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Relationship Between Routine Analysis/Sperm Function and Fertility Tests of Cattle Bull Semen

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ABSTRACT

Success of artificial insemination in bovine industry depends upon quality of frozen-thawed semen. Therefore, proper analysis of the post-thaw semen can provide insights into the fertilizing capacity of the cryopreserved semen. Frozen-thawed semen of 14 cross-bred and 12 pure-bred bulls was analyzed for capacitation/acrosome reaction and cervical mucus penetration test. Correlations between routine tests/sperm function and fertility tests were calculated. Mean values for individual motility, viability, abnormalities, hypo-osmotic swelling test, acrosome integrity and capacitated/acrosome reacted spermatozoa in frozen-thawed semen were 41.5±1.1, 59.9±1.9, 19.3±1.1, 32.6±0.8, 79.9±3.8, 33.7±2.3 and 40.3±1.1, 76.8±2.5, 17±1.1, 48.4±3.5, 92.9±2.4 and 32.9±2.8% in cross-bred and pure-bred bulls, respectively. It indicated low mean values of percent viability, acrosome integrity and distance covered by spermatozoa/30 min in cervical mucus in pure-bred as compared to cross-bred bulls. Viability and hypo-osmotic swelling test showed an average correlation with acrosome reacted spermatozoa (+0.58, +0.47). However, there was very weak correlation between acrosome reaction and viability (+0.06)/hypo-osmotic swelling test (+0.11)/acrosome integrity (+0.02). Sperm abnormalities were negatively correlated with acrosome reaction. Weak positive correlation was obtained between number of spermatozoa penetrated in peak 0.5 cm of cervical mucus and motility (+0.07)/viability (+0.12)/hypo-osmotic swelling test (+0.14). During the present study 23, 96 and 53.8% of tested bulls exhibited >40% post-thaw motility, >55% viability and >35% hypo-osmotic swelling test, whereas, only 46.1, 65.8 and 50% bulls were with >35% acrosome reacted spermatozoa, >25 mm distance covered/30 min and >350 sperms penetrated in peak 0.5 cm in cervical mucus penetration test respectively. Therefore on the basis of functional and fertility tests, it can be predicted that semen of about 50% tested bulls was of good quality. Motility of sperm is required to reach the site of fertilization and sperm membrane/acrosome integrity are important for adhesion, penetration and fertilization of sperm, therefore, it is concluded that sperm function and fertility tests like HOST, CMPT, acrosome integrity and *in vitro* capacitation/acrosome reaction are better indicators for assessment of quality of frozen-thawed semen and should also be performed before selecting breeding bulls.

Key words: Cattle bull, sperm, function, fertility tests, relationship

INTRODUCTION

The semen is evaluated conventionally on the basis of motility, morphology and viability (Zubair *et al*., 2013). Conventional parameters used for evaluation of semen have limited application because they only help to assess the structural integrity of the cell (Neild *et al*., 1999). Post-thaw motility is of significant value in order to discard frozen semen of poor quality but appear a little value to predict the fertility of the individual males (Christensen *et al*., 1999). Proper assessment of the post-thaw quality of spermatozoa can provide insights into the fertilizing capacity of the cryopreserved spermatozoa (Januskauskas and Zilinskas, 2002). So, a number of laboratory evaluation tests measuring the physical and functional integrity/fertility (HOST, CMPT, intact acrosomes, *in vitro* capacitation/acrosome reaction, zona free hamster assay etc.) of spermatozoa *in vitro* have been devised over the past few decades. Frozen-thawed semen of 14 cross-bred and 12 pure-bred bulls was analyzed for individual motility, viability, morphology, HOST, acrosome integrity, CMPT, *in vitro* capacitation/acrosome reaction and correlations between routine tests/sperm function and fertility tests were calculated.

MATERIALS AND METHODS

Procurement of semen: Frozen semen of 14 cross-bred and 12 pure-bred cattle bulls was procured from three different freezing labs of Punjab, India.

Individual motility: A drop of frozen-thawed semen was placed on micro slide, covered with cover slip and progressively motile spermatozoa were observed under bright field microscope (400 X) at 37°C. A total of 200 spermatozoa were counted in different fields and percentage motility was calculated.

