QUALITY TRAITS OF FRESH, REFRIGERATED AND CRYOPRESERVED BUFFALO BULL SEMEN AND THEIR INTERRELATIONSHIPS

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ABSTRACT

A study was carried out on semen ejaculates (40) of five healthy Surti buffalo bulls during favourable breeding season. The ejaculates with >70% IM were diluted @ 100 million sperms/ml in Tris-fructose-egg yolk-glycerol (TFYG) diluent supplemented with L-cysteine @ 1 mg/ml, and examined for quality parameters such as sperm progressive motility, viability, morphology, acrosomal and plasma membrane integrity. A part of the extended semen was preserved at 5°C and another was processed for ultra-low temperature (-196°C) preservation in LN_2 using biofreezer after filled in French mini straws. The mean values of ejaculate volume, sperm concentration, mass activity (0-5 score), individual sperm motility, live sperm, abnormal sperm, intact acrosome and HOS reactive sperms observed in fresh semen were 3.04±0.11 ml, 963.05±50.97 million/ml, 2.97±0.06, 75.00±0.69 %, 88.22 ±0.48 %, 4.40±0.26 %, 93.67±0.31 % and 89.17±0.57 %, respectively. Just after dilution the percentages of progressively motile, live & abnormal sperms, intact acrosomes and HOS reactive sperms were 80.51±0.65, 85.95±0.65, 4.92±2.20, 91.00±2.30, 85.72±0.67, respectively. The corresponding values after 48 hrs of refrigeration (5°C) were 61.75±0.85, 67.85±0.85, 6.45±0.27, 89.05±0.32, 67.30±0.96%, the values at pre-freeze stage (after equilibration) were 72.62±0.69, 78.97±0.93, 5.45±0.25, 90.90±0.35, 78.12±0.79% and at post-thaw stage (37°C/30 sec) 46.50±0.72, 52.97±0.79, 7.10±0.26, 85.73±0.18, 51.62 ±0.82%, respectively. The mean motile spermatozoa observed after 1, 2 and 3 h of post-thaw incubation at 37°C in water bath were 32.75±0.82, 18.88 ±0.70 and 8.88±0.66% ($P<0.01$), respectively. The semen quality parameters, fresh and cryopreserved were acceptable for artificial breeding use. The seminal traits in initial, 48 hrs refrigerated, pre-freeze and post-thawed samples revealed significant ($p<0.01$) interrelationships ($r = 0.44$ to 0.84) between progressive motile sperm, live sperm and HOST reactive sperm directing more emphasis on these quality parameters for better semen evaluation.

KEY WORDS: Sperm quality, Surti buffalo, Refrigeration, Cryopreservation, Interrelationships

INTRODUCTION

The main purpose of research in the laboratory evaluation of semen, either fresh, refrigerated or frozen thawed, has been ultimately to predict its fertility. It is well established that characteristics of bull semen vary widely, not only between the bulls, but also between the ejaculates within bulls and from time to time or season to season (Raval and Dhami, 2010). Sperm progressive motility, proportion of dead and abnormal spermatozoa, and acrosomal and plasma membrane integrity of spermatozoa are recognized as important indices of semen quality and significantly correlated with keeping quality, freezability and/or fertility of bovine semen (Fiaz *et al.,* 2010; Chaudhari *et al.,* 2014). The evaluation of interrelationships of above spermatozoal attributes of fresh, refrigerated and cryopreserved 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, this study was designed to differentiate the quality parameters of fresh, refrigerated and cryopreserved Surti buffalo semen, and to know their interrelationships.

MATERIALS AND METHODS

The study was undertaken on five sexually mature healthy pedigreed breeding bulls of Surti breed of buffaloes, aged 4 to 6 years during the favourable breeding season at Central Sperm Station of the College in Anand, Gujarat (India). All these bulls were in good health and under veterinary care and were in regular twice a week morning semen collection schedule using artificial vagina. Eight ejaculates were studied from each bull (total 8 x 5 = 40 ejaculates) at weekly interval.

