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Seminal Attributes, Freezability and their Interrelationships in Zebu Cattle and Buffalo Bulls from Central Gujarat

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Abstract

A study was carried out on nine healthy mature breeding bulls (3 each of Gir, Surti and Murrah breed) to evaluate their fresh and frozen semen quality and their interrelationships. The ejaculates immediately after collection were evaluated for routine physico-morphological attributes, including HOS test. The ejaculates (n=72) having >75% initial motility were diluted @ 80 million sperm/ml using TFYG extender and the French mini straws filled were frozen in liquid nitrogen vapour using a programmable biofreezer. Thawing of straws was done at 37°C for 30 sec and assessed for freezability by conventional technique. All the cattle and buffalo bulls donated consistently normal thick creamy yellow and thick milky white semen, respectively. In Gir, Suti and Murrah bulls (n=24 ejaculate each) the seminal attributes such as ejaculate volume (6.69±0.17, 3.12±0.10 and 3.96±0.16 ml, $p<0.01$); initial motility (80.21±0.88, 84.58±0.60 and 84.38±0.76 %, $p<0.01$); total sperm output/ejaculate (9013.85±265.32, 3935.49±259.63 and 5366.48±332.99 million, $p<0.01$) and live sperm (84.71±0.83, 86.17±0.78 and 86.79±0.79 %, $p<0.05$) differed significantly. The mean percentages of post-thaw motile sperm (53.29±1.56, 58.33±1.43 and 59.58±1.20, $p<0.01$); live sperm (59.00±1.95, 67.00±1.59 and 68.42±1.66 %, $p<0.01$); and HOS reactive sperm (48.25±0.78, 44.21±1.29 and 51.54±1.29 %, $p<0.01$) in Gir, Surti and Murrah bulls semen also differed significantly. The variation among the bulls was significant for buffalo breeds in most of their fresh seminal attributes, except HOST, and for post-thaw motility, but not among Gir bulls. The important seminal attributes like motility, live sperm and HOS reactive sperm of fresh and frozen-thawed semen were significantly and positively interrelated in all three breeds of bulls ($r = 0.40$ to 0.81, $p<0.05$ to 0.01), suggesting that motility and HOST of fresh semen were good predictors of freezability of bovine semen.

Introduction

Male fertility is an important factor in bovine reproduction, since a single bull is generally bred to numerous cows, particularly through AI. Therefore semen analysis is the most valuable diagnostic tool to evaluate male fertility potential (Patel *et al.,* 2012). However, no single test or

combinations of tests have been proved to be totally reliable for accurate prediction of semen quality in relation to fertility. Most of the tests that are used for evaluation of semen are based on physical characteristics of spermatozoa. The correlations of these physical attributes with fertility are highly variable and relatively poor (Dhami *et al*., 1990; Shelke and Dhami, 2001; Tiwari *et al*., 2009). There are numerous factors that may affect the motility, plasma membrane integrity, morphology and viability of semen. Hypo-osmotic swelling (HOS) test is used to evaluate sperm plasma membrane integrity as *in vitro* fertility test as it is of fundamental importance in the fertilization process (Jeyendran *et al.,* 1984; Lodhi *et al.,* 2008). The evaluation of interrelationships of spermatozoal attributes of fresh and cryopreserved bovine semen would help to select a few most valid simple traits of fresh semen to predict freezability and even fertility of such ejaculates, instead of going through a plethora of time consuming unpredictable cumbersome tests. Hence, this study was planned to evaluate the comparative physico-morphological and functional attributes of fresh and frozen-thawed semen of cattle and buffalo bulls and their interrelationships in order to select the test(s) that are predictive of semen freezability.

Materials and Methods

This investigation included nine healthy mature breeding bulls (3 each of Gir, Surti and Murrah breed), aged 5-8 years, stationed at Sperm Station of the College of Veterinary Science, AAU, Anand-388 001 during September 2017 to May 2018. All these bulls were in good health and under optimal veterinary care. They were maintained in nearly identical nutritional and managerial conditions throughout the period of study with twice a week semen collection schedule. Semen was collected using artificial vagina from each bull in the morning hours between 7.30 and 8.30 am over a dummy buffalo bull. Immediately after collection, the tubes containing semen were placed in a water-bath maintained at 34ºC and evaluated for various physico-morphological attributes (Salisbury *et al.,* 1978) including hypo-osmotic swelling (HOS) test (Jeyendran *et al.,* 1984). In all, 72 semen ejaculates from 9 bulls (8 ejaculates/bull) were studied at weekly intervals.

