Buffalo, through their potential for producing milk, meat and draft power, contribute significantly to the agricultural economy of many developing countries including India. Artificial insemination (AI) in buffaloes on large scale using semen from bulls with superior germplasm can solve the problem of low productivity as well as reproductive efficiency. During last three decades, extensive research work has been carried out both in India and abroad on various aspects for improving the freezing technology of buffalo bull semen. In spite of this improvement, the post-thaw semen of buffalo bulls is not as freezable as that of cattle (Dhami et al. 1995). The viability and fertility of frozen–thawed buffalo bull spermatozoa is considerably lower as these are more susceptible to hazards during freezing and thawing than cattle spermatozoa which create hurdles in extensive exploitation of production potential of buffalo. Therefore, a better understanding of the fundamental principle of cryopreservation of buffalo spermatozoa is necessary as per its specific requirements.

One of the many possible causes of lower freezability of buffalo bull semen compared to cattle bull can be due to the differences in the lipid ratio of the spermatozoa (Andrabi 2008). Freezing-thawing of buffalo spermatozoa is known to cause considerable damage to DNA, motility apparatus, plasma membrane and acrosomal cap (Rasul et al. 2001), leakage of intracellular enzymes (Dhami and Kodagali 1990) and thus, reduced fertility. Although some of the bulls may apparently donate freezable semen but the freezability may be non-freezable adversely affecting fertility, whereas not so non-freezable semen may have acceptable freezability and fertility (Dhami and Shelke 2005). Hence periodic detailed andrological investigation is prerequisite to successful breeding programme.

Evaluation of plasma membrane integrity, motility, vigour and morphology of fresh and frozen thawed semen along with the seminal characteristics and their interrelation might establish a basis for the predicting the fertility potential of semen. Hence the objectives of this investigation were to compare seminal attributes between the buffalo bulls of freezable and non-freezable semen and further, establishment of correlation between microscopic evaluation parameters.

MATERIALS AND METHODS

The study was conducted on 12 apparently healthy Murrah buffalo breeding bulls, aged between 5–7 years, maintained at Frozen Semen Bank, RCDF Limited, Bassi, Jaipur. The selected bulls were divided into 2 groups, each comprising of 6 bulls. Group 1 comprised those bulls, which were donating semen of excellent quality with freezable freezability and fertility parameters, whereas, Group 2 included those bulls which were frequently donating either initial non-freezable semen or higher degree of damage during processing (during equilibration or cryopreservation).
but were otherwise healthy. Total 96 ejaculates (8 ejaculates from each of 12 bulls), were collected twice a week by artificial vagina method. The collected semen was subjected to initial examination of volume, colour, concentration and initial motility qualifying after which they were further processed as per standard laboratory procedures. The minimum initial standards were a volume of 1 ml, colour ranging from milky white to creamy, minimum concentration of 500 million/ml. Samples having +3 or more mass movement were considered for further processing in case of freezable ejaculates and below +3 were also considered in case of non-freezable ejaculate evaluation.

The selected ejaculate was diluted with tris egg yolk extender to attain a final concentration of 80 million/ml after which they were filled into French mini straws. These straws were finally shifted to liquid nitrogen containers and stored till use. These were analyzed for percent viability, progressive motility, reaction to 100 mOsmol hypo-osmotic solution (Pant et al. 2002) and acrosomal integrity (Watson and Stewart 1979) at four stages of processing namely post-dilution (34°C), post-equilibration (4°C), post-thaw (37°C) and 1 h after incubation at 37°C. Thawing of frozen semen straws for post thaw evaluation was done in water bath at 37°C for 30 sec. The data obtained was analysed using SAS statistical package version 9.2. Multivariate analysis was used to determine the correlations and to frame regression equations and their significance was tested by ANOVA.

**RESULTS AND DISCUSSION**

Significant differences (P<0.05) were found during the study for various parameters when freezable and non-freezable semen was compared (Table 1). Perusal of the Table 2 indicates that sperm viability was significantly (P<0.01) correlated with progressively motile spermatozoa, HOS responsive spermatozoa and acrosomal integrity in freezable and non-freezable ejaculates. Also, progressive motility was significantly (P<0.01) correlated with HOS reactive and acrosomal integrity in freezable and non-freezable ejaculates, respectively (Table 2). Our findings also indicated that HOS reactive spermatozoa percentage was significantly (P<0.01) correlated with acrosomal integrity in freezable and non-freezable ejaculates, respectively (Table 2). The results of the present study were in agreement with some previous work on cattle (Lodhi et al. 2008, Sharma et al. 2012), human (Jeyendran et al. 1984), equine (Mantovani et al. 2002), ram (Boholooli et al. 2012) and fresh goat spermatozoa (Fonseca et al. 2005).

