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Semen Ejaculates Characteristics, *in vitro* Fertility and Their Interrelationships in Sahiwal Bull Semen

Research Article

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ABSTRACT

This study aimed at determining the physico-morphological attributes**,** *in vitro* fertility and their interrelationships in semen of Sahiwal bulls. Semen samples were collected weekly for 6 weeks from ten mature Sahiwal bulls divided randomly into 5 groups. The mean (±SEM) of ejaculate volume (mL), mass activity (score 0-5), progressive sperm motility (%), sperm concentration (10^6/mL), live sperm (%), abnormal sperm (%), intact acrosome (%)**,** hypo-osmotic swelling (HOS) positive sperm (%), sperm penetration distance (SPD, mm) were 4.28, 3.17, 76.73, 1185.53, 83.37, 9.97, 82.70, 79.00 and 56.85, respectively. The bulls varied significantly (P<0.05) in all their ejaculates characteristics Moreover, HOS positive sperm also had significant (P<0.01) positive correlation with mass activity (r=0.756), progressively motile sperm (r=0.374), live sperm (r=0.395) and SPD (r=0.681), and negative correlation with abnormal sperm (r=-0.463). Sperm penetration distance has significant (P<0.05) positive correlation with mass activity (r=0.776), progressively motile sperm (r=0.653), sperm concentration (r=0.365) and HOS positive sperm (r=0.681), and negative correlation with intact acrosome (r=-0.415). Overall semen quality of Sahiwal bulls was found optimum for use in breeding programme.

KEY WORDS hypo-osmotic swelling test, *in vitro* fertility, physico-morphological, Sahiwal, semen, sperm penetration distance.

INTRODUCTION

Sahiwal, originated from zebu cattle, is one of the indigenous dairy cattle breeds of South Asia which is considerably declining in number (Dahlin *et al*. 1998) because of indiscriminate crossbreeding with exotic breeds and it needs special considerations to conserve the valuable germplasm of this indigenous breed (Dahlin *et al*. 1998). Sahiwal cattle are well known for their disease resistance, heat tolerance and adequate performance at low quality roughages (Nay and Hayman, 1956; Dahlin *et al*. 1998). Due to its promising adaptability to tropical and subtropical environment and reasonable dairy performance, both semen and cows of Sahiwal breed have been exported to many countries. Hence, preservation of this breed is urgently needed.

The first generation and most successful reproductive biotechnology that has made a profound contribution to improve productivity of dairy animals is artificial insemination (AI). For preservation, evaluation of semen is needed and several tests become necessary to judge the quality of semen.

The semen quality evaluations are the most important factor in estimation of fertility in domestic animals. Nowadays, *in vitro* tests including acrosome reaction, hypo-osmotic swelling test and cervical mucus penetration tests have been used for quality determination of semen.

Evaluation of sperm membrane functional status is of vital importance since an intact and functionally active membrane is required for metabolism, capacitation, acrosome reaction and the event of sperm being connected to the oocyte surface (Jayendran *et al.* 1984; Brito *et al.* 2003).

Fertilization of oocyte will not happen if the sperm membrane is biochemically inactive even if it stays structurally intact, so the hypo-osmotic swelling (HOS) test is a good indicator of fertilization potential. Because of the great importance of sperm membrane in fertilization, considerable attention is given to the membrane integrity in spermiogram evaluation.

Assessment of spermatozoal movement in the bovine cervical mucus is helpful in understanding the fundamental mechanism of *in vitro* sperm transport. *In vitro* cervical mucus penetration test (CMPT) was performed in order to investigate infertility problems and to gain information regarding the functions and quality of spermatozoa (Murase *et al.* 2001; Anilkumar *et al.* 2001). Cervical mucus acted as a barrier which eliminates the spermatozoa with abnormal morphology and allows only the spermatozoa with normal morphology to pass and that the spermatozoa which cannot penetrate into the mucus lack the ability to fertilize the ovum (Keel and Schalue, 2000; Robayo *et al.* 2008)

Assessment of male fertility in animal by mating or AI is expensive and protracted, and only allows a limited number of male animals to be tested. Hence, interest has not long been focused on methods related to *in vitro* fertilization technique (Larson and Rodriguez Martinez, 2000). The information on the seminal physico morphological attributes and *in vitro* fertility of such bulls and their interrelationships is totally lacking in India. Hence, the present work was carried out with the objective to study sperm functional parameters in relation to *in vitro* fertility in assessing semen of Sahiwal breed.

