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**Research Article**

# Effect of certain additives on the freezability of crossbred bull semen*

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## ABSTRACT

Thirty two ejaculates (8 each from FH and FxH bulls) were subjected to freezing. Cervical mucus penetration test and hypo-osmotic swelling test used for quality assessment of fresh and frozen thawed spermatozoa. Sperm penetration distance (SPD) travelled by vanguard spermatozoa of HF and FxH bulls were 45.06 ± 3.32 and 39.94 ± 2.98mm of fresh semen and 19.69 ± 0.47 and 18.38 ± 0.59mm of fresh semen in 60 min. SPD traveled by vanguard spermatozoa differed significantly (P<0.01) among the bulls and between the breed. HOS positive sperm for HF and FxH semen was 49.38 ± 2.80 and 42.06 ± 3.57 of fresh semen and 21.88 ± 0.44/hr and 21.00 ± 0.57/hr of frozen thawed sperm. A significant difference (P<0.01) in total swollen sperm was observed between breeds and between the two crossbred bulls significantly (P<0.01) between HF bulls in frozen semen. A significant positive correlation of HOS positive sperm and SPD with mass motility, initial progressive motility, live count, per cent intact acrosome, post-thawed motility, post thaw livability and post thaw intact acrosome and negative correlation (P<0.01) with total sperm abnormalities were recorded. A significant (P<0.01) improvement was recorded after incorporation of ascorbic, caffeine and chloroquine in diluter. Maximum improvement was recorded in presence of ascorbic acid. Since SPD and HOS tests measure the functional and structural normality and subsequent fertility of spermatozoa. these tests can be used as predictor for fertility of frozen semen and ascorbic acid can be supplemented in the freezing media for obtaining better post-thaw recovery.

**Key words:** Semen, freezing, additives, penetration distance, motility, and hypo-osmotic response

The increasing demand of crossbred bull semen in breeding program has necessitated the production and preservation of good quality semen with high fertilizing ability. To obtain this, detailed information about quality and freezability of semen is required. It would be better if prediction of fertility of frozen semen were also known. In order to establish relationship between lab tests and the fertility of semen. Some *in vitro* fertility tests need to be for formed in addition to assessing the fresh and post-thaw semen quality. Cervical mucus penetration test (CMPT) and hypo osmotic swelling test (HOST) bears a positive correlation with fertilizing ability and membrane integrity of the spermatozoa (Prasad, 1997) and hence are of promising importance.

Ascorbic acid is known to improve post – thaw motility and live per cent of buffalo spermatozoa (Singh *et al.*, 1995). Caffeine, a metabolic stimulant enhances motility, respiration and fructolysis of epididymal and ejaculated spermatozoa of cattle and buffalo bulls (Garber *et al.*, 1973, Fattouh, *et al.*, 1985). It has also been reported to improve progressive motility of both fresh and preserved sperms (Schill, 1982). Chloroquine diphosphate, a membrane stabilizer is known to stimulate the respiration and motility of fresh and aged spermatozoa *in vitro* (Norman and Gombe, 1975) and to improve the post thaw quality of frozen semen (Sidhu *et al.*, 1996).

Increasing evidences of beneficial effects of above compounds inspired us to investigate further in a more comprehensive way their effect on structural and functional status of the frozen semen of Holstein Friesian and crossbred (Holstein Friesian x Haryana) cattle bull with an objective to ascertain their role, if any, in improving the potential fertility of frozen- thawed sperm.

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## MATERIALS AND METHODS

Two purebred Holstein Friesian (HF) and two crossbred (FxH) bulls of 5-7 years of age, maintained under identical feeding and managemental regimes were used for the study. Semen from each bull was collected twice a weak by artificial vagina over a male partner after a false mount during morning hour, before feeding and thirty two ejaculates (8 each from 4 bulls) were studied. All the ejaculates were evaluated for routine cytomorphological seminal attributes, cervical mucus penetration test (CMPT) and hypo-osmotic swelling test (HOST) in fresh semen sample. The semen samples after initial evaluation were extended so as to maintain 50 million motile sperms/ml of diluted semen. Ascorbic acid, caffeine and chloroquine were incorporated as an additives in diluters (Tris-fructose-yolk-glycerol) in the concentration of 10mM, 7mM and 0.54mM, respectively. The extended semen was filled in medium french-straws and frozen in $LN_2$ vapor, 24 hours after freezing, post thaw evaluation of semen was done.

