaw semen characteristics were compared among different storage time in individual bull. Post-thaw motility, viability, percentage of spermatozoa with intact acrosome and percentage of spermatozoa with intact functional membrane in frozen-thawed semen of all three bulls consistently and significantly decreased from 4 months onward at 8, 12, 16, 20 and 24 months ($P< 0.01$) during long-term storage of semen. One bull recorded less than 50 per cent post-thaw motility at 8 months of storage period onward up to 24 months in contrast to other two bulls with post-thaw motility ≥ 50 per cent up to 16 months of storage. From the present study, it may be concluded that post-thaw characteristics of frozen semen stored for long period of time varied not only among different storage time but also among the bulls. Hence, assessment of post-thaw semen quality should be made prior to dispatch of long-term stored frozen semen to be used for insemination under field conditions.

Accepted : 02.12.15

Key words: Crossbred bulls, Frozen semen, Long term storage, Post-thaw semen characteristics

Frozen semen of crossbred bulls is required for intense breeding to obtain crossbred cows with genetic potential for higher milk production on commercial basis and to prevent greater exotic inheritance in resultant progeny. High viability and motility of spermatozoa are important factors for successful artificial insemination as significant correlation has been reported between the post-thaw sperm viability and the subsequent conception rate (Wongtawan *et al.*, 2006). Length of the storage period of frozen semen may have influence on quality and fertility of frozen-thawed bull semen (Larsson and Rodriquez-Martinez, 2000). Moreover, freezing and subsequent thawing procedures render the remaining surviving spermatozoa physiologically different from spermatozoa before cryopreservation. Cryo-injury and handling during storage result in large number of infertile sperms due to physical, osmotic and chemical damages. Freezing affect not only post-thaw motility but may have functional and structural changes on sperm, which might affect the fertility of semen. Fertility could be negatively affected by long-term storage of frozen semen in liquid nitrogen (Haugan *et al.*, 2007) with decrease in motile spermatozoa and non-return rate. Therefore, present study was

* Part of MVSc Thesis.

[1]Post-graduate student,
[2]Professor and corresponding author,
[3]Veterinary Asstt. Surgeon, Central Semen Station, Livestock Development Deptt.,
[4] Professor& Head
[5, 6] Assistant Professor
[2]Corresponding Author,
Corresponding Addresses: B-16, Adarsh Nagar, DURG (CG) 491 001; Tel. ®0788-2211818, Mobile: 075871-69135, 098271-09150, Fax: 0788-2623326,
E-mail address: awasthimk1963@rediffmail.com

undertaken to assess the effect of long-term storage on post-thaw characteristics of frozen semen of HF crossbred bulls.

The experimental study was conducted on three HF crossbred bulls aged between 4 to 6 years and maintained at Central Semen Station Anjora (Durg). 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. Frozen semen samples of three HF crossbred bulls stored for 4, 8, 12, 16, 20 and 24 months were used to study the effect of long-term storage on post-thaw characteristics. Three straws of frozen semen sample of same batch and storage period of each bull were thawed at 37°C for 30 seconds, semen samples were pooled and 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 with modified method of Watson (1975). The functional membrane integrity was evaluated based on hypo-osmotic swelling test (HOST) using millipore water (osmolarity 100 m.osm., Bhosrekar, 2005). The mean of three observations of each parameter was taken for statistical analysis. Post-thawed semen characteristics were compared among different storage time in individual bull. Data were subjected to two-way ANOVA for analysis of variance and significant difference was analyzed by Duncan's multiple range test (Snedecor and Cochran, 1967).

Post-thaw motility, sperm viability, percentage of spermatozoa with intact functional membrane and percentage of spermatozoa with intact acrosome of frozen-thawed semen of all three bulls consistently and significantly ($P< 0.01$) decreased from 4 months onward at 8, 12, 16, 20 and 24 months during long-term storage of semen. The present findings are in agreement with the previous observations that reported a significant decrease in proportion of motile spermatozoa with storage on the 4, 5 and 6 years and viability with storage on the 3, 4, 5 and 6 years (Malik *et al.*, 2015). The fertility of frozen semen could be negatively affected by a long storage period in liquid nitrogen of more than 1950 days of storage (Haugan *et al.*, 2007). With alteration in integrity of acrosomal membrane during long-term storage of frozen semen, the percentage of spermatozoa with intact functional membrane also reduced. Present finding is consistent with report of Lemma (2010) who documented that high proportion of spermatozoa in frozen-thawed semen with altered acrosome also lost functional membrane integrity.