Various conventional evaluation parameters have been used to assess semen quality. Evaluating a relationship between different semen evaluation parameters is important, as we can judge other parameters on the basis of evaluation of one parameter. El-Sisy et al. (2010) showed a significant correlation between live spermatozoa per cent with HOS reactive (r=0.681) and acrosomal abnormality (r=0.220) in buffalo bull spermatozoa which is in agreement to our findings. The percentage of live sperms and acrosome intact sperms has also been shown to be highly correlated with percentage of motile sperms (Kumar 2004, Kirk et al. 2005).

**Table 1. Comparative functional parameters (Mean±SE) of freezable (n=48) versus non-freezable (n=48) semen during various stages of processing in Murrah buffalo bulls**

| Parameter                  | Type of semen | Stage of semen processing | Fresh Diluted | Equilibration | 0 h Post-thaw | 1 h Post-thaw |
|----------------------------|---------------|---------------------------|---------------|---------------|--------------|--------------|
| Live spermatozoa (%)       | Freezable     |                           | 90.88±0.26   | 94±0.94       | 78.29±0.31   | 74.00±0.36   |
|                           |               |                           | (86–94)       |               | (73–83)      | (69–80)      |
|                           | Non-freezable |                           | 64.42±0.91   | 79–90         | 48.77±1.11   | 10.75±0.78   |
|                           |               |                           | (49–79)       |               | (32–63)      | (02–25)      |
| Progressive motile spermatozoa (%) | Freezable  |                           | 86.15±0.34   | 90–90         | 73.17±0.38   | 40.46±0.50   |
|                           |               |                           | (79–90)       |               | (68–80)      | (34–49)      |
|                           | Non-freezable |                           | 44.48±0.75   | 30–54         | 31.25±0.69   | 5.42±0.58    |
|                           |               |                           | (30–54)       |               | (20–43)      | (00–13)      |
| HOS Reactive Spermatozoa (%) | Freezable  |                           | 89.19±0.26   | 92–98         | 85.27±0.23   | 72.90±0.39   |
|                           |               |                           | (84–92)       |               | (81–89)      | (68–79)      |
|                           | Non-freezable |                           | 63.17±0.93   | 77–81         | 47.58±1.09   | 9.94±0.79    |
|                           |               |                           | (47–77)       |               | (31–61)      | (00–24)      |
| Intact acrosome (%)       | Freezable     |                           | 91.21±0.30   | 95–97         | 85.48±0.37   | 70.79±0.32   |
|                           |               |                           | (87–95)       |               | (80–90)      | (67–76)      |
|                           | Non-freezable |                           | 65.3±0.88    | 86–90         | 49.65±1.04   | 15.88±0.64   |
|                           |               |                           | (46–80)       |               | (34–61)      | (05–24)      |

A,B,C,DMeans with different superscript within rows differ significantly (P<0.05). a,b Means with different superscript between columns differ significantly (P<0.05); n= Number of ejaculates.
Relationship between parameters | Type of semen coefficient | Correlation Estimate | Regression Equation | Regression Coefficient Estimate |
--- | --- | --- | --- | --- |
Viability | Motility | Freezable | 0.92738** | 0.38±0.01 | y=57.39+0.38x |
 | | Non-freezable | 0.99934** | 1.31±0.00 | y= 5.76+1.31x |
HOST | Freezable | 0.98934** | 1.01±0.01 | y= 0.43+1.01x |
 | Non-freezable | 0.99934** | 1.00±0.00 | y= 0.79+1.00x |
Acrosome | Freezable | 0.92725** | 0.81±0.02 | y=16.52+0.81x |
 | Non-freezable | 0.98273** | 1.07±0.01 | y= −5.43+1.07x |
Progressive motility | HOST | Freezable | 0.92052** | 2.30±0.07 | y=−120.32+2.30x |
 | | Non-freezable | 0.94212** | 0.68±0.02 | y=−0.62+0.68x |
Acrosome | Freezable | 0.94709** | 2.01±0.05 | y= −98.40+2.01x |
 | Non-freezable | 0.95266** | 0.75±0.02 | y= −5.67+0.75x |
HOST | Acrosome | Freezable | 0.91623** | 0.78±0.24 | y= 17.38+0.78x |
 | Non-freezable | 0.98272** | 1.07±0.01 | y= −6.15+1.07x |

***(P<0.01).

Sisy et al. (2010) reported a significant correlation of motility with live (r= 0.728), HOS reactive (r=0.918) and abnormal acrosome (r=0.277) in buffalo bull spermatozoa. Kumar (2004) reported a significantly positive correlation between sperm motility, live percentage and HOST reactive spermatozoa. Raval and Dhami (2006) also reported a between sperm motility, live percentage and HOST reactive spermatozoa.

Lodhi et al. (2008) reported a positive and significant (P<0.05) correlation between initial motility with live sperm (r=0.54) which is in concurrence with our findings.

In conclusion, non-freezable semen was having least viability, progressive motility, reaction to hypo-osmotic solution and acrosomal integrity, so, it can be considered as poor quality semen. As far as correlation between various semen evaluation parameters is concerned, there was a positive correlation for all the parameters in semen of freezable and non-freezable quality.

