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**Research Article**

# FUNCTIONAL AND BIOCHEMICAL ATTRIBUTES OF BULL SEMEN WITH REFERENCE TO FREEZABILITY AT DIFFERENT STAGES OF SEMEN PROCESSING

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## ABSTRACT

The present study was undertaken to measure the functional and biochemical profile of freezable and non-freezable ejaculates of bull semen. Total sixty four ejaculates (eight ejaculates from each bull) were collected from six Jersey and two Sahiwal X H.F crossbred bulls and assessed for initial parameters (volume, mass activity and sperm concentration), functional parameters (progressive motility, per cent live spermatozoa, hypo-osmotic swelling test, acrosomal integrity, morphologically abnormal spermatozoa including estimation of enzymes such as Alanine transaminase (ALT), Aspartate transaminase(AST) and Alkaline phosphatase (ALP), and minerals (Calcium and Magnesium) in seminal plasma at four stages of semen processing viz. post-dilution, post-equilibration, post thaw and 1 hr post thaw incubation. The result revealed a significantly higher ($p<0.05$) mass motility, progressive motility, per cent livability, HOS reactive sperm, ALP and lower ALT activity in freezable ejaculates at all the four stages of semen processing.

**Key words:** Biochemical parameters, Ejaculates, Enzymes, Freezable, Seminal plasma

Semen evaluation along with artificial insemination has made rapid stride in the genetic improvement of the cattle. Artificial insemination is the most effective breeding tool that affords widespread propagation of genes carried by superior males (Khalifa *et al.*, 2014). Assessment of semen parameters provides an insight regarding spermatogenesis, functional proficiency of spermatozoa and also about secretary pattern of accessory sex glands (Moazzam *et al.*, 2015). Screening of the semen at initial stages followed by its preservation allows elimination of poor quality semen while proper assessment of the post thaw quality of the spermatozoa can provide insight into the fertilizing capacity of the cryopreserved spermatozoa (Januskauskas and Zilinskas, 2002). Several semen evaluation tests measuring the physical and functional integrity of spermatozoa has been coined over the past few decades. These tests are considered powerful tool for predicting the quality of the semen, whether it's freezable or non-freezable so, that superior quality germplasm can be disseminated at farmers' doorsteps. The major objective of this study was to compare the functional and the biochemical attributes of bulls of variable freezability.

## MATERIALS AND METHODS

Semen samples were collected at Sperm Station Palampur, India (32.6º N, 76.3º E, altitude 1290.8 m) from six Jersey and two Sahiwal × H.F. crossbred bulls. Semen was collected twice a week from each bull by artificial vagina method. The semen ejaculates were subjected to initial examination of volume, spermatozoa concentration, mass activity and initial motility. Minimum initial standards (volume of 2 ml, spermatozoa concentration of 500 million/ml and initial motility of above 70 percent) qualifying ejaculates were further processed. After initial assessment of semen, the selected samples were diluted with prewarmed (37°C) tris citric acid egg yolk extender so as to pack 20 millions spermatozoa in 0.25 ml French mini straws. Equilibration was done in the cold handling cabinet (IMV, France) maintained at 4° C for 3 hours by bringing down the temperature from 37° C to 4° C by gradual cooling. Straws were then shifted to programmable bio-freezer (IMV Digit cool 500) where nitrogen vapours were used to bring down the temperature to -140°C within 10 minutes. Finally straws were shifted to goblets and plunged into liquid nitrogen where they could be stored for definite period of time for future use.

Total 64 ejaculates (8 per bull) were evaluated at four stages of the semen processing viz. post-dilution, post-equilibration, post thaw stage and 1hr post thaw incubation stage at 37°C for per cent livability, progressive motility, reaction to 150 m Osmol hypo-osmotic solution, acrosomal integrity and morphological abnormalities. Ejaculates were classified into freezable (n=49) and non-freezable (n=15) ejaculates on the basis of initial motility (≥ 70 per cent) and morphological abnormalities (10-15 per cent). At post thaw stage, freezable ejaculates should have motility (≥50 per cent), acrosomal integrity (≥65 per cent) and HOST (≥ 40 per cent). Thawing of frozen semen was done at 37° C for 30 seconds. For estimation of enzymes and minerals, separation of the seminal plasma was done at each of these four stages by centrifugation at 4000 rpm for 15 minutes at 37°C and seminal plasma was stored at -20°C till further estimations were made.

