NORMS AND INTERRELATIONSHIPS OF QUALITY ATTRIBUTES OF FRESH, REFRIGERATED AND CRYOPRESERVED BUFFALO SEMEN

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ABSTRACT

This study was undertaken on semen ejaculates (n=48) of six mature Surti buffalo bulls. Following evaluation the ejaculates were extended in standard TFYG (Tris-citric acid-fructose-egg yolk-glycerol) diluent at 34°C temperature keeping 100 x 106 sperm per ml. The fractions of extended semen samples were preserved at refrigeration preservation (5°C, 72 hrs) as well as at ultra low (-196°C) temperature using split sample technique. The semen filled in French mini straws was frozen in LN2 vapour using programmable biofreezer. The mean ± SE values of various spermatozoal attributes obtained of fresh, refrigerated and cryopreserved semen were within normal acceptable limit for the buffalo semen. The correlation matrix analysis was worked out for sperm motility, viability, normal morphology, intact acrosome and plasma membrane integrity in fresh semen, semen preserved at 5°C for 24 and 72 hrs and in post-thawed samples. There were significant (P<0.01) interrelationships (r = 0.29 to 0.94) observed between sperm motility, viability, 5normal morphology, intact acrosome and plasma membrane integrity in fresh, refrigerated and cryopreserved buffalo semen, which proved that initial motility and membrane integrity can be used as predicative measures of cryopreservability of buffalo semen in routine evaluation.

KEY WORDS: Buffalo, Fresh semen, Refrigeration, Cryopreservation, Sperm parameters, Interrelationships.

INTRODUCTION

The literature on the ejaculate quality as well as keeping quality and freezability of bovine semen in different extenders is quite large (Dhami *et al*., 1993; Rana *et al*., 2003; Lodhi *et al.,* 2008; Patel *et al.,* 2012; Mahmoud *et al.,* 2013), but the information about the interrelationships of various spermatozoal attributes of fresh, refrigerated and frozen-thawed bovine semen is meager both in cattle and buffaloes (Rana and Dhami, 2003; Raval and Dhami, 2010). The evaluation of interrelationships of spermatozoa attributes of fresh, refrigerated and cryopreserved bovine semen would help to select a few most valid simple traits of fresh or refrigerated semen to predict future keeping quality, freezability and even fertility of such ejaculates, instead of going through a plethora of time consuming unpredictable cumbersome tests. Hence an attempted was made to study such correlations and report the same for Surti buffalo bulls semen.

MATERIALS AND METHODS

The present study was undertaken during the favourable breeding season on semen of six sexually mature healthy Surti buffalo bulls (*Bubalus bubalis*), 4-6 years old. The bulls were maintained under uniform standard nutritional and managerial practices at the Central Sperm Station of the College in Anand, Gujarat (India). The bulls were under regular (twice a week) semen collection schedule using artificial vagina. Semen collections were made in the early morning between 7.30 and 8.30 hrs. Two consecutive ejaculates were obtained and pooled samples were used for further study.

The ejaculates (8 per bull, total 48) after collection were immediately transferred in to a water bath at 34ºC and evaluated for gross quality, motility and sperm concentration (by Accucell photometer). Only the pooled ejaculates with >70% initial motility were used for further dilution and storage.

The standard Tris-citrate-fructose-egg yolk-glycerol (TFYG) extender prepared fresh daily as per FAO (1979) added with antibiotics benzyl penicillin 1000 IU/ml and streptomycin sulphate 1000 µg/ml was used for semen dilution. The qualifying ejaculates from each bull were diluted at the concentration of 100 $\times 10^6$ spermatozoa ml^{-1} at 34°C and evaluated for progressive sperm motility. The fractions (2 ml) of extended semen ejaculates were taken in 5 ml sterilized glass tubes for refrigeration and the rests were frozen. The tubes containing extended semen samples were properly labeled, covered with aluminum foil and placed in plastic box half filled with warm water (32-34°C), which was then transferred to a refrigerator for gradual cooling and storage at 5°C. The individual sperm motility, viability, morphology, acrosome integrity and plasma membrane integrity (HOST) were assessed at 24 hourly interval up to 72 hrs of preservation. The remaining aliquots of diluted samples were filled in French mini straws using IS4 system of IMV France, gradually cooled to 5°C and equilibrated for 4 hrs in cold handling cabinet and then frozen in LN2 vapour using programmable biofreezer. Thawing of straws was done at 37°C in water bath for 30 sec. The frozen-thawed samples were also evaluated for all above parameters, including 1-hr incubation test at 37°C water bath.