Following evaluation, the ejaculates were extended at 32-35ºC with Tris-citric acid-fructose-egg yolk-glycerol (TFYG) diluent keeping 80 million sperm per ml. The extended semen was filled and sealed in French mini straws by using IS4 machine (IMV, France). The straws were gradually cooled to 5ºC over 60-90 minutes in a cold handling cabinet and then equilibrated for 4 hrs. The freezing of straws was carried out in liquid nitrogen vapour using a programmable bio-freezer (Digicool, IMV, France). The straws were thawed in water bath at 37°C for 30 sec, and were assessed for sperm motility, viability, morphology and HOS test. The seminal attributes of fresh and frozen-thawed sperm traits were analyzed statistically using completely randomized design to derive mean ± SEs and ANOVA. The mean differences among bulls and among breeds were statistically tested by employing SPSS version 20.00. The Pearson correlations of fresh and frozen thawed sperm parameters were also worked out (Snedecor and Cochran, 1994).

Results and Discussion

All the bulls of cattle (Gir) and buffalo (Surti and Murrah) breeds donated consistently normal thick creamy yellow and thick milky white semen, respectively. This was in accordance with many previous reports on different breeds (Rana and Dhami, 2004; Chaudhari *et al.,* 2014; Bhakat *et al.,* 2015; Chaudhary *et al.,* 2017[a]). The breed-wise means (±SE) of various seminal attributes observed in freshly ejaculated and frozen-thawed semen of Gir, Surti and Murrah bulls are presented in Table 1 and 2.

Ejaculate Volume, Mass Activity and Sperm Concentration

The mean values of ejaculate volume and sperm output per ejaculate were significantly ($p<0.01$) higher in Gir bulls than Surti and Murrah buffalo bulls, however, the latter two breeds did not differ significantly (Table 1). The individual bull variation was significant among the buffalo breeds, but not in cattle. However, the mass activity scores and sperm concentration/ml neither varied significantly between breeds nor between bulls within the breed, except sperm concentration in Surti bulls. The absolute values and significantly higher ejaculate volume and

Table 1: Comparative sperm output in Gir cattle and Surti and Murrah buffalo bulls under middle Gujarat climate (Mean ± SE)

Seminal attribute	Breed of bull		
	Gir (n=24)	Surti (n=24)	Murrah (n=24)
Ejaculate volume (ml)	6.69^{y}±0.17	3.12^{x}±0.10	3.96^{x}±0.16
Mass activity (score 0-5)	3.75±0.06	3.63±0.08	3.63±0.08
Sperm concentration (million/ml)	1348±21.85	1246±58.40	1343±42.93
Sperm Output/Ejaculate (million)	9014^{y}±265.3	3936^{x}±259.6	5366^{x}±332.9

Means bearing uncommon superscripts within the row differ significantly ($p<0.05$).

Table 2: Mean (±SE) sperm quality parameters of fresh and frozen-thawed semen of Gir cattle and Surti and Murrah buffalo bulls

Seminal attribute	Stage	Gir (n=24)	Surti (n=24)	Murrah (n=24)
Sperm motility (%)	Fresh	80.21^{x}±0.88	84.58^{y}±0.60	84.38^{y}±0.76
	Post-thaw	53.29^{x}±1.56	58.33^{y}±1.43	59.58^{y}±1.20
Live sperm (%)	Fresh	84.71±0.83	86.17±0.78	86.79±0.79
	Post-thaw	59.00^{x}±1.95	67.00^{y}±1.59	68.42^{y}±1.66
Total sperm abnormality (%)	Fresh	4.00±0.16	3.63±0.12	3.79±0.17
	Post-thaw	7.04±0.37	6.75±0.31	7.33±0.26
HOS reactive sperm (%)	Fresh	82.54±0.91	82.63±0.73	83.50±1.02
	Post-thaw	48.25^{x}±0.78	44.21^{y}±1.29	51.54^{z}±1.29

Means bearing uncommon superscripts within the row differ significantly between breeds ($p<0.05$).

sperm output obtained for cattle bulls than buffalo bulls were in harmony with those reported by Dhami and Sahni (1994) and Dhami *et al.* (2001), Chowdhury *et al.* (2013), Bhakat *et al.* (2015) and Chaudhary *et al.* (2017[a]). Further, the findings on mass activity were in accordance with those of Chaudhary *et al.* (2017[a]), while Shelke and Dhami (2001) and Rana and Dhami (2004) recorded higher mass activity in cattle than the buffalo semen. Like present trend of non-significantly higher sperm concentration per ml and significantly higher total sperm output in Gir cattle than buffalo semen has been documented earlier by Shelke and Dhami (2001), Rana and Dhami (2004), Chowdhary *et al.* (2013) and Chaudhary *et al.* (2017[a]), while Dhami and Sahni (1994) and Bhakat *et al.* (2015) reported higher values in Murrah than HF or Karan Fries bulls. Patel *et al.* (2012) reported significantly ($p<0.01$) higher mean sperm concentration per ml in Jafarabadi and Mehsana buffalo bulls than the crossbred bulls, but the total sperm output per ejaculate did not differ between them.