For measuring volume, each ejaculate was weighed using the integrated SMILE system which automatically

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got converted into the volume measure. Mass activity was graded as described by Salisbury *et al.* (1978). The concentration of the spermatozoa (millions/ml) was determined by bovine photometer (IMV 7407). Progressive motility, per cent livability and acrosome integrity were assessed as per Sharma (2011); HOST test was performed as described by Pant *et al.* (2002); morphological abnormalities were assessed by Rose Bengal Stain as per Blom (1972). Enzyme ALT, AST and ALP were estimated using the diagnostic kit (Agappe Diagnostics) and hyaluronidase was estimated using the ELISA kit (Qayee-Bio) based on the double-antibody enzyme-linked immunosorbent one-step assay. Calcium and magnesium were estimated using the atomic absorption spectrophotometer (Perkin Elmer Analyst) at a wavelength of 285.2 nm. The results were analysed statistically with the Students' 't' test using SPSS between the freezable and non-freezable ejaculates.

## RESULTS AND DISCUSSION

The results of present study are summarized in table 1, 2 and 3. Data of present study revealed that mass activity, forward progressive motility, livability, HOS reactive spermatozoa, ALP were significantly higher ($p < 0.05$) in the freezable ejaculates as compared to non-freezable ejaculates at all the four stages of semen evaluation whereas per cent intact acrosome was significantly higher in freezable ejaculates at post-dilution, while no difference was observed at other three stages of processing. However, morphological abnormalities, AST, ALT were significantly ($p < 0.05$) higher in non-freezable as compared to freezable ejaculates at all the stages. No significant difference ($p < 0.05$) was observed for hyaluronidase and calcium at all the stages.

The average ejaculate volume corresponds to Chauhan *et al.* (2017). Contrarily, lower semen volume was reported by Gopinathan *et al.* (2016). Semen volume is probably a breed characteristic, which further depends upon the scrotal size and weight, reproductive health condition and age of the bulls, methods and frequency of collection, pooled volume, nutrition, season and management (Vettical, 2016). The concentration of spermatozoa was in accordance with Gopinathan *et al.* (2016) and Chauhan *et al.* (2017). Concentration of ejaculates has been considered vital for the production of maximum number of the semen straw in order to get maximum number of doses for artificial insemination. Variation in mass activity could be attributed to higher spermatozoa concentration, low spermatozoa abnormalities, age of the bulls (Javed *et al.*, 2000), season, management and collection procedure (Sharma *et al.*, 1991).

**Table 1**
**Comparison of initial parameters (mean±SE) of freezable and non-freezable semen of bulls**

| Parameter1s | Volume (ml) | Mass motility | Concentrations (millions/ ml) |
|---|---|---|---|
| Freezable (n=49) | 5.62±0.25 (4-9) | 3.0±0.08[a] (3-4) | 1064.96±61.77 (529-1875) |
| Non-freezable (n=15) | 5.68±0.33 (2.5-7.7) | 2.5±0.09[b] (2-3) | 860.46±64.76 (225-1324) |

Values with different superscripts within the same column differ significantly ($P<0.05$)

Livability of the spermatozoa is an important criterion while selecting ejaculates for freezing so as to get more number of post thaw live spermatozoa (Perumal *et al.*, 2015). In our study, per cent livability was significantly higher ($p<0.05$) in freezable than in non-freezable ejaculates at all the stages of semen evaluation (Table 2). Gebreselassie *et al.* (2012) reported the similar finding which was in concurrence with our study. Variation in viability could be attributed to breed difference, age and technical skill of the observer (Tomar *et al.*, 1985). Motility is considered as the important indicator of fertilizing ability of the spermatozoa and as an expression of their viability and structural integrity (Kathiravan *et al.*, 2011). Per cent progressively motile spermatozoa was significantly higher ($p<0.05$) in freezable than in the non-freezable ejaculates (Table 2). Similar observation was reported by Gebreselassie *et al.* (2012). The drop in motility from fresh to equilibrated semen may be due to osmotic shock during equilibration and also due to decline in the per cent livability.