REFERENCES

Andrabi S M H. 2008. Factors affecting the quality of cryopreserved buffalo (Bubalus bubalis) bull spermatozoa. Reproduction in Domestic Animals 44: 552–69.

Barnabe V H, Barnabe R C, Appuda R P, Visintin J A and Freitas M T L. 1992. Seasonal behaviour of semen collected by electro ejaculation from buffaloes raised in SAO Paulo State (Southeast Brazil). p. 484. 12th International Congress on Animal Reproduction and Artificial Insemination, Netherlands.

Brito L F C, Barth A D, Bilodeau-Goessel S, Panich P L and Kastelic J P. 2003. Comparison of methods to evaluate plasmalemma of bovine sperm and their relationship with in vitro fertilization rate. Theriogenology 60(8): 1539–51.

Correa J R and Zavos P M. 1994. The hypoosmotic swelling test: its employment as an assay to evaluate the functional integrity of the frozen-thawed bovine sperm membrane. Theriogenology 42(2): 351–60.

Dhami A J and Kodagali S B. 1990. Freezability, enzyme leakage and fertility of buffalo spermatozoa in relation to the quality of semen ejaculates and extenders. Theriogenology 35(3): 853–63.

Dhami A J and Shelke V B. 2005. Investigations into the causes of poor semen quality and freezability in Jafarabadi buffalo bulls. Indian Journal of Animal Sciences 75(8): 925–29.

Dhami A J, Jani V R, Sahni K L and Mohan G. 1995. Freezability and fertility of heterospermic semen of Friesian and Murrah bulls using Tris and milk extenders. Indian Veterinary Journal 72: 1273–76.

El-Sisy G A, El-Sheshtawy R I, Mohamed A A and El-Nattat W S. 2010. Correlations between semen parameters and conception rate in buffaloes. Global Veterinaria 5(1): 15–21.

Fonseca J F, Torres C A A, Maffili V V, Borges A M, Santos A D F, Rodrigues M T and Oliveira R F M. 2005. The hypoosmotic swelling test in fresh goat spermatozoa. Animal Reproduction 2(2): 139–44.

Graham E F. 1978. Fundamentals of the preservation of spermatozoa. The Integrity of frozen spermatozoa (The National Research Council eds.), pp. 4–44, National Academic Science, Washington DC.

Jeyendran R S, Van der Ven H H, Perez-Pelaez M, Crabo B G and Zaneveld L J D. 1984. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to the other semen characteristics. Journal of Reproduction and Fertility 70(1): 219–28.

Kale M M, Manik R S and Tomer O S. 2000. In vitro assessment of crossbred buck fertility. Indian Journal of Animal Sciences 70(1): 25–29.

Kirk E S, Squires E L and Graham J K. 2005. Comparison of in
vitro laboratory analyses with the fertility of cryopreserved stallion spermatozoa. *Theriogenology* 64(6): 1422–39.

Kumar S. 2004. Studies on the effect of certain additives on quality and fertility of cryopreserved semen of jersey bulls maintained under subtropical climate. M.V.Sc Thesis, CSKHPKV, Palampur, India. p. 47.

Lodhi L A, Qureshi Z M, Ahmad Z I and Jamil I H. 2008. Correlation between hypo osmotic swelling test and various conventional semen evaluation parameters in fresh Nili-Ravi buffalo and Sahiwal cow bull semen. *Pakistan Veterinary Journal* 28(4): 186–88.

Mantovani R, Rota A, Falomo M E, Bailoni L and Vincenti L. 2002. Comparison between glycerol and ethylene glycol for the cryopreservation of equine spermatozoa: semen quality assessment with standard analyses and with the hypoosmotic swelling test. *Reproduction Nutrition and Development* 42(3): 217–26.

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 Journal of Animal Reproduction* 23(1): 8–11.

Prasad J K, Kumar S, Mohan G, Shanker U and Agarwal S K. 1999. Hypo-osmotic swelling test (HOST) and its response in fresh and freeze-thawed semen. *Indian Journal of Animal Sciences* 69(10): 766–69.

Rasul Z, Ahmad N and Anzar M. 2001. Changes in motion characteristics, plasma membrane integrity and acrosome morphology during cryopreservation of buffalo spermatozoa. *Journal of Andrology* 22: 278–83.

Raval R J and Dhami A J. 2006. Physico-biochemical attributes of semen and their interrelationships in triple crossbred (HF X J X K) bulls. *International Journal of Cow Science* 2(2): 34–40.

Sharma M, Singh M, Kapoor S and Jasial S. 2012. Inter relationship between some routine semen evaluation parameters in Jersey × local hill cattle crossbred bulls. *Open Veterinary Journal* 2: 26–31.

Sharma M. 2011. Correlation between certain quality evaluation parameters and fertility of frozen-thawed Jersey crossbred bull semen. M.V.Sc. Thesis, CSKHPKV, Palampur, India. p. 42.

Watson R C and Stewart D L. 1979. The effect of cooling to 5°C and freezing and thawing on the ultrastructure of bull spermatozoa. *Journal of Reproduction and Fertility* 56(1): 233–38.