The standard TFYG extender was prepared just before collection as per FAO and supplemented with an antioxidant *L-cysteine HCl* @ 1 mg/ml. It was kept in thermo-regulatory water bath at 34°C until used for extension. Following gross evaluation, the ejaculates with >70% initial motility were diluted @ 100 million sperms/ml at 34°C and evaluated for sperm quality parameters such as motility, viability, morphology (eosin-nigrosin stain), acrosomal integrity (Giemsa stain) and plasma membrane integrity (HOS test) as per standard procedures considering as an initial or 0-hr evaluation. The part of extended semen (at least 2 ml each) was transferred to a refrigerator for gradual cooling and storage at 5°C. The individual sperm motility, viability, morphology, acrosomal integrity and plasma membrane integrity (HOST) were assessed at 24 hourly intervals up to 72 hrs of preservation. The remaining 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 using programmable biofreezer (Digitcool 5300, IMV, France). The thawing of straws was done in water bath at 37°C for 30 seconds, and post-thaw evaluation of samples were done for all above parameters, including longevity of sperms at 1, 2 and 3 hrs of post-thaw incubation at 37°C.

The data generated were analyzed statistically using ANOVA and critical different test or Duncan's new multiple range test by employing IBM SPSS Statistics version 20.00 to know the variation at different stages of preservation. The interrelationships between various sperm parameters at different stages were worked out through correlation matrix analysis (Snedecor and Cochran, 1994).

RESULTS AND DISCUSSION

Quality of Fresh, Refrigerated and Cryopreserved Spermatozoa

The mean ejaculate volume, density (1-4 score), sperm concentration and mass activity (0-5 score) of fresh Surti buffalo semen were found to be 3.04±0.11 ml, 3.06±0.08, 963.05±50.97 million/ml and 2.97±0.06, respectively.

Additionally, the percentages of individual motile sperm, live sperm, abnormal sperm, intact acrosome sperm and HOS reactive sperms observed in fresh (un-diluted) semen, and, at different stages of refrigerated and cryopreserved semen are given in Table 1. The values of motile, live, intact acrosome and HOS reactive sperm declined, while abnormal sperm increased gradually and significantly ($P<0.01$) with advancement of the refrigeration preservation time as well as pre-freeze to post-thaw semen. Further, the post-thaw incubation (37 °C) survival study revealed 46.75±0.71 %, 32.75±0.82 %, 18.88±0.70 % and 8.88±0.66 % progressive sperm motility after 0, 1, 2 and 3 hrs of incubation in water bath.

The values and trend of observations of various spermatozoa attributes of Surti bulls found in the present study corroborated well with several of the earlier reports on bovine semen (Sharma *et al.,* 1992; Dhami *et al.*, 1993; Shelke and Dhami, 2001; Lodhi *et al.,* 2008; Tiwari *et al.,* 2009; Khawaskar *et al.*, 2012; Mahmoud *et al.,* 2013, Chaudhari *et al.,* 2014) and were within normal physiological acceptable limits for use in artificial breeding services.

Table 1: Overall quality (Mean ± SE) of Surti buffalo semen in fresh, and at different stages of refrigeration and cryopreservation in TFYG diluent supplemented with Cysteine

Spermatozoal Traits / Preservation	Motile sperm (%)	Live sperm (%)	Abnormal sperm (%)	Intact acrosome (%)	HOST reactive sperm (%)
Fresh (Neat semen)	79.00±0.69	88.22±0.48	4.40±0.26	93.67±0.31	89.17±0.57
0-h (Just after dilution)	80.51±0.65	85.95±0.65	4.92±2.20	91.00±2.30	85.72±0.67
24-h of refrigeration	70.00±0.69	75.87±0.69	5.35±0.24	91.72±0.32	75.30±0.93
48-h of refrigeration	61.75±0.85	67.85±0.85	6.45±0.27	89.05±0.32	67.30±0.96
72-h of refrigeration	55.00±0.85	60.50±0.85	7.78±0.26	86.32±0.31	59.85±1.01
Pre-freeze (Post-equilib)	72.62±0.69	78.97±0.93	5.45±0.25	90.90±0.35	78.12±0.79
Post-thaw (Post-freeze)	46.50±0.72	52.97±0.79	7.10±0.26	85.73±0.18	51.62±0.82