### Cervical Mucus Penetration Test (CMPT):

This test was carried out as per Kremer (1965) with a slight modification. Cervical mucus was collected from estrus cow under aseptic condition. Semen sample (0.5 ml) were taken in a 2 ml micro-centrifuge tube into which the loaded capillary tubes in duplicate were placed so that the free end remained dipped in the semen just above the bottom and incubated at 37°C for 60 min. After incubation the capillary tubes were removed, placed on a graduated glass slide and the distance travelled (in mm) by the vanguard spermatozoa was measured under a phase contrast microscope. The grading was done as per the method of Matousek *et al.* (1989).

### Hypo-osmotic Swelling Test (HOST):

The test was performed after slight modification in the method described by Jayendran *et al.* (1984). A solution having the osmolarity of 150 milli-osmol/ Kg was used for the purpose and the suspension was incubated at 37°C for 60 min. Spermatozoa with swollen tail were observed under higher power magnification (400x) of phase contrast microscope. Two hundred spermatozoa were examined and classified in 4 different classes according to presence of the various tail swelling patterns. (Vanden Saffele *et al.*, 1992).

## RESULTS AND DISCUSSION

The HF bull semen had significantly higher mass motility, individual motility, per cent live sperm count and per cent intact acrosome and lower sperm abnormalities than crossbred bull semen. Sperm penetration distance traveled by freshly ejaculated spermatozoa of HF and crossbred (FxH) was 45.06±3.32 and 39.94±2.98 mm, respectively. A significant ($P<0.01$) difference was recorded among the bulls and between breeds. Variation in SPD between bulls and breeds, as reported in present study was expected as variation in initial quality was recorded.

SPD of Jersey bull spermatozoa (45.17 mm /30 min., Kumar and Devanathan, 1996) and crossbred bull sperm; 34.71±2.51 and 29.92±2.72 mm /hr, respectively for FxH and FxJxH; Prasad *et al.*, 1999) has been reported. The variation in SPD value is affected due to initial motility, kinetics and quality of progression and sperm concentration (Suttiyovin *et al.*, 1995). SPD was significantly positively ($P<0.01$) correlated with mass motility (0.74), individual motility (0.87), per cent livability ($r=0.74$), per cent intact acrosome ($r = 0.74$) and per cent HOS positive sperm (0.93) and negatively correlated ($P<0.01$) with abnormalities in both the breeds. A high correlation of sperm concentration with SPD is reported. (Sidhu *et al.*, 1996).

A significant reduction in the sperm penetration distance after post thaw evaluation was recorded for both the breeds. A slightly higher SPD of frozen thawed spermatozoa observed in HF bulls may be attributed due to higher initial and post thaw motility. Reduction in SPD is attributed to cryoinjury (Matousek *et al.*, 1989). A still lower SPD (13.75 mm /hr and 10.83 mm /hr in FxH and FxJxH bulls) than our findings is reported (Prasad *et al.*, 1999).

SPD after incorporation of additives was significantly higher in all the treatment groups than the control (Table-2), which differed significantly ($P<0.01$) among all the treatments, between bulls and between both the breeds. Significant increase in SPD after

**Table 1: Physico-morphological seminal attributes, SPD value and per cent HOS positive sperm of HF and crossbred bull fresh semen (Mean ± SE)**

| Seminal Characteristics | HF | CB |
|---|---|---|
| Mass motility (0-5) | 4.00±0.57 [a] | 3.06±0.25 [b] |
| Volume (ml) | 4.90±1.87 [b] | 5.66±1.35 [a] |
| Per cent Individual motility | 82.19±3.64 [a] | 68.13±6.24 [b] |
| Per cent live sperm | 87.38±11.46 | 84.13±2.31 |
| Per cent total abnormal sperm | 10.06±1.57 | 12.63±2.34 |
| Per cent intact acrosome | 89.94±1.57 | 87.38±2.34 |
| Sperm concentration ($10^6$/ml) | 962.50±118.77 | 968.13±111.31 |
| Sperm penetration distance (mm) | 45.06±3.32 [a] | 39.94±2.18 [b] |
| Per cent HOS positive sperm | 49.38±2.80 [a] | 42.06±3.57 [b] |