The HOS-reactive spermatozoa per cent was significantly ($p<0.05$) higher in the freezable ejaculates than the non-freezable ejaculates. Gebreselassie *et al.* (2012) reported that freezable ejaculates have higher value of the plasma membrane integrity than non-freezable ejaculates. The percentage of acrosomal integrity of spermatozoa was significantly higher in freezable ejaculates at post-dilution stage, however, no significant difference was observed at other stages (Table 2). Gebreselassie *et al.* (2012) also recorded significantly higher per cent intact acrosome in the freezable (79.43±1.10) than non-freezable ejaculates (64.17±1.42). Variation in acrosomal integrity could be attributed to the factors like age of the bulls (Javed *et al.*, 2000), temperature (Chandra *et al.*, 1999), frequency of semen collection and sexual excitement before collection (Badaway *et al.*, 1973).

The morphologically abnormal spermatozoa were significantly ($p<0.05$) lower in freezable ejaculates as compared to non-freezable ejaculates at all the stages of

**Table 2**
**Comparative functional parameters (mean±SE) of freezable and non-freezable semen of bulls during various stages of processing**

| Parameter (n=64) | Quality | Stage of semen processing | | | |
|---|---|---|---|---|---|
| | | Fresh Diluted | Equilibration | 0 hr Post-thaw | 1 hr Post-thaw |
| Livability (%) | Freezable (n=49) | 85.16±0.70$^{aA}$ (73-95) | 80.51±0.76$^{aB}$ (66-90) | 67.92±1.30$^{aC}$ (42-88) | 53.33±1.56$^{aD}$ (26-83) |
| | Non-freezable (n=15) | 80.80±1.97$^{bA}$ (63-92) | 71.87±2.41$^{bB}$ (56-85) | 56.87±2.50$^{bC}$ (44-75) | 42.13±2.17$^{bD}$ (28-56) |
| Progressive motile spermatozoa (%) | Freezable (n=49) | 80.31±0.72$^{aA}$ (67-91) | 74.45±0.86$^{aB}$ (60-85) | 57.33±1.76$^{aC}$ (22-79) | 38.78±1.55$^{aD}$ (10-60) |
| | Non-freezable (n=15) | 58.87±4.21$^{bA}$ (33-86) | 53.20±4.42$^{bA}$ (26-79) | 36.07±2.43$^{bB}$ (20-48) | 24.27±1.90$^{bB}$ (11-33) |
| HOS Reactive Spermatozoa (%) | Freezable (n=49) | 78.51±1.00$^{aA}$ (54-90) | 72.27±1.10$^{aB}$ (54-83) | 60.00±1.60$^{aC}$ (26-77) | 45.29±1.51$^{aD}$ (20-66) |
| | Non-freezable (n=15) | 73.93±2.43$^{bA}$ (61-86) | 64.00±2.81$^{bB}$ (40-78) | 49.00±2.91$^{bC}$ (35-72) | 37.40±1.89$^{bD}$ (19-48) |
| Intact Acrosome (%) | Freezable (n=49) | 84.84±0.87$^{aA}$ (69-95) | 78.82±1.03$^{B}$ (55-93) | 67.39±1.37$^{C}$ (46-88) | 54.12±1.38$^{D}$ (33-77) |
| | Non-freezable (n=15) | 87.60±1.45$^{bA}$ (73-95) | 78.07±1.06$^{B}$ (72-89) | 66.40±2.90$^{C}$ (45-80) | 55.27±3.42$^{D}$ (25-74) |
| Morphological abnormalities (%) | Freezable (n=49) | 7.35±0.62$^{aA}$ (2-19) | 7.61±0.59$^{aA}$ (2-20) | 8.16±0.56$^{aB}$ (3-18) | 9.14±0.63$^{aC}$ (2-21) |
| | Non-freezable (n=15) | 9.27±1.18$^{bA}$ (1-15) | 9.87±0.98$^{Ba}$ (4-16) | 10.00±1.00$^{bA}$ (4-17) | 11.27±1.14$^{bB}$ (4-19) |