The ejaculate volume of bull semen depends upon the body/scrotal size and weight, apart from general & genital health and quality of ejaculatory thrust by the bull. Ejaculate volume together with sperm concentration and motility are of great importance in frozen semen production for wider application in AI industry. Higher the motility and sperm concentration greater will be the swirls, with faster waves and eddies giving better mass activity score, which reflects the initial quality of semen. The differences

in ejaculate volume and sperm concentration in bovine and bubaline species could be due to variation between breeds, individual, age, libido, climate, testicular health, accessory sex glands function, and frequency and method of semen collection etc.

Sperm Motility and Viability of Fresh and Frozen-Thawed Semen

The percentages of motility and live sperm in both fresh and frozen-thawed semen were significantly ($p<0.01$) higher in buffalo bulls of both the breeds than the Gir bulls (Table 2), and the bull variation was significant only in buffalo breeds. Dhami and Sahni (1994), Bhakat *et al.* (2015) and Chaudhary *et al.* (2017[a]) reported similar higher initial sperm motility and viability in Murrah or Surti buffalo than Friesian, Gir or Karan Fries bulls' semen. On the contrary, Shelke and Dhami (2001), Rana and Dhami (2004) and Chowdhury *et al.* (2013) recorded higher sperm motility in fresh and frozen-thawed semen of cow bulls than buffalo bulls. The present findings on significantly higher live sperm per cent reported in buffalo breeds than in Gir breed concurred with Shelke and Dhami (2001) and Chaudhary *et al.* (2017[a]). Chowdhury *et al.* (2013) however reported comparatively lower post-thaw motility and viability in both cattle and buffalo semen as compared to present observations.

The individual sperm motility and viability are an essential parameters for assessment of semen quality and freezability, and can yield a reliable picture of semen potency, because they give clue concerning acceptance or rejection of the ejaculate for advance processing & use, and both are positively correlated with freezability of semen sample (Shelke and Dhami, 2001; Rana and Dhami, 2004; Patel *et al.*, 2012; Chaudhary *et al.,* 2017[a,b]). Sperm motility is essential during their transportation in the oviduct and oocyte penetration. However, it swings between breeds, species, individuals, age groups, seasons and the evaluation techniques employed. The variation in post-thaw motility and viability of sperms may be due to variation in initial quality of semen, extender, equilibration and freezing-thawing protocol used, and the stain and staining technique followed in different studies.

Sperm Abnormalities and HOST of Fresh and Frozen-Thawed Semen

The percentages of total abnormal sperms in both fresh and frozen-thawed semen of Gir cattle and Surti and Murrah buffalo bulls were quite low, and did not differ statistically between them. The bull variation was significant only for fresh sperm abnormalities in Gir breed. The mean post-thaw HOS reactive sperm per cent were significantly ($p<0.01$) lower in Surti bulls compared to Murrah buffalo, but no such difference was seen in fresh semen between cattle and buffalo breeds. The post-thawed sperms of Murrah buffalo bulls were better HOS resistant, while Surti buffalo sperms showed greater fragility. Similar trend of non-significantly higher sperm abnormalities in Gir than Surti bulls (Chaudhary *et al.,* 2017[a]), or in HF than Murrah bulls (Dhami and Sahni, 1994), in Jafarabadi & Mehsana buffalo than crossbred bulls (Patel *et al.,* 2012) and in Karan Fries than Murrah (Bhakat *et al.,* 2015) has been reported in fresh and/or frozen-thawed semen, while Rana and Dhami (2004) noted identical and much higher total sperm abnormalities in Gir and Jafarabadi bulls (22-23% in fresh and 32-33% in frozen-thawed semen). In contrast, Shelke and Dhami (2001) and Chowdhury *et al.* (2013) reported greater sperm abnormalities in buffalo than cow bulls. For semen sample to be accepted for use in AI, it should have less than 20 per cent total sperm abnormalities. This is because only the live and morphologically normal sperm can drift in the forward direction to reach the site of fertilization in the oviduct after being deposited in the reproductive tract of the female animal in estrus.