Interrelationships of Fresh, Refrigerated and Cryopreserved Spermatozoa

The correlation matrix analysis of various sperm parameters studied in fresh, 48-hr refrigerated as well as pre- & post-freezing samples of Surti buffalo semen are presented in Table 2.

Ejaculate volume had significant ($p<0.05$; ≥ 0.28) positive correlations with sperm concentration and intact acrosome per cent in fresh semen (0.29, 0.31). Mass activity had significant ($p<0.01$; ≥ 0.42) positive correlations with sperm concentration, individual sperm motility and abnormal sperm per cent in fresh semen (0.36, 0.41, 0.50), with abnormal sperm per cent at 48-hr of refrigeration (0.28), and with pre-and post-freeze stage (0.36, 0.39) and with per cent sperm motility, HOS reactive sperm, and live sperm at post-thaw stage (0.47, 0.56, 0.53, resp.). The individual sperm motility in fresh semen had significant positive correlations with per cent HOS reactive and live sperm in fresh semen (0.48, 0.30) as well as motile and HOS reactive sperm at pre-freeze (0.37, 0.44), and post-thaw stage (0.36, 0.31). The HOS reactive sperm per cent in fresh semen showed significant positive correlations with per cent live sperm in fresh semen (0.51), and with pre-freeze sperm motility and HOS reactive sperm (0.40, 0.38).

Live sperm per cent in fresh semen had significant negative correlations with abnormal sperm per cent in fresh (-0.48) and pre-freeze semen (-0.31), and positive correlation with intact acrosome per cent post-freezing (0.28). The abnormal sperm per cent in fresh semen had significant positive correlations with abnormal sperm per cent at 48-hr of refrigeration (0.68) as well as at pre-freeze and post-thaw stage (0.78, 0.60), and with HOS reactive and live sperm at post-thaw stage (0.31, 0.30), and it had negative correlations with per cent intact acrosome in fresh (-0.35) and pre-freeze and post-thawed semen (-0.48, -0.35). Intact acrosome in fresh semen had highly significant positive correlations with per cent intact acrosome at 48-hr of refrigeration (0.65) as well as pre-freeze and post-thaw stage (0.77, 0.60), and with pre-freeze and post-thaw motility (0.38, 0.39), 2 hr post-thaw incubation survival (0.28), and HOS reactive and live sperm at pre-freeze stage (0.40, 0.41).

The individual sperm motility at 48-hr of refrigeration had significant positive correlations with per cent HOS reactive sperm and live sperm at 48-hr of refrigeration (0.81, 0.84) and with live sperm per cent post-freeze (0.29). The HOS reactive sperm at 48-hr of refrigeration had significant positive correlations with live sperm per cent at 48-hr of refrigeration and at post-thaw stage (0.83, 0.36). Live sperm per cent at 48-hr of refrigeration showed significant positive correlations with per cent post-thaw motile, live and HOS reactive sperms (0.29, 0.29, 0.30). The abnormal sperm per cent

Table 2: Interrelationships of seminal attributes with sperm parameters of refrigerated and cryopreserved buffalo semen