The sperm progressive motility was determined at 37°C temperature under high power magnification (40 X) of phase contrast microscope (Longshou, USA) fitted with a biotherm stage and a closed circuit television. The viability and morphology of spermatozoa were assessed with eosin-nigrosin stained semen smears (Campbell *et al.,* 1953) under oil emulsion lens of a phase contrast microscope (100x; Olympus BX20, Tokyo, Japan). The percentages of sperm with intact acrosome were assessed using Geimsa stain (Watson, 1975). The plasma membrane integrity of spermatozoa was assessed using a hypo-osmotic swelling (HOS) test employing 150 mOs/L solution of sodium citrate and fructose with 30 minutes of incubation at 37°C (Jeyendran *et al.,* 1984; Rasul *et al.* (2000). The wet preparations of semen were then evaluated using a phase contrast microscope (40 X) for swelling and coiling of tail. Nearly 200 spermatozoa were assessed from different fields for each trait and expressed as per cent.

The data generated were analyzed statistically using ANOVA and critical different test by employing IBM SPSS Statistics version 20.00. The interrelationships between various sperm parameters evaluated in fresh, refrigerated and cryopreserved semen were worked out through correlation matrix analysis (Snedecor and Cochran, 1994).

RESULTS AND DISCUSSION

The mean ± SE values and the correlation matrix analysis between various sperm parameters studied in fresh semen, in 24- & 72-hr refrigerated samples as well as in post-thaw samples of Surti buffalo semen are presented in Tables 1 and 2, respectively.

Quality of Fresh, Refrigerated and Cryopreserved Spermatozoa

The mean ejaculate volume, density score, sperm concentration and mass activity score of Surti buffalo semen examined averaged 3.43±0.10 ml, 2.14±0.11, 841.38±61.32 million per ml and 3.45±0.07, respectively. Further, the initial motility, live sperm, abnormal sperm, intact acrosome and HOS reactive sperm noted in fresh semen of Surti buffalo bulls were 78.54±0.51, 90.48±0.33, 6.15±0.15, 94.40±0.20 and 79.35±0.42 per cent, respectively. The values of motile and live sperm, intact acrosome and HOS reactive sperm declined while abnormal sperm increased gradually and significantly in pre-freeze to post-thaw semen as well as in refrigerated samples at every 24 hourly intervals till 72-hrs (Table 1). The values and trend of observations of various attributes found corroborated well with several of the earlier reports on bovine semen (Sharma *et al.,* 1992; Dhami *et al.*, 1993; Shelke and Dhami, 2001; Taraphder *et al*., 2001; Rana *et al.,* 2003; Lodhi *et al.,* 2008;

Tiwari *et al.,* 2009; Khawaskar *et al*., 2012; Mahmoud *et al.,* 2013) and were within normal physiological acceptable limits.

Table 1: Overall initial quality, freezability (at -196°C) and storage ability (at 5°C) of Surti buffalo bulls semen in TFYG diluent (Mean ± SE)

Spermatozoal Traits	Initial quality	Freezability (at -196°C) / Steps		Storage ability (at 5°C) / Periods		
		Pre-freeze	Post-thaw	24-hr	48-hr	72-hr
Motile sperm (%)	78.54 ±0.51	69.48 ±0.37	47.71 ±0.79	68.33 ±0.45	59.79 ±0.58	52.50 ±0.68
Live sperm (%)	90.48 ±0.33	79.21 ±0.39	57.19 ±0.79	77.35 ±0.46	69.90 ±0.54	62.56 ±0.65
Abnormal sperm (%)	6.15 ±0.15	7.90 ±0.14	12.33 ±0.17	9.35 ±0.15	11.08 ±0.11	12.65 ±0.14
Head abnormality (%)	1.83 ±0.09	2.27 ±0.08	3.42 ±0.09	2.31 ±0.07	2.85 ±0.08	3.13 ±0.09
Midpiece abnormality (%)	0.88 ±0.06	1.23 ±0.06	2.08 ±0.07	1.75 ±0.06	2.02 ±0.04	2.42 ±0.08
Tail abnormality (%)	3.44 ±0.08	4.40 ±0.07	6.83 ±0.10	5.29 ±0.09	6.21 ±0.09	7.10 ±0.10
Intact acrosome (%)	94.40 ±0.20	89.54 ±0.18	76.83 ±0.23	88.15 ±0.18	84.63 ±0.18	81.67 ±0.23
HOST reactive sperm (%)	79.35 ±0.42	67.96 ±0.32	45.02 ±0.84	66.04 ±0.50	57.92 ±0.55	51.35 ±0.60