Figures within parenthesis indicate range
$^{a,b}$Values with different superscripts within the column for a particular parameter differ significantly ($p<0.05$)
$^{A,B,C,D}$Values with different superscripts within the row differ significantly ($p<0.05$)

semen evaluation (Table 2). The morphological abnormalities were in accordance to Ghirardosi *et al.* (2017). In contrary to our findings, higher morphological abnormalities has been reported earlier (Khalil *et al.*, 2017). The difference between the freezable and non-freezable ejaculates may be attributed to the factors like method of semen collection, temperature shock and technique employed (Bishop *et al*., 1954).

Enzymes such as AST, ALT, Lactate dehydrogenase, cholinestrases and phosphatase (Roberts, 1971), hyaluronidase (Bhosrekar *et al.*, 1994) have been recognised to be intimately related to the sperm cell and are essential for various metabolic processes of spermatozoa. These enzymes are good indicators of semen quality as they measure the plasma membrane stability of spermatozoa (Corteel, 1980).

Estimation of the hyaluronidase has assumed a great importance due to its presence in the acrosomal system of spermatozoa. Integrity of acrosome is directly involved in the fertilizing capacity of spermatozoa so the estimation of this enzyme gives an idea regarding the fertilizing capacity of spermatozoa present in the ejaculates. No significant ($p>0.05$) difference was observed between concentration of hyaluronidase for freezable and non-freezable ejaculates at all the four stages of semen evaluation. Contrarily, significant difference was observed for mean hyaluronidase concentration at different stages of semen evaluation in buffalo bull semen (Dhaka, 2013).

Significant difference ($p<0.05$) for AST was observed at post thaw stages whereas ALT concentration varies significantly at all the stages of semen evaluation for freezable and non-freezable ejaculates (Table 3). Concentration of the transaminases in the seminal plasma of the freezable ejaculates was lower than the non-freezable ejaculates giving indication of less damage in former. Transaminase in the semen plays a vital role in the catabolism of glutamate in bovine spermatozoa (Flip and Anderson, 1964). Increased concentration of the transaminases in the seminal plasma could be attributed to increased spermatozoa abnormalities and damage to the spermatozoa plasma membrane (Dogan *et al.*, 2009).

Alkaline phosphatase is a widely distributed phospomonoesterase enzyme, originating from seminal vesicles and to the less extent from the testes and

**Table 3**
**Comparative concentrations of various enzymes and minerals (mean±SE) in seminal plasma of freezable and non-freezable semen of bulls during various stages of processing**