The present HOS reactive sperms in fresh semen were in accordance with those reported by different workers either in cattle or buffalo bulls (Lodhi *et al.,* 2008; Chaudhary *et al.,* 2017[a]; Kapadiya *et al.,* 2018) without species variation. However, several authors reported significantly lower HOS reactive sperms in cattle than buffalo bulls semen (Rana and Dhami, 2004; Chowdhury *et al.,* 2013; Bhakat *et al.,* 2015) with much lower absolute values of 40 to 60 per cent only, while other recorded value around 80 per cent (Tiwari *et al.,* 2009) in buffalo semen. Brahmkshatri (1995) found significantly higher post-thaw HOS reactive sperm in Murrah than crossbred bulls

(52 vs 41 %) using distilled water, while Prasad *et al.* (1999), Rana and Dhami (2004), Zubair *et al.* (2013) and Chaudhary *et al.* (2017[b]) documented very low value (24 to 28 %) in Gir, Surti, Jafarabadi and crossbred bulls. Assessment of sperm membrane function appears to be a significant marker for the fertilizing capacity of spermatozoa, since it is involved in metabolic changes with the surrounding medium and in the process of capacitation, acrosome reaction and fusion with the oocyte membrane (Brahmkshatri, 1995). Greater post-thaw HOS reactivity is generally positively correlated with better sperm longevity and fertility.

Interrelationships of Sperm Traits of Fresh & Frozen-Thawed Semen

The ejaculate volume in Gir bull semen (Table 3) had highly significant ($p<0.01$) positive correlations only with post-thaw motility (r=0.62) and live sperm (r=0.54) and negative correlation with post-thaw abnormal sperm (-0.71). Sperm concentration/ml had highly significant ($p<0.01$) positive correlations with mass activity (0.54), live sperm (0.43) and post-thaw HOS reactive sperm (0.52). Mass activity score revealed significant ($p<0.01$) positive correlations with motile (0.47), live (0.70) and HOS reactive (0.60) sperm in fresh ejaculates, and with post-thaw HOS reactive sperm (0.41). Initial sperm motility had significant ($p<0.01$) correlations with initial live (0.78), abnormal (-0.71) and HOS reactive (0.72) sperm. The HOS reactive sperm in the fresh semen showed significant ($p<0.01$) correlations with live (0.71) and abnormal (-0.58) sperm per cent in fresh semen and with post-thaw HOS reactive sperm (0.62). The post-thaw motility had significant correlations with post-thaw live (0.71) and abnormal (-0.77) sperms.

Among Surti buffalo bull semen (Table 4), ejaculate volume showed highly significant ($p<0.01$) positive correlations only with post-thaw sperm motility (r=0.63) and live sperm (r=0.66) per cent and negative correlation with post-thaw HOS reactive (r= -0.41) and abnormal (r= -0.52) sperm. Sperm concentration per ml had similar highly significant ($p<0.01$) positive correlations with post-thaw sperm motility (0.59) and live sperm (0.48) per cent and negative correlation with post-thaw HOS reactive sperm (-0.41). Mass activity score revealed significant ($p<0.01$) positive correlations with initial motile (0.61), live (0.76) and HOS reactive (0.77) sperm, and with post-thaw HOS reactive sperm (0.48). The HOS reactive sperm in the fresh semen showed significant ($p<0.01$) correlations with live (0.79) and abnormal (-0.43) sperm in fresh semen and with post-thaw HOS reactive (0.69) and live (0.49) sperm. Initial live sperm had negative correlation with abnormal sperm in fresh semen (-0.52), and with post-thawed HOS reactive (0.64), live (0.47) and abnormal (-0.53) sperm. Post-thaw motility had significant positive correlations with post-thaw live (0.81) and HOS reactive (0.52) sperm and negative correlation with abnormal sperm (-0.62). Post-thaw HOS reactive sperm showed significant correlations with post-thaw live (0.55) and abnormal (-0.52) sperms. Almost similar correlations were also noted among Murrah bulls' semen (Table 4).

The present correlation findings in ox and buffalo bull semen corroborated well with many previous reports, particularly of Brahmkshatri (1995), Prasad *et al.* (1999), Shelke and Dhami (2001), Lodhi *et al.* (2008), Patel *et al.* (2012), Zubair *et al*. (2013), Chaudhari *et al.* (2014) and Chaudhary *et al.* (2017[b]). Dhami and Sahni (1994) found all the seminal attributes, except volume, to be significantly interrelated in Murrah and HF bulls (r = 0.32 to 0.86). Patel *et al.* (2012) found significant ($p<0.01$) positive correlation for sperm motility and HOS test. Rana and Dhami (2004) found significant ($p<0.01$) interrelationships for percentages of motile, live, abnormal spermatozoa and HOS reactive sperms in fresh and post-thawed semen of bovine and bubaline species (r = 0.17 to 0.90). Patel and Siddiquee (2013) found positive correlations of ejaculate volume with mass motility and sperm concentration, and mass motility was positively correlated with motility and live sperm count in fresh semen of Kankrej bulls. The present findings and those of others suggested that these traits could be of practical utility in routine semen evaluation to predict semen quality, freezability and fertility. Under the conditions of the present study, it is inferred that assessment of sperm motility and HOS test could be a valuable and practical tool to know the functional capacity of fresh and cryopreserved bull and buffalo spermatozoa.