Interrelationships of Fresh, Refrigerated and Cryopreserved Spermatozoa

The ejaculate volume had significant (P<0.05) negative and positive correlation with post-thaw live sperm and abnormal sperm (-0.33, 0.36). Sperm concentration had significant (P<0.05) positive correlations with mass activity and individual sperm motility (0.44, 0.36) in fresh semen and with abnormal sperm per cent (0.36) at 72-hr of refrigeration preservation. It had significant (P<0.05) negative correlations with HOS reactive sperm, live sperm and intact acrosome percentage in fresh semen (-0.29, -0.33, -0.34).

Mass activity had significant (P<0.01) positive correlations with individual sperm motility in fresh semen (0.77), intact acrosome at 24-hr of refrigeration (0.32) and with post-thaw motility, HOS reactive sperm, live sperm and incubation survival at 60-min (0.33, 0.34, 0.43, 0.38, resp.). It had significant (P<0.05) negative correlations with abnormal sperm at 24-hr of refrigeration and at post-thaw stage (-0.34, -0.30). The individual sperm motility in fresh semen had significant (P<0.05) positive correlations with sperm motility and live sperm at 72-hr of refrigeration (0.32, 0.30), and negative correlations with abnormal sperm in fresh and post-thaw semen (-0.32, -0.29).

Live sperm per cent in fresh semen had significant positive correlations with intact acrosome in fresh semen (0.65, P<0.01), with individual sperm motility and live sperm per cent at 24-hr of refrigeration (0.32, 0.31, P<0.05), and with HOST and live sperm per cent at 72-hr of refrigeration (0.31, 0.31). It had significant negative correlation with abnormal sperm in fresh semen (-0.36). Abnormal sperm in fresh semen showed significant (P<0.01) positive correlations with abnormal

sperm motility; HOST = Hypo-osmotic swelling test; LSP = Live sperm %; AbSP = Abnormal sperm %;
IA = Intact acrosome %; PTM0 =Post-thaw motility 0 min; PTM60= Post-thaw incubation motility 60 min.

sperm at 24-hr and 72-hr of refrigeration (0.57, 0.43), as well as at post-thaw stage (0.43). It had significant negative correlations with intact acrosome (-0.50, P<0.01) in fresh semen, with individual sperm motility, HOS reactive sperm, live sperm and intact acrosome at 24-hr of refrigeration (-0.36, -0.35, -0.41, -0.50, resp.), with HOS reactive sperm and live sperm at 72-hr of refrigeration (-0.34, -0.29) and with post-thaw HOST reactive sperm and intact acrosome (-0.28, -0.36).

The HOS reactive sperm in fresh semen showed significant (P<0.01) positive correlations with live sperm and intact acrosome in fresh semen (0.49, 0.40) and with post-thaw intact acrosome (0.44). It had significant negative correlation with abnormal sperm (-0.34) at 72-hr of refrigeration. Intact acrosome in fresh semen had highly significant (P<0.01) positive correlations with sperm motility, HOS reactive sperm, live sperm and intact acrosome at 24-hr of refrigeration (0.42, 0.33, 0.43, 0.53, resp.), with live sperm and intact acrosome at 72-hr of refrigeration (0.30, 0.38) and with post-thaw intact acrosome (0.32).

The individual sperm motility at 24-hr of refrigeration had significant (P<0.01) positive correlations with HOS reactive sperm, live sperm and intact acrosome at 24-hr of refrigeration (0.75, 0.86, 0.39) and with individual motility, HOS reactive sperm, live sperm and intact acrosome at 72-hr of refrigeration (0.54, 0.59, 0.56, 0.37). Live sperm at 24-hr of refrigeration had significant (P<0.01) positive correlations with individual sperm motility, HOS reactive sperm, live sperm and intact acrosome at 72-hr of refrigeration (0.54, 0.56, 0.58, 0.34). The abnormal sperm per cent at 24-hr of refrigeration had significant (P<0.01) positive correlations with abnormal sperm at 72-hr of refrigeration (0.39) and at post-thaw stage (0.35). It had significant (P<0.05) negative correlations with intact acrosome at 24-hr of refrigeration (-0.32) and with post-thaw HOS reactive sperm, live sperm and 1-hr incubation motility (-0.33, -0.30, -0.34). The HOS reactive sperm at 24-hr of refrigeration had significant (P<0.01) positive correlations with live sperm at 24-hr of refrigeration (0.81), and with individual sperm motility, HOS reactive sperm, live sperm and intact acrosome at 72-hr of refrigeration (0.55, 0.63, 0.60, 0.38, resp.).