| Parameter (n=64) | Quality | Stage of semen processing | | | |
|---|---|---|---|---|---|
| | | Fresh Diluted | Equilibration | 0 hr Post-thaw | 1 hr Post-thaw |
| Hyaluronidase (ng/mL) | Freezable (n=8) | 6.483±0.16$^{A}$ (5.90-7.19) | 6.440±0.32$^{A}$ (5.141-8.15) | 6.231±0.26$^{A}$ (5.11-7.52) | 5.981±0.47$^{A}$ (3.15-7.32) |
| | Non-freezable (n=8) | 6.096±0.47$^{A}$ (3.07-7.22) | 5.726±0.18$^{A}$ (5.22-6.64) | 5.787±0.16$^{A}$ (5.10-6.43) | 6.06±0.23$^{B}$ (4.95-7.19) |
| ALP(U/L) | Freezable (n=49) | 759.77±18.78$^{aA}$ (506-987) | 807.63±17.20$^{aB}$ (605-1034) | 945.34±17.22$^{aC}$ (717-1178) | 1180.53±17$^{aD}$ (1016-1428) |
| | Non-freezable (n=15) | 523.00±21.5$^{bA}$ (398-678) | 580.26±20.80$^{bA}$ (605-1034) | 851.53±34.48$^{Bb}$ (590-1050) | 1039.00±23.75$^{bC}$ (915-1213) |
| AST(U/L) | Freezable (n=49) | 156.81±11.35 (44-296) | 166.04±10.78 (48-280) | 174.69±9.06$^{a}$ (60-296) | 176.32±9.54$^{a}$ (60-296) |
| | Non-freezable (n=15) | 164.00±15.40 (56-280) | 177.86±16.40 (56-296) | 192.53±13.68$^{b}$ (112-296) | 199.73±14.20$^{b}$ (120-300) |
| ALT (U/L) | Freezable (n=49) | 14.16±0.733$^{aA}$ (6-26) | 18.12±0.678$^{aB}$ (10-28) | 25.04±0.519$^{aC}$ (18-32) | 33.22±1.233$^{aD}$ (26-39) |
| | Non-freezable (n=15) | 24.86±1.233$^{bA}$ (18-34) | 31.06±1.140$^{bB}$ (25-39) | 37.13±0.735$^{bC}$ (33-42) | 44.00±1.018$^{bD}$ (39-52) |
| Calcium (mg/L) | Freezable (n=49) | 114.85±1.53 (96.48-142.4) | 114.64±1.31 (86.81-139.8) | 116.29±1.34 (102.2-140.2) | 113.39±1.48 (92.95-137.6) |
| | Non-freezable (n=15) | 115.00±3.49 (96.47-142.3) | 112.30±2.95 (101.8-136.3) | 111.40±3.59 (77.66-139.4) | 113.40±3.17 (95.33-144.4) |
| Magnesium (mg/L) | Freezable (n=49) | 27.77±0.71$^{a}$ (19.46-42.03) | 28.45±0.76$^{a}$ (16.15-44.86) | 28.23±0.77 (18.67-40.99) | 29.13±0.78 (18.16-43.77) |
| | Non-freezable (n=15) | 31.62±1.01$^{b}$ (27.08-142.3) | 32.37±2.09$^{b}$ (17.72-49.2) | 30.49±1.99 (9.34-42.16) | 31.95±0.944 (28.81-42.17) |

Figures within parenthesis indicate range, $^{a,b}$Values with different superscripts within the column for a particular parameter differ significantly $p<0.05$), $^{A,B,C,D}$Values with different superscripts within the row differ significantly ($p<0.05$)

epididymis in bull (Bucci *et al.*, 2016). Thus, the estimation of ALP activities in seminal plasma reflects the functional state of accessory sex glands, metabolic activity of spermatozoa and sperm membrane integrity that are helpful in differentiating the reproductive biology of bulls of different breeds. In the present study, ALP was significantly ($p<0.05$) decreased in the non-freezable ejaculates than the freezable ejaculates which was in accordance to report of Perumal *et al.* (2015) in Mithun semen.

For calcium, no significant ($p>0.05$) difference was found between freezable and non-freezable ejaculates at all the stages of evaluation. However, for magnesium significant ($p<0.05$) difference was found at post-dilution and post-equilibration stage in freezable and non-freezable ejaculates, respectively. Mean calcium and magnesium concentration was lower than as reported by Eghbali *et al.* (2010). Calcium and magnesium act as a regulator of many physiological processes in all the living cells, including the spermatozoa. For the last stage of capacitation, $Mg^{2+}$ and $Ca^{2+}$ are required. For successful fertilization, calcium flux control through the spermatozoal membrane is necessary (Bailey and Buhr, 1993).