MATERIALS AND METHODS

Study Animals

The present study was conducted on ten mature and healthy purebred Sahiwal (SW) bulls. Animals were randomly divided into five groups comprising two animals in each group. The age of the bulls ranged from 36 to 60 months. All the experimental animals were maintained identically at university farm complex, WBUAFS, West Bengal with optimal conditions of feeding (12-15% of protein in the diet) and management throughout the experimental period. The bulls were made sure of breeding soundness before bringing in use and semen samples were collected weekly by sterilized artificial vagina (AV) in aseptical conditions from each bull for 6 occasions respectively. A total of 60 ejaculates were collected.

Semen evaluation

Immediately after collection, the ejaculate was placed in a 37 ˚C water bath and the volume was recorded. The mass activity score and percent of progressively motile sperms were evaluated immediately after collection. Mass activity was scored from 0 to 5 on a wet mount of neat semen at 100x magnification (0=cells present without motion; 5=very rapid dark swirls) according to method.

The percent of progressively motile spermatozoa was estimated by microscopic examination at 400 x magnification on a pre-warmed slide (37 ˚C), and a subjective assessment of the progressive statement was recorded according to the procedure of Ax *et al.* (2000). Sperm concentration was measured using Neubaur haemocytometer (Salisbury *et al.* 1978), the percent of viable spermatozoa was estimated by viewing 200 spermatozoa under 1000x magnification using eosin-nigrosin staining method (Sidhu and Guraya, 1985a). The same method was used to evaluate sperm abnormal morphology.

The percent of normal and / or intact acrosomal cap i.e. acrosomal integrity was evaluated from each sample by standard Giemsa staining method. The proportion of spermatozoa only with normal apical ridge (NAR) was considered and estimated by viewing 200 spermatozoa under 1000x magnification and their mean results were expressed in % intact acrosome.

Fertility of bulls was assessed based on *in vitro* fertility test on (HOST) and CMPT. Hypo-osmotic swelling test was performed as described by Jayendran *et al.* (1984). The stained slides were then observed under light microscope by viewing 200 spermatozoa of different types of tail coiling under 450x magnifications and expressed as % HOS positive sperm.

The CMPT (Kremer, 1980) was performed by using “Penetrak kit” (Serono Diagnostic, USA). The test uses a flat capillary tube (10 cm long). The sealed capillary tubes filled with bovine cervical mucus were stored at -20 ˚C. For the test, 40 µL of each semen samples, from the neat semen was taken separately into different 2 mL of round-bottomed glass test tubes.

Sealed capillary tubes were thawed at room temperature and broken carefully avoiding any bubble. The cut end of each capillary tube was inserted vertically into each test tube immediately.

All the prepared sets were incubated at 37 ˚C for 60 min in an incubator containing 5% CO_2 in air. Each sample was run in duplicate.

After incubation, capillary tubes were withdrawn from the test tubes, cleaned and placed individually on a measuring scale. The distance traveled in millimeter by the vanguard spermatozoa was measured with under phase-contrast microscope (200x; 450x).

The semen samples were graded on the basis of the distance traveled by the vanguard spermatozoa in the capillary tubes after incubation. For the penetration density, i.e. numbers of spermatozoa able to enter into the mucus column after the treatment were recorded.

The numbers of spermatozoa were counted from the scale where the ability of sperm cells to migrate towards maximum divisions in maximum numbers. The total numbers of spermatozoa in each group were determined, and were expressed as the number per mm^2. The results were considered and recorded from the mean reading of each corresponding set.

Data analysis

The obtained data were analyzed by the General Linear Model using SPSS software (Version 10.0 for Windows; SPSS Inc., Chicago, IL, USA) computer program. It included descriptive statistics by using Multivariate tests of Pillai's trace, Wilks' Lambda, Hotelling's trace and Roy's largest root. Results are quoted as arithmetic mean ± standard error of mean (SEM) and the means were compared using *Duncan's* Multiple Range Tests. A probability of $P<0.05$ was considered to be statistically significant. Pearson's correlation coefficient (two tailed) test was used to examine the association between all the parameters of the semen.

RESULTS AND DISCUSSION

The findings (means±SEM) on the seminal attributes of Sahiwal bulls observed during the study are presented in Table 1 and their interrelationships in Table 2.