The reduction in post-thaw quality of frozen semen with increasing storage time in the present study might be due to detrimental effects of cryopreservation including induction of premature acrosomal reaction, altered mitochondrial function, destabilization of plasma membrane and failure of chromatin decondensation (Wongtawan *et al.*, 2006). Suggested possible damage to spermatozoa after a number of months of storage at –196°C include i) production of reactive oxygen species during freezing, which may cause abnormalities of chromatin structure and DNA integrity, reduction in sperm membrane fluidity and decrease the sperm function following cryopreservation (Chatterjee and Gagnon, 2001); and ii) loss of sperm surface proteins, which are necessary for fertilization. All these events influence the viability and fertility of the frozen semen (Chaveiro *et al.*, 2006 and Wongtawan *et al.*, 2006). Cooling is a major stressor, as a result of which membrane bound

phospholipids reorient themselves into a different configuration that disrupt membrane function and permeability (Lessard *et al.*, 2000). Frozen-thawed semen sample with higher number of spermatozoa having damaged acrosome and lower number of functional membrane intact spermatozoa resulted in reduced pregnancy rate (Deneke *et al.*, 2010). This indicates the significance of the use of combination of semen evaluation methods to assess potential fertility of semen as suggested by Selvaraju *et al.* (2008).

The studies on effect of long-term storage on post-thaw characteristics of spermatozoa are possible only when the same sire is used over a longer period of time (Lemma, 2010). In the present study, frozen semen samples of three sires stored for 4, 8, 12, 16, 20 and 24 months were used to assess the post-thaw quality of spermatozoa. Presently, frozen semen straws contain 20 million motile spermatozoa as per minimum standard protocol of Government of India. Therefore, frozen semen sample with post-thaw motility of 50 per cent and above is considered suitable for the use of artificial insemination as minimum 10 million motile spermatozoa are required for successful conception in bovines. In the present study, variations in the post-thaw semen quality were observed among the bulls; bull no. AH 567 recorded less than 50 per cent post-thaw motility at 8 months of storage onward in contrast to other two bulls, in which post-thaw motility was recorded as ≥ 50 per cent up to 16 months of storage. The fertility of frozen-thawed spermatozoa varies considerably among individuals of the same species and breed. These findings further suggest that a combination of semen evaluation tests should be used to assess post-thaw semen quality prior to dispatch of frozen semen that has been stored for long period of time and to be used for insemination in the field.

From the present study it may be concluded that frozen semen straws should be used at the earliest possible time after its production for artificial insemination under field conditions to obtain better conception rate.

REFERENCES

Bhosrekar, M. R. (2005). Semen production in farm animals and A.I. Bookmark Publishers, Pune, Maharshtra, India, p. 194.

Campbell, R.G., Hancock, J.L. and Rothschild, L. (1953). Counting live and dead bull spermatozoa. *J. Expt. Biol.*, **30**: 44-49.

Deneke, N., Lemma, A. and Yilma, T. (2010). Study on the efficiency of conventional semen evaluation procedure employed at Kaliti National artificial insemination center and fertility of frozen-thawed bull semen. As part of MSc thesis, Faculty of Veterinary Medicine, Addis Ababa University

Dhami, A. J., Sahni, K. L. and Mohan G. (1991). Effect of pre freeze holding time (at 5°C) and thawing rates on post thaw Motility and Thermoresistance of Bubaline and Taurine Spermatozoa. *Indian J. Anim. Reprod.*, **12**(2):129-134.

Haugan, T., Grohn, Y.T., Kommisrud, E., Ropstad, E and Reksen, O. (2007). Effects of sperm concentration at semen collection and storage period of frozen semen on dairy cow conception. *Anim. Reprod. Sci.*, **97:** 1-11.

Larsson, B. and Rodriquez-Martinez, H. (2000). Can we use in vitro fertilization tests to predictsemen fertility? *Anim. Reprod. Sci.*; **60**: 327-336.

Lemma A. (2010). Effect of Cryopreservation on Sperm Quality and Fertility in Artificial Insemination in Farm Animals Ed. Milad Manafi, Pub. In Tech, URL-*http://www.intechopen.com/articles/show/title/effect-of cryopreservation-on-sperm-quality-and-fertility*

Lessard, C., Parent, S., Leclerc, P., Bailey, J.L. and Sullivan, R. (2000). Cryopreservation Alters the Levels of the Bull Sperm Surface Protein P25b. *J Androl.*, **21**:700-707.

Malik, A., Laily, M., Zakir, M.I. (2015). Effects of long term storage of semen in liquid nitrogen on the viability, motility and abnormality of frozen thawed

Frisian Holstein bull spermatozoa. *Asian Pacific J. Reprod.* URL-http://www.sciencedirect.com/science/article/pii/S230505001460052X

Selvaraju, S., Ravindra, J.P., Ghosh, J., Gupta, P.S.P. and Suresh, K.P. (2008). Evaluation of sperm functional attributes in relation to *in vitro* sperm-zona pellucida binding ability and cleavage rate in assessing frozen thawed buffalo (*Bubalus bubalis*) semen quality. *Anim. Reprod. Sci.*, 106: 311–321.

Snedecor, G.W. and Cochran, W.G. (1967). *Statistical methods.* 6th edn. The Oxford and IBH Publ. Co., 66, Janpath, New Delhi, India.

Watson, P.F. (1975). Use of Giemsa stain to detect changes in the acrosome of frozen ram spermatozoa. *Vet. Record.*, **97:**12-15.

Wongtawan, T., Saravia, F., Wallgren, M., Caballero, I. and Rodriguez-Martinez, H. (2006). Fertility after deep intra-uterine artificial insemination of concentrated low-volume boar semen doses. *Theriogenology*, **65**: 773-787.