It was concluded from the study that most of the functional and biochemical parameters were higher in the freezable ejaculates than the non-freezable. Significantly higher mass motility, per cent livability, progressive motile, HOS reactive spermatozoa and alkaline phosphatase were recorded in freezable ejaculates compared to non-freezable at all the stages of semen processing.

## REFERENCES

Badaway, A.M., Yaseen, A.M., Elbashary, A.S. and Ibrahim, M.A. (1973). Effect of sexual preparation on some characteristics of the semen of buffalo and cattle bulls. *Alexandria J. Agric. Res.* **21(2)**: 185-191.

Bailey, J.L. and Buhr M.M. (1993). Cryopreservation alters the $Ca^{2+}$ flux of bovine spermatozoa. *Can. J. of Anim. Sci.* **74(1)**: 45-51.

Bhosrekar, M.R., Char, S.N., Patil, B.R. and Rangnekar, D.V. (1984). Effect of thawing temperature and time on the forward motility, live count and acrosomal maintenance of bovine spermatozoa. *Indian J. Anim. Sci.* **54(12)**: 1126-1130.

Bishop, M.W.H., Campbell, R.C., Hancock, J.L. and Watson, A. (1954). Semen characteristics and fertility in bulls. *J. Agric. Sci.* **44**: 227-248.

Blom, E. (1972). The ultrastructure of some characteristics sperm defects and a proposal for a new classification of bull spermatozoa. In: Proceedings of the VII Simposio international di Zootechnia Milan, pp. 125-139.

Bucci, D., Giaretta, E., Spinaci, M., Rizzato, G., Isani, G., Mislei, B., Mari, G., Tamanini, C. and Galeati, G. (2016). Characterization of alkaline phosphatase activity in seminal plasma and in fresh and frozen-thawed stallion spermatozoa. *Theriogenol.* **85**: 288-295.

Chandra, M., Srivastava, V.K. and Shukla, A.K. (1999). Study of effect of temperature on semen quality (volume) and quality (motility) using fuzzy approach. *Buff J.* **15(1)**: 105-113.

Chauhan, H.S., Kumar, R., Kumar, S., Tyagi, S. and Rajkumar. (2017). Physical and morphological characteristics of Frieswal bull semen. *Int. J. Chem. Stud.* **5(3)**: 865-867.

Corteel, J.M. (1980). Effets du plasma seminal sur la survie et la fertilite des spermatozoids conserves *in vitro. Reprod. Nutr. Dev.* **20**: 1111-1123.

Dhaka, Y. (2013). Comparative study on certain functional and biochemical parameters in fresh and frozen semen of buffalo bulls with varying reproductive performance. M.V.Sc. thesis submitted to CSKHPKV, Palampur, India.

Dogan, I., Polat, U. and Nur, Z. (2009). Correlations between seminal plasma enzyme activities and semen parameters in seminal fluids of Arabian horses. *Iranian J. Vet. Res.* **10**: 27.

Eghbali, M., Alavi-Shoushtari, S.M., Arsi-Rezaei, S. and Ansari, M.H.K. (2010). Calcium, Magnesium and Total Antioxidant Capacity (TAC) in seminal plasma of water buffalo (BubalusBubalis) bulls and their relationships with semen characteristics. *Vet. Res. Forum.* **1(1)**: 12-20.

Flipse, R.J. and Anderson, W.R. (1964). Metabolic fate of glutamic acid C in bovine spermatozoa. *J. Dairy Sci.* **47**: 686.

Gebreselassie, G.A., Srivastava, S.K., Gosh, S.K., Suman, C.L. and Tripathi, R.P. (2012). Physico-morphological characteristics of crossbred bull semen. *Indian Vet. J.* **89**: 91-93.

Ghirardosi, M.S., Fischman, M.L., Jorge, A.E., Chan, D. and Cisale, H. (2017). Relationship between morphological abnormalities in commercial bull frozen semen doses and conception rate. Andrologia. DOI: 10.1111/and.12884.