Ejaculate volume

The ejaculate volume of semen in Sahiwal bulls varied from 3.20 to 6.00 mL with a mean of 4.28 ± 0.08 mL. The bulls among different groups also varied significantly ($P<0.01$) in their mean ejaculate volume. These findings coincided with those of Singh *et al.* (2000) and Shanmugavel and Singh (2002) in Sahiwal bulls but are in contrast to those of Patel *et al.* (1989a), Dhami *et al.* (1998), Javed *et al.* (2000), Chander *et al.* (2004) and Srivastava and Kumar (2006) in bulls of different cattle breeds. However, comparatively lower ejaculate volume was reported by Raval and Dhami (2006) and Swain and Singh (2007) than the present one in crossbred bulls and Sahiwal bulls, respectively.

Ejaculate volume is probably a breed characteristic, which depends upon the body / scrotal size and weight, reproductive health condition of bulls, age of bulls, method and frequency of collection, pooled volume, nutrition, season and management (Nazir, 1988).

Mass activity, progressive sperm motility and sperm concentration

The mass activity score (0-5 scale) and per cent progressive sperm motility noted in Sahiwal bulls' semen varied from 2.7 to 3.6 and 65.0 to 85.0 percent with the overall means of 3.17 ± 0.07 and 76.73 ± 0.43%, respectively. The sperm concentration per ml of semen recorded varied from 1125 to 1250 million with a mean of 1185.53 ± 4.17 million. The bulls also varied significantly ($P<0.01$) between themselves in their progressive sperm motility percent and sperm concentration. The present findings were in accordance with the observations of Raju and Rao (1982), Shelke and Dhami (2001), Chander *et al.* (2004), Swain and Singh (2007) and Rao *et al.* (2011) for mass activity, progressive sperm motility and sperm concentration.

However, Patel *et al.* (1989a), Dhami *et al.* (1998), Singh *et al.* (2000), Raval and Dhami (2006) and Srivastava and Kumar (2006) reported comparatively lower sperm concentration and higher mass activity and progressive sperm motility than the present one. Initial sperm motility is also an important attribute for acceptance or rejection of the ejaculate for further processing and use in AI, and it is positively correlated with keeping quality, freezability and fertility of that sample (Gopalkrishna and Rao, 1979; Belorkar *et al.* 1991; Shelke and Dhami, 2001). Progressively motile spermatozoa, provides an accurate prediction of semen quality as well as required for fertilization also (Lasley, 1951; Tomar *et al.* 1966). One of the most reliable parameter to evaluate the fertilizing capacity of bovine spermatozoa is motility (Correa *et al.* 1997; Zhang *et al.* 1998; Verberckmoes *et al.* 2002). According to Donnelly *et al.* (1998) motility is a strong predictor of the ability of a given sample to achieve fertilization *in vitro*. Sperm motility is also known to be prerequisite for penetration through cervical mucus (Mortimer *et al.* 1986), the cumulus (Tesarik *et al.* 1990) and Zona pellucida (Green, 1988). Similarly, sperm concentration per unit volume has a great bearing in semen processing / freezing and preservation, since along with the initial motility; the dilution rate depends upon this vital trait. Sperm concentration in semen collection could be considered as an initial indicator of semen quality in semen used for cryopreservation also (Belorkar *et al.* 1988; Shelke and Dhami, 2001).

Spermatozoa viability and morphological defects of spermatozoa

The percentages of live and abnormal spermatozoa in fresh semen of Sahiwal bulls varied from 79 to 88 and 7.00 to 14 percent with the overall means of 83.37 ± 0.34 and 9.97 ± 0.28 percent, respectively. The bulls also varied in their live and abnormal sperm counts, but variations are significant ($P<0.05$) for both the characters.

Table 1 Comparative study of semen ejaculates of Sahiwal bulls

Parameters	GR I	GR II	GR III	GR IV	GR V	df	SEM	F-vlue	Overall	P-vlue
Volume (mL)	4.88^{a}	3.88^{bc}	4.17^{b}	3.60^{c}	4.88^{a}	4	0.077	5.62	4.28	0.000
Mass activity (0-5)	3.38	3.32	3.03	3.07	3.07	4	0.069	1.13	3.17	0.366
Progressive motility (%)	78.83^{a}	79.0^{a}	70.0^{c}	81.50^{a}	74.33^{b}	4	0.425	23.04	76.73	0.000
Concentration (10^{6}/mL)	1251.0^{a}	1163.3^{b}	1157.8^{b}	1125.5^{c}	1240.0^{a}	4	4.17	1.12	1185.5	0.000
Live (%)	81.83^{c}	82.33^{c}	82.67^{bc}	84.83^{ab}	85.17^{a}	4	0.377	4.10	83.37	0.011
Abnormality (%)	11.33^{a}	10.50^{ab}	10.00^{ab}	9.17^{b}	8.83^{b}	4	0.279	2.61	9.97	0.059
Intact acrosome (%)	73.17^{b}	84.67^{a}	85.50^{a}	84.67^{a}	85.50	4	0.394	36.80	82.70	0.000
HOS positive sperm (%)	79.50	80.33	80.17	78.50	76.50	4	0.547	1.65	79.00	0.193
SPD (mm)	60.67^{a}	58.83^{ab}	54.67^{c}	56.50^{bc}	53.60^{c}	4	0.500	6.80	56.85	0.067