Table 3: Correlation (r) matrix of sperm quality parameters of fresh and frozen-thawed semen of Gir bulls

Trait	Ejacul Volume	Sp Count	Mass Activity	Initial Motile	Initial HOST	Initial Live	Initial Abn	PT Motile	PT HOST	PT Live
Sp Count	-0.07	---								
M Activity	-0.02	0.54**	---							
Fr Motility	0.23	0.34	0.47*	---						
Fr HOST	0.26	0.30	0.60**	0.72**	---					
Fr Live Sp	0.07	0.43*	0.70**	0.78**	0.71**	---				
Fr Abn Sp	-0.13	-0.19	-0.28	-0.71**	-0.58**	-0.60**	---			
PT Motility	0.62**	0.03	-0.10	0.40*	0.22	0.06	-0.33	---		
PT HOST	0.12	0.52**	0.41*	0.39	0.62**	0.33	-0.39	0.24	---	
PT Live Sp	0.54**	0.07	-0.29	0.16	0.06	0.02	-0.01	0.71**	0.08	---
PT Abn Sp	-0.71**	0.19	0.10	-0.25	-0.17	-0.01	0.22	-0.77**	-0.01	-0.78**

N = 24; Fr = Fresh/Initial, PT = Post-thaw; *Significant at p<0.05 level; **Significant at p<0.01 level.

Table 4: Correlation (r) matrix of quality parameters of fresh and frozen-thawed semen of Surti and Murrah buffalo bulls

Trait	Ejacul Volume	Sp Count	Mass Activity	Fresh Motile	Fresh HOST	Fresh Live	Fresh Abn	PT Motile	PT HOST	PT Live	PT Abn
Correlations: Surti buffalo semen											
Ej. Volume	--	0.36	0.00	0.26	-0.14	-0.19	-0.07	0.63**	-0.41*	0.66**	-0.52**
Sp Count	0.33	--	0.10	0.39	-0.31	-0.16	-0.06	0.59**	-0.41*	0.48*	-0.33
M Activity	-0.17	0.37	--	0.61**	0.77**	0.76**	-0.45*	-0.12	0.48*	-0.22	0.37
Fr Motility	0.22	0.31	0.46*	-	0.36	0.32	-0.23	0.34	0.10	0.22	0.02
Fr HOST	0.06	-0.05	0.60**	0.35	--	0.79**	-0.43*	-0.40	0.69**	0.49*	-0.41*
Fr Live Sp	-0.11	-0.21	0.34	-0.03	0.61**	--	-0.52**	-0.29	0.64**	0.47*	-0.53**
Fr Abn Sp	0.43*	0.25	-0.27	0.10	-0.38	-0.62**	--	-0.05	-0.22	-0.07	-0.06
PT Motility	0.60**	0.24	-0.03	.54**	0.36	0.11	0.12	--	0.52**	0.81**	-0.62**
PT HOST	-0.11	0.17	0.53**	0.27	0.70**	0.37	-0.33	0.29	--	0.55**	-0.52**
PT Live Sp	0.30	-0.04	-0.40*	0.34	-0.07	-0.07	0.01	0.65**	0.07	--	-0.72**
PT Abn Sp	-0.45*	-0.25	0.00	-0.61**	-0.19	0.02	-0.01	-0.74**	-0.20	-0.74**	--
Correlations: Murrah buffalo semen											

N = 24; Fr = Fresh/Initial; PT = Post-thaw; *Significant at p<0.05 level; **Significant at p<0.01 level.

Conclusion

The bulls of Gir, Surti and Murrah breeds perform well in middle Gujarat climate in terms of ejaculate quality and freezability. In this region, the buffalo bulls produce good quality semen than cattle. The significant positive correlations between mass activity and progressive motility (%) as well as between HOST score and progressive motility for all three breeds, suggest that motility estimation and HOST in fresh and post-thawed semen can be adopted for routine assessment of semen quality.

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Conflict of Interest

Authors declare that they have no conflict of interest.

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