Gopinathan, A., Sivaselavam, S.N., Karthickeyan, S.M.K. and Kulasekar, K. (2016). Effect of body weight and scrotal circumference on semen production traits in crossbred Holstein Friesian bulls. *Indian J. Anim. Reprod.* **39(1)**: 24-27.

Januskauskas, A. and Zilinskas, H. (2002). Bull semen evaluation post thaw and relation of semen characteristics to bull's fertility. *Vet. Zootech. Lith.* **17**: 29-36.

Javed, M.T., Khan, A. and Kausar, R. (2000). Effect of age and season on some semen parameters of Nili-Ravi buffalo (*Bubalus bubalis*) bulls. *Vet. Arhiv.* **70(2)**: 83-94.

Kathiravan, P., Kalatharan, J., Karthikeya, G., Rengarajan, K. and Kadirvel, G. (2011). Objective sperm motion analysis to assess dairy bull fertility using computer-aided system: A Review. *Reprod. Domest. Anim.* **46**: 165-172.

Khalifa, T., Rekkas, C., Samartzi, F., Lymberopoulos, A., Kousenidis, K. and Dovenski, T. (2014). Highlights on artificial insemination (AI) technology in pigs. *Mac. Vet. Rev.* **37**: 5-34.

Khalil, W.A., EI-Harairy, M.A., Zeidan, A.E.B. and Hassan, M.A.E. (2017). Evaluation of bull spermatozoa during and after cryopreservation: Structural and ultrastructural insights. *Int. J. Vet. Sci. Med.* https://doi.org/10.1016/j.ijvsm.2017.11.001.

Moazzam, A., Choudhary, M.N., Muhammad, I., Sarwat, J. and Ijaz, A. (2015). From basic to contemporary semen analysis. Limitations and variability. *J. Anim. Plant Sci.* **25(2)**: 328-336.

Pant, H.C., Mittal, A.K., Patel, S.H., Shukla, H.R., Kasiraj, R. and Prabhakar, J.H. (2002). The hypoosmotic swelling test: an assay of cell membrane integrity and quality of frozen semen straw. *Indian J. Ani. Reprod.* **23(1)**: 8-11.

Perumal, P., Srivastava, S.K., Vupru, K., Khate, K., Nahak, A.K. and Rajkhowa, C. (2015). Semen quality parameters of freezable and non-freezable ejaculates of Mithun Bulls. *Adv. Anim. Vet. Sci.* **1(3)**: 11-18.

Roberts, S.J. (1971). Veterinary Obstetrics and Genital Disease (2nd edn.), CBS publishers and Distributors, pp: 612-750.

Saha, S., Mishra, G.K., Singh, R.B., Shukla, M.K., Tiwari, R. and Saxena, S.K. (2011). Effect of FMD vaccination on semen characteristics in Holstein Friesian bulls. *Indian J. Ani. Reprod.* **32(2)**: 49-51.

Salisbury, G.W., Van Denmark, N.L. and Lodge, J.R. (1978). Principles and techniques of freezing spermatozoa. In: Physiology of Reproduction and Artificial Insemination of Cattle. Saliburg, G.A. (edt.), San Francisco: W.H Freeman Co. pp: 454-459.

Sharma, M. (2011). Correlation between certain quality evaluation parameters and fertility of frozen-thawed Jersey crossbred bull semen. M.V.Sc. thesis submitted to CSKHPKV Palampur, India.

Sharma, M.L., Mohan, G. and Shanti, K.L. (1991). Characteristics and cryopreservation of semen of Holstein Friesian bulls under tropics. *Indian J. Ani. Reprod.* **61**: 977-979.

Tomar, N.S., Sharma, K.C. and Shukla, S.N.(1985). Semen production in relation to the age of HF×Hariana bulls. *Indian Vet. J.* **62**: 499-501.

Vettical, B.S. (2016). Evaluation of Cauda epididymal semen quality of crossbred bulls in the tropics. *Int. J. Appl. Sci. and Biotechnol.* **4(1)**: 130-132.