Traits		Initial								Ref 48					Pre-freeze					Post-thaw					
		Vol	Conc	MA	IM	HOST	LSP	AbSP	IA	IM	HOST	LSP	AbSP	IA	IM	HOST	LSP	AbSP	IA	PTM0	HOST	LSP	AbSP	IA	PTM1
Initial	Conc	0.29*	1.00																						
	MA	0.18	0.36*	1.00																					
	IM	-0.09	0.00	0.41*	1.00																				
	HOST	0.07	-0.12	0.12	0.48**	1.00																			
	LSP	0.01	0.04	-0.18	0.30*	0.51**	1.00																		
	AbSP	0.04	0.08	-0.50**	0.07	-0.18	-0.48**	1.00																	
	IA	0.31*	0.16	0.11	0.13	0.23	0.23	-0.35*	1.00																
48-hr Ref	IM	-0.03	0.06	0.14	0.11	0.08	0.14	0.11	-0.04	1.00															
	HOST	0.06	0.15	0.14	-0.07	-0.09	0.01	0.15	0.02	0.81**	1.00														
	LSP	0.10	0.17	0.11	-0.05	0.12	0.19	0.06	0.11	0.84**	0.83**	1.00													
	AbSP	0.15	-0.01	0.28*	0.05	0.00	-0.31*	0.68**	-0.26	-0.07	0.01	-0.10	1.00												
	IA	0.18	0.09	-0.09	-0.11	0.04	0.05	-0.18	0.65**	-0.15	0.07	0.06	-0.25	1.00											
Pre-freeze	IM	0.04	-0.18	0.11	0.37*	0.40*	0.16	-0.11	0.38*	0.24	0.07	0.12	-0.25	0.15	1.00										
	HOST	0.14	-0.11	0.16	0.44**	0.37*	0.26	-0.08	0.40*	0.18	0.02	0.03	-0.09	0.22	0.77**	1.00									
	LSP	0.21	0.07	0.03	0.20	0.15	0.26	-0.26	0.41*	-0.19	-0.24	-0.17	-0.18	0.33*	0.35*	0.63**	1.00								
	AbSP	-0.11	-0.03	0.36*	0.13	-0.08	-0.24	0.78**	-0.48**	0.13	0.12	0.05	0.81**	-0.33*	-0.22	-0.11	-0.20	1.00							
	IA	0.05	0.18	-0.02	0.20	0.11	0.28*	-0.48**	0.77**	-0.01	0.05	-0.01	-0.50**	0.57**	0.36*	0.35*	0.25	-0.61**	1.00						
Post-thaw	PTM0	0.13	0.25	0.47**	0.36*	0.13	0.07	0.23	0.39*	0.19	0.20	0.29*	0.09	0.20	0.15	0.23	0.12	0.07	0.24	1.00					
	HOST	-0.04	0.19	0.56**	0.31*	0.17	0.01	0.31*	0.10	0.27	0.26	0.29*	-0.01	-0.03	0.27	0.32*	0.02	0.02	0.07	0.65**	1.00				
	LSP	0.01	0.28	0.53**	0.27	0.11	-0.05	0.30*	0.14	0.29*	0.36*	0.30*	-0.03	0.08	0.19	0.21	-0.09	-0.03	0.15	0.61**	0.79**	1.00			
	AbSP	-0.06	-0.02	0.39*	0.16	0.17	-0.16	0.60**	-0.38*	0.02	-0.06	0.00	0.70**	-0.24	-0.15	-0.02	-0.10	0.80**	-0.55**	0.06	0.13	0.05	1.00		
	IA	0.22	0.11	0.07	-0.03	0.24	0.22	-0.35*	0.60**	0.02	-0.03	0.03	-0.19	0.37*	0.30*	0.35*	0.27	-0.32*	0.51**	0.17	0.11	0.15	-0.22	1.00	
	PTM1	0.08	0.00	0.00	0.17	-0.11	-0.04	-0.06	0.45**	-0.20	-0.06	-0.12	-0.03	0.34*	0.10	0.25	0.33*	-0.19	0.44**	0.37*	0.19	0.19	-0.13	0.24	1.00
	PTM2	0.08	-0.05	0.06	0.03	0.00	-0.14	-0.04	0.28	-0.16	-0.06	-0.12	0.00	0.20	0.22	0.15	0.25	-0.17	0.17	-0.03	0.08	0.10	-0.14	0.31*	0.58**

*P<0.05; **P<0.01; Number of observations=40.