Means having different superscript in a row differ significantly (P>0.05).

incorporation of additives may be attributed to enhanced motility. Although concentration of spermatozoa affects the SPD (Suttiyovin *et al.*, 1995) but in our study since concentration was kept constant this increase could only be attributed to additives.

The sperm penetration distance was more in presence of ascorbic acid than caffeine and chloroquine, which could be due, increase post-thaw motility. A strong significantly positive correlation of SPD with post-thaw motility, livability, acrosomal integrity and percent HOS positive spermatozoa is self-explanatory. More the number of normal motile cells more distance they will travel in the mucus. Ascorbic acid, a well-known antioxidant is reported to prevent damage spermatozoa during freezing (Maxwell and Stoyanov, 1988). Beneficial effect of caffeine in dilutor for improvement post-thaw motility is reported (Tesarik *et al.*, 1992). Through inhibiting CAMP and CGMP responsible for enhancing sperm motility Garber *et al.*, (1973)

Chloroquine a membrane stabilizer and anti-inflammatory agent is reported to stimulate respiration and motility (Norman and Gombe, 1975) through directly or indirectly acting on adenyl cyclase system (Garber *et al.*, 1973).

Hypo-osmotic swelling test can assess the functional and physiological aspects of spermatozoa, which is based on the membrane potentiality. Spermatozoa with damage or inactive membranes are unable to support osmotic swelling and hence they will not respond to HOS test on the other hand spermatozoa with intact membrane, which will respond to HOST (Jayendran *et al.*, 1984).

Per cent swollen sperm (freshly ejaculated) for HF and FxH bulls was 49.34±2.80 and 42.66 ± 3.57, respectively. A significant difference (P<0.01) was observed between breeds and crossbred bulls. Variable responses of sperm towards hypo-osmotic solution, for different bulls and breeds depend on the initial quality and membrane integrity. Per cent HOS positive sperm was significantly (P<0.01) positively correlated with mass

**Table 2: Effect of treatments on physico morphological characters, sperm penetration distance and Hypo—osmotic swelling positive spermatozoa of HF and crossbred semen (Mean±SE).**

| Seminal characteristics | Holstein Friesian Bull Semen | | | | Crossbred bull semen | | | |
|---|---|---|---|---|---|---|---|---|
| | Tris (control) | Tris + AA | Tris + Caffeine | Tris + Chl. | Tris | Tris + AA | Tris caffeine | Tris + Chl. |
| Post-thaw Motility (%) | 30.31±1.25 | 50.31±1.25 | 40.31±0.96 | 37.81±1.20 | 28.13±1.36 | 45.31±1.40 | 37.50±1.29 | 34.38±1.32 |
| Post-thaw Livability (%) | 55.50±1.01 | 72.50±0.47 | 65.00±0.65 | 61.56±0.93 | 54.94±1.32 | 72.75±1.01 | 67.13±1.05 | 64.63±1.05 |
| Post-thaw intact acrosome (%) | 70.50±0.79 | 81.56±0.63 | 75.19±0.50 | 73.75±0.77 | 67.75±1.25 | 78.88±0.98 | 73.06±1.35 | 71.861.15 |
| S.P.D. (mm) | 19.69±0.47 | 29.50±0.52 | 25.25±0.63 | 23.88±0.62 | 18.38±0.59 | 27.19±0.58 | 24.25±0.54 | 23.50±0.71 |
| Per cent HOS positive sperm | 21.88±0.44 | 31.50±0.77 | 27.25±0.66 | 26.19±0.65 | 21.00±0.57 | 29.38±0.66 | 26.50±0.55 | 25.25±0.56 |

AA=Ascorbic Acid; Chl. = Chlorpromazine Hydrochloride

motility (r = 0.78), initial motility (0.91), percent live sperm count (r=0.70) and per cent intact acrosome (r = 0.71) and SPD value (r = 0.93) and negatively (P<0.01') correlated with total sperm abnormalities (r = 0.71) in both the breeds. Our study signifies that increase in motile live and intact acrosome sperm is always reflected through HOS test, which gives a fair indication of superiority of the semen.