Individual sperm motility at 72-hr of refrigeration had highly significant (P<0.01) positive correlations with HOS reactive sperm, live sperm and intact acrosome at 72-hr of refrigeration (0.89, 0.95, 0.46). Live sperm per cent at 72-hr of refrigeration was significantly (P<0.01) and positively correlated with intact acrosome at 72-hr of refrigeration (0.48). The abnormal sperm per cent at 72-hr of refrigeration had significant negative correlation with post-thaw intact acrosomes (-0.37). The HOS reactive sperm at 72-hr of refrigeration showed significant (P<0.01) positive correlations with live sperm and intact acrosome at 72-hr of refrigeration (0.93, 0.49).

The post-thaw motility immediately after thawing had significant (P<0.01) positive correlations with HOS reactive sperm, live sperm and motility after 1-hr of incubation (0.94, 0.93, 0.90), while it had significant negative correlation with post-thaw abnormal sperm (-0.42). The post-thaw HOS reactive sperm showed significant (P<0.01) positive correlations with live sperm and 1-hr incubation motility (0.93, 0.88), and negative correlation with post-thaw abnormal sperm (-0.44). Post-thaw live sperm had significant (P<0.01) positive correlation with 1-hr incubation motility (0.83). Post-thaw abnormal sperm had significant negative correlation with post-thaw live sperm (-0.47), intact acrosome (-0.34) and 1-hr post-thaw incubation motility (-0.36, Table 2).

These findings on correlation coefficients observed corroborated well with many of the reports, particularly of Dhami *et al.* (1993), Dhami and Sahni (1994), Shelke and Dhami (2001), Rana and Dhami (2003), Tiwari *et al.* (2009), Raval and Dhami (2010) and Patel *et al.* (2012) in bovine semen. Raval and Dhami (2010) recorded highly significant (P<0.01) positive correlations for ejaculate volume with abnormal sperm, initial motility with mass activity and live sperm per cent, and negative correlations for abnormal sperm with initial motility and live sperm per cent in triple crossbred bulls. Patel *et al.* (2012) found significant (P<0.01) positive correlation for sperm individual motility and hypo-osmotic swelling test.

Our findings also corroborated with those of Rana and Dhami (2003) and Raval and Dhami (2010), who found significant (P<0.01) and positive interrelationships for the percentages of motile spermatozoa in fresh, post–thawed and refrigerated semen of bovine and bubaline species. Similar were the findings for the percentages of live sperms, abnormal sperms, intact acrosome and damaged acrosome (r = 0.17 to 0.90). The findings suggested that these traits could be of practical utility in routine semen evaluation to predict its keeping quality, freezability and fertility.

Further, the results obtained in this study show that motility may be a candidate marker for semen quality, considering that significant correlations were found between motility and both sperm abnormalities and acrosome as well as plasma membrane integrity, which is in accordance with the opinion of Mahmoud *et al.* (2013). Dhami *et al.* (1993) recorded highly significant positive correlations (0.68 to 0.98) for the sperm motility traits of liquid and frozen-thawed semen of HF bulls at various storage intervals/processing steps, and concluded that freezability of semen could be predicted based on its keeping quality at 5°C.

The results of the present study showed highly significant correlation between HOST score and progressive motility (%), sperm viability (%) and morphologically normal spermatozoa (%) for Surti buffalo bull semen. The findings, thus in general, suggest that motility estimation in fresh, post-thawed and refrigerated semen is a fairly good indicator of live and abnormal sperm and acrosomal integrity of spermatozoa at various steps of semen processing / freezing / preservation and hence, this trait alone can be adopted in routine assessment of semen quality, instead of going into the time consuming cumbersome staining procedures for assessment of viability, morphology and acrosomal integrity, which in fact are not always correlated with *in vivo* fertility.

Sperm motility was also correlated with sperm abnormalities and membrane integrity (HOST). Under the conditions of the present study, it was inferred that HOS test in addition to motility could be a valuable and practical tool to know the functional capacity of fresh and preserved buffalo spermatozoa. It could be added in the routine analysis of semen samples for artificial insemination.

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