SPD: sperm penetration distance and HOS: hypo-osmotic swelling.
SEM: standard error of the means.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).

Table 2 Correlations between different parameters of semen ejaculates of Sahiwal bulls

Parameters	1	2	3	4	5	6	7	8	9
1. Volume	1.000								
2. Mass activity	0.485**	1.000							
3. Progressive motility	0.011	0.530**	1.000						
4. Concentration	-0.908**	0.437*	0.026	1.000					
5. Live	0.350	0.390*	0.347	0.186	1.000				
6. Abnormality	-0.340	-0.435*	-0.245	-0.156	-0.921**	1.000			
7. Intact acrosome	-0.282	-0.099	-0.208	-0.448*	0.486**	-0.524**	1.000		
8. HOS positive	0.304	0.756**	0.374*	0.124	0.395*	-0.463*	0.090	1.000	
9. SPD	0.331	0.776**	0.653**	0.365*	0.153	-0.191	-415*	0.681**	1.000

* P<0.05 and ** P<0.01.
SPD: sperm penetration distance and HOS: hypo-osmotic swelling.

Our findings were in conformity with the reports of previous studies in different breeds of bovines (Patel *et al.* 1989a; Veeraiah *et al.* 1999; Dhami *et al.* 1998; Raval and Dhami, 2006; Srivastava and Kumar, 2006; Swain and Singh, 2007).

However, comparatively higher abnormal sperm values were also reported in the bovines (Raju and Rao, 1982; Bhavsar *et al.* 1990; Shelke and Dhami, 2001).

High level of morphological abnormal sperms in semen is associated with low fertility in cattle (Thundathil *et al.* 1998).

Studies on live and physico-morphological attributes of spermatozoa will help in identifying good quality ejaculates.

Sometimes many structural abnormalities can occur in the spermatozoa due to faulty spermatogenesis caused by heredity, disease, adverse environmental effects and improper semen handling procedures.

Accurate morphological screening of the ejaculates allows elimination of bulls with a potential low fertility, prior to the entrance of bulls into a progeny testing programme and the preservation of semen, thus contributing to a major savings for AI enterprises.

The assessment of sperm concentration and morphology is based on the direct relation between the incidence of abnormal spermatozoa and the type of certain morphological defects with the *in vivo* fertility of the bull (Söderquist *et al.* 1991).

Acrosomal integrity, hypo-osmotic swelling test and cervical mucus penetration test

The percentages of intact acrosome and HOS positive spermatozoa in fresh semen of Sahiwal bulls varied from 70 to 88 and 74 to 84 percent with the overall means of 82.70 ± 0.39 and 79.00 ± 0.55%, respectively.

The bulls also varied significantly (P<0.01) in their intact acrosome sperm count but no significant variation was found between bulls regarding HOS positive spermatozoa. Thundathil *et al.* (2002) and Rana and Dhami (2003) and Srivastava and Kumar (2006) reported comparatively lower HOS positive spermatozoa percent in other bovines than the present one.

The value of HOST test in the present study partially corroborates the findings of the other workers.

Acrosomal integrity is a necessary further indicator of potential sperm function since in order to perform normally at artificial insemination; spermatozoa must demonstrate a full range of functions. These include the ability to survive, reach the oviduct, interact with the oviductal epithelium, attach to and penetrate the zona pellucida and interact with the oocyte. Almost similar value regarding intact acrosome was reported by Swain and Singh (2007) in Sahiwal bulls' semen.

An intact plasma membrane is required for normal sperm function and intactness of the plasma membrane is of utmost importance and it is tested using HOS tests (Kumi-Diaka, 1993).