The post thawed HOS responsive sperm were 21.88±0.44 and 21.00±0.57, respectively in HF and FxH bulls. No significant difference in HOS positive sperm was observed between breeds and between the crossbred bulls. However, it differed significantly (P<0.01) between two HF bulls. A significant reduction in the per cent swollen sperm after freezing due to alterations in the membrane permeability and structures evidenced by decreased and altered motion characteristics, inactivation of intracellular enzyme decreased ability to penetrate the cervical mucus and Zona-free Hamster egg penetration and diminished pregnancy rates in human (Centola *et al.*, 1992) is reported. In our study post-thawed HOS positive sperm were significantly positively correlated with motility, livability and acrosomal intactness, which clearly indicate the role of osmolarity on the spermatozoal membrane damage during cryopreservation and its further implication in fertilization.

The overall mean (±SE) HOS positive spermatozoa after incorporation of additives are given in table 2. Significant increase in post-thawed HOS positive sperm recorded after incorporation of additives is justifiable on the lines that they help sperm cells in some or other way. Ascorbic acid reduces the damages caused by free radicals (Jackson and Edwards 1988), caffeine regulates the plasma membrane calcium channel (Mann and Lutwak-Mann, 1981) and chloroquine diphosphate (Norman and Gomber 1975) is responsible for protecting the membrane damage to sperm cells.

It could be inferred from the findings of the present study that these additives have a very promising role in preventing the damages to sperm cells during freezing and either of these can be used (preferably ascorbic acid) in the freezing media for obtaining better post-thaw recovery rate more particularly sperm with better fertilizing potential.

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## REFERENCES

Centola, G.M., Raubertas, R.F. and Mattox, J.I.I. (1992). Cryopreservation of human semen: composition of cryoprotectives, sources of variability and prediction of post-thaw survival. J. Andrology, **13**: 283-288.

Fattouh, El-S.M., Seida, A.A., Nasir, M.T. and Abdou-Ahmed, M.M. (1985). Effect of caffeine on the motility of ejaculated and epididymal buffalo spermatozoa. Vet. Med. J. Egypt, **33**: 261-271.

Garbers, D.L., First, N.L. and Lardy, H.A. (1973). The stimulation of bovine epididymal sperm metabolism by cyclic nucleotidase inhibitors. Biol. Reprod., **8**: 589-598.

Jackson, M.J. and Edwards, R.H.T. (1988). Free radicals, Muscle damage and muscular dystrophy. In: Reactive oxygen species in Chemistry, Biology and Medicine. Series A : Life Sciences, Vol. 146 (ed. A. Quintanilha). Plenum Press, New York, pp 197–210.

Jayendran, R.S., Vandervent, H.H., Perez-peleae, Z.M., Crabo, B.G., Zanevald, L.J.D. (1984). Development of assay to assess the functional integrity of human sperm membrane and its relationship to other semen characteristics. J. Reprod. Fertil., **70**: 219-225.

Kremer, J.A. (1965). Simple sperm penetration test. International J. fertil., **10**: 209.

Kumar, R.A. and Devnathan, T.G. (1996). Effect of spermatozoal quality on sperm progression speed in cervical mucus. Indian Vet. J.,**73**: 645-648.

Mann, T. and Lutwak-Mann, C. (1981). Male reproductive function and semen. 1st Ed. Springer-verlag, Berlin, Heidetberg, New York pp 262–267.

Matousek, J., Riha, J., Sarsen, V., Veseldky and Londa, F. (1989). Penetration of cervical mucus and other body fluids by bull sperm in capillary tubes. Anim. Reprod. Sci., **18**: 161–166.