Vol = Ejaculate volume; Conc = Sperm Count/ml; MA= Mass activity score; IM = Individual sperm motility; HOST = Hypo-osmotic swelling test; LSP = Live sperm %; AbSP = Abnormal sperm %;

IA = Intact acrosome %; PTM0 =Post-thaw motility 0 min; PTM1, PTM2= Post-thaw incubation motility 1 hr and 2 hr.

at 48-hr of refrigeration had significant positive correlations with abnormal sperm per cent pre-freeze and post-thaw (0.81, 0.70), and negative correlation with post-thaw intact acrosome per cent (-0.50). Intact acrosome per cent at 48-hr of refrigeration were significantly and positively correlations with pre-freeze live sperm and intact acrosome (0.33, 0.57) as well as post-thaw intact acrosome and incubation motility (0.37, 0.34), and negative correlation with pre-freeze abnormal sperm per cent (-0.33).

Further, the pre-freeze sperm motility had significant positive correlations with per cent pre-freeze HOS reactive sperm, live sperm and intact acrosome (0.77, 0.35, 0.36) and post-thaw intact acrosome (0.30). The pre-freeze HOS reactive sperm had significant positive correlations with live sperm and intact acrosome pre-freeze (0.63, 0.35) and post-thaw (0.32, 0.35). The pre-freeze live sperm per cent had significant positive correlation with post-thaw incubation motility (0.33). The pre-freeze abnormal sperm per cent showed significant negative correlation with pre-freeze and post-thaw intact acrosome (-0.61, -0.32), and positive correlation with post-thaw abnormal sperm (0.80), while pre-freeze intact acrosome showed highly significant positive interrelationships with post-thaw motility and intact acrosome (0.44, 0.51) and negative correlation with post-thaw abnormal sperm per cent (-0.55).

Post-thaw motility soon after thawing had significant positive correlations with per cent post-thaw HOS reactive sperm, live sperm and motility after 1-hr of incubation (0.65, 0.61, 0.37). The post-thaw HOS reactive sperm showed significant positive correlations with per cent post-thaw live sperm (0.79), and post-thaw incubation motility at 2 hrs revealed significant positive correlations with post-thaw intact acrosome and motility after 1 hr of incubation (0.31, 0.58). The rest of the interrelationships of various traits studied in fresh, refrigerated and cryopreserved buffalo semen were found to be statistically insignificant or even negligible.

These findings on correlation coefficients observed corroborated well with many of the reports reviewed earlier, particularly of Sharma *et al.* (1992), Dhami *et al.* (1993), Shelke and Dhami (2001), Rana and Dhami (2003), Lodhi *et al.* (2008), Patel *et al.* (2012) and Chaudhari *et al.* (2014) for bull and buffalo semen. Raval and Dhami (2010) also recorded highly significant ($P<0.01$) positive correlations for ejaculate volume with total abnormal sperm, initial motility with mass activity and live sperm per cent, and negative correlations for total 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 corroborated with those of Rana and Dhami (2003), Raval and Dhami (2010) and Chaudhari *et al.* (2014), who also 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. 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. Sperm motility was also correlated with sperm abnormalities and membrane integrity (HOST). Under the conditions of the present study, it was inferred that assessment of sperm motility and HOS test could be a valuable and practical tool to know the functional capacity of fresh and preserved buffalo spermatozoa.

CONCLUSION

The seminal characteristics of Surti buffalo bulls studied revealed that the sperm quality at different stages of preservation were acceptable for artificial breeding purpose. The interrelationships of

seminal attributes in initial, 48 hr refrigerated, pre-freeze and post-thawed samples were found significant (r=0.34 to 0.84, p<0.01) between sperm motility, live sperm and HOST reactive sperm. Therefore, it was inferred that assessment of sperm motility and HOS test could be a valuable and practical tool to know the functional capacity of fresh and preserved buffalo spermatozoa.

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