According to England and Plummer (1993) and Srivastava and Kumar (2006), HOS test help in measuring the different aspect of sperm membrane behaviour separately, especially the biochemical activity and integrity of membrane, since the number of swollen spermatozoa was shown to be inversely proportional to the number of spermatozoa with damaged membranes. Sperm penetration distance (SPD, mm) in fresh semen of Sahiwal bulls varied from 51 to 65 mm with the overall means of 56.85 ± 0.50 mm. The bulls also varied significantly (P<0.05) between different groups in their SPD. These values are in agreement with those reported by Thundathil *et al.* (2002) and Rana and Dhami (2003) in bovine. Kumar and Devnathan (1996) reported comparatively lower percentage of intact acrosome spermatozoa in other bovines than the present one. Estimation of spermatozoal movement in the bovine cervical mucus is of vital importance in the clinical management of bovine infertility. The sperm penetration provides a reliable data for fertility assessment, allows great discrimination of sperm function and act as a useful tool for the diagnosis and management of fertility (Alexander, 1981). These values of SPD were in harmony with those reported by Thundathil *et al.* (2002) and Rana and Dhami (2003) in bovine. The variation in SPD value is reportedly affected due to initial motility, quality of progression of spermatozoa and sperm concentration (Suttiyovin *et al.* 1995). Differences in SPD among the bulls studied might be contributed by abnormality and acrosome integrity. This was in accordance to Bals Pratsch *et al.* (1988) who found that the seminal parameters allowed a correct prediction of the penetration test (sperm concentration, abnormality and motility) in 54% cases.

However, Tardif *et al.* (1999) indicated that fertility trials are the ultimate test of fertility, but they are expensive and time consuming, and results can be influenced by the insemination dose or the number of females used. Owing to these constraints, *in vitro* tests are often used to test sperm function in the hope that their outcomes can estimate fertility after AI. However, no single sperm laboratory test has been found to accurately predict fertility *in vivo*, although statistically significant estimations can be made by combining several *in vitro* assays (Larsson and Rodriguez Martinez, 2000).

Interrelationships among physio morphological attributes of semen

The correlation matrix (correlation coefficient=r) analysis of physico-morphological attributes of semen (Table 2) revealed that the ejaculate volume had significant positive correlation with the mass activity (r=0.485) and negative correlation with the sperm concentration (r=-0.908), while mass activity score showed significant positive correlation with the percentage of progressively motile sperms (r=0.530), sperm concentration (r=0.437), live sperm (r=0.390), HOS positive sperm (r=0.756) and SPD (mm) (r=0.776) and negative correlation with abnormal sperm (r=-0.435). Progressively motile sperm percentage in semen of Sahiwal bulls of this study had significant (P<0.01) positive correlation with SPD (mm) (r=0.653) and HOS positive sperm (r=0.374). Sperm concentration had significant positive correlation with SPD (mm) (r=0.365) and negative correlation with intact acrosome sperm percentage (r=-0.448). The live sperm had highly significant (P<0.01) negative correlation with abnormal sperm (r=-0.921) and positive correlation with intact acrosome sperm percentage (r=0.486) and HOS positive sperm (r=0.395). Abnormal sperm percentage had significant (P<0.01) negative correlation with intact acrosome sperm percentage (r=-0.524) and HOS positive sperm percentage (r=-0.463). Intact acrosome sperm percentage had significant negative correlation with SPD (mm) (r=-0.415) and HOS positive sperm also had significant (P<0.01) positive correlation with SPD (mm) (r=0.681). These findings to some extent corroborated well with the reports of Gopalkrishna and Rao (1979) and Dhami and Sahni (1994). However, Saxena and Tripathi (1978) and Saxena and Tripathi (1979) reported that sperm concentration was positively correlated with initial sperm motility, live spermatozoa, and negatively correlated with abnormal spermatozoa in Red Dane bulls. It was also reported by (Patel *et al.* 1989a; Patel *et al.* 1989b) that significant and positive correlations of mass activity with sperm concentration, initial motility, live sperm and negative correlation with abnormal sperm percent existed. In another study, Srivastava and Kumar (2006) also postulated a significant positive correlation of HOS positive sperm and SPD with mass motility, initial progressive motility, live count, percent intact acrosome and negative correlation with total sperm abnormalities.

CONCLUSION

On the basis of the present result it is concluded that a good correlation observed between CMPT and HOS test was probably because of the fact that both tests assessed the functional integrity of sperm plasma membrane and indirectly provided information about membrane associated cell functions. Overall semen quality of Sahiwal breed was found optimum for use in breeding programme.

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