Maxwell, W.M.C. and Stoyanov, T. (1988). Liquid storage of ram semen in absence of some antioxidants. Reprod. Fertil. Dev., **8**: 1013-20.

Norman, C. and Gombe, S. (1975). Stimulatory effect of the lysosomal stabilizer, chloroquine, on the respiration and motility of fresh and aged spermatozoa. J. Reprod. Fertil., **44**: 481-486.

Prasad, J.K. (1997). Studies on enzymatic changes and in vitro fertility tests in relation to cryopreservation of crossbred bull semen. M. V. Sc. Thesis, IVRI, Izatnagar, U.P., India.

Prasad, J.K., Kumar, S., Mohan, G., Agarwal, S.K. and Shankar, U. (1999). Simple modified method for cervical mucus penetration test for quality assessment of bull semen. Indian J. Anim. Sci., **69**: 103 – 105.

Schill, W.B. (1982). Therapies der, Idiopathischen Astheno und oligozoospermite mit Penroxifylline Fortschr. Med., 100: 696-700.

Sidhu, S.S., Pangawakar, G.R. and Chaudhry, R.K. (1996). Effect of some additives on the release of enzymes from buffalo spermatozoa during cryopreservation. Indian Vet. J., **73**: 154 – 158.

Singh, B., Chand, D., Yadav, N.K. and Singh, P. (1995). Effect of vitamins C addition in the diluent on the quality of refrigerated Murrah buffalo bull (Bubalus bubalis) semen. Indian J. Anim. Res., **29**: 79-84.

Singh, B., Chand, D., Yadav, N.K. and Singh, P. (1995). Effect of vitamins C addition in the diluent on the quality of refrigerated Murrah buffalo bull (Bubalus bubalis) semen. Indian J. Anim. Reprod., **29**: 79-84.

Singh, B., Singh, D., Singh, P., Yadav, N.K., Singh, B. and Chand, D. (1996). Effect of vitamin C addition in the diluent of the quality of deep-frozen Muraah buffalo bull (Bubalus Bubalis) semen. International J. Anim. Sci., **11**: 131-132.

Suttiyotin, P., Thwaetes, C.J., Sanchez-Partida, L.G. and Setchell, B.P. (1995). Evaluation of a modified sperm penetration test I ram semen. Theriogenology, **44**: 29-40.

Tesarik, J., Mendoza, C., and Carreras, A. (1992). Effects of phosphodisterase inhibitors, caffeine and pentaxyphylline on spontaneous and stimulus induced acrosome reactiosn in human sperm. Fertil. Steril., **58**: 1185-1190.

Vanden Soffele, J., Vermeulen, L., Schoojan, S. and Courhaire, F.H.(1992). Evaluation of the hypo-osmotic swelling in relation with advanced method of semen analysis. Andrologia, **24**: 213-217.

## ISSAR NEWS

1. Department of AGRO Veterinary College Bangalore organized Fourth Annual Conference of ISSAR (Karnataka Chapter) and one day seminar on "Management of Infertility on December 9, 2005 with Dr. M. Devaraj, Professor and Head as the organizing secretary at Central Frozen Semen Production and Training Institute Hessaraghatta Bangalore. About 80 Veterinarians from the State Dept. of AH&VS KMF and KVAFSU participated. Prof. R.N. Srinivas Gowda Hon. Vice-Chancellor, Dr. S.S. Honnapagol, Registrar, Karnataka Veterinary. Animal and Fisheries Sciences University (KVAFSU), Bidar, Dr. M.G. Govindaiah, Dean Veterinary College Bangalore and Dr. G. Krishnappa, Director, Institute of Animal Health & Veterinary Biologicals, Bangalore were the guests of honor. A compendium of scientific papers of the seminar was released on the occasion. Dr. S.A. Ashokan, Professor, Dept. of ARGO, Madras Veterinary College, Chennai was the guest speaker.

2. Karnataka chapter of ISSAR conducted the election of execution committee on 9/12/2005 and unanimously nominated the following.

| | | | |
|---|---|---|---|
| 1) | **President** | : | DR. K.S. Gangadher former, Director of Extension, KVAFSU, Bidar. |
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