Sperm viability and abnormalities: Frozen-thawed semen was evaluated for viability and abnormalities through eosin nigrosin staining method (Blom, 1950). A total of 150 sperms were counted in different fields under oil immersion (1000 X) and percent live sperm was calculated. Spermatozoa with various abnormalities of head, tail and mid-piece were also observed in eosin-nigrosin stained slides.

Hypo-Osmotic Swelling Test (HOST): HOST was performed as per the method of Correa and Zavos (1994). A total of 150 spermatoazoa were counted at 400 X in different fields and total number of coiled tailed sperms was calculated. The number of coiled tailed spermatozoa in PBS was deducted from the number in hypo-osmotic solution and the resultant figure was taken as the HOS-reactive spermatozoa.

Cervical Mucus Penetration Test (CMPT): CMPT was performed as per the method of Murase and Braun (1990). Cervical mucus was collected from a normal cycling cow in estrus and was filled in a capillary by capillary action. Capillary was sealed from one side with polyvinyl alcohol powder and pre-heated at 37°C for 10 min. Approximately 100 μL of frozen thawed semen was placed at the bottom of an eppendorf tube and a capillary tube was placed with its open end in the semen. After 30 min of incubation at 37°C, the capillary tube was fixed on scaled glass slide and viewed under microscope at 400 X. The length of the tube was then scanned to establish the distance furthest from the semen reservoir attained by spermatozoa. The maximum distance of migration of spermatozoa after 30 min of incubation was defined as the migration distance. Number of migrated spermatozoa was counted in the peak 0.5 cm.

Acrosomal integrity: Sperm smears stained with giemsa were examined microscopically under oil immersion at 1000 X. About 200 spermatozoa with intact acrosomes (stained dark purple) and damaged acrosome (without stain) was counted in different fields and percentage of spermatozoa with intact acrosomes was calculated (Sarma, 1995).

***In vitro* capacitation and acrosome reaction:** According to parrish *et al.* (1989) about 200×10^6 spermatozoa/mL were incubated in TALP medium (100 mM NaCl, 3.1 mM KCl, 25 mM $NaHCO_3$, 0.3 mM Na_2HPO_4, 21.6 mM Na lactate, 2 mM $CaCl_2$, 0.4 mM $MgCl_2$. $4H_2O$, 10 mM Hepes, 1 mM Na pyruvate, 0.6% BSA, 5 mM glucose and heparin 10 μg mL^{-1}) at 37°C in an incubator for 4-6 h. Motility was checked every hour and sperm smears were prepared at 0, 4 and 6 h of incubation. Sperm smears were stained with giemsa to observe different stages of acrosome reaction. Giemsa stained slides were observed under bright field microscope at 1000 X and about 200 spermatozoa with swollen heads, vesiculated and shedded acrosomes were counted. A few slides were also stained with chlortetracycline cysteine (Ward and Storey, 1984) to confirm different stages of acrosome reaction.

Statistical analysis: The data obtained was analyzed statistically according to Independent Sample t-test and One-Way ANOVA using difference between means of two groups and means of different group application at 5% level of significance (SPSS, Version 16.0).

RESULTS

A considerable variation in percent motility, viability, abnormalities, membrane integrity (HOST), acrosome integrity, acrosome reaction and Cervical Mucus Penetration Test (CMPT) was found in the frozen-thawed semen of 26 tested cattle bulls irrespective of the breed (Table 1). Individual motility, viability, abnormalities, HOST, acrosome integrity, capacitation/acrosome reaction ranged from 40-50, 51.9-74.4, 14.4-24.3, 29.8-38.7, 66.2-94.6 and 23.3-50% in the frozen-thawed semen of pure-bred cattle bulls, whereas, mean values were 41.5±1.1, 59.9±1.9, 19.3±1.1, 32.6±0.8, 79.9±3.8 and 33.7±2.3%. Mean values for individual motility, viability, abnormalities, HOST, acrosome integrity and capacitation/acrosome reaction in frozen-thawed semen of cross-bred cattle bulls were 40.3±1.1, 76.8±2.5, 17±1.1, 48.4±3.5, 92.9±2.4 and 32.9±2.8%, which were in the range of 30-45, 59.1-84.8, 9.7-23.2, 29.5-66, 61.3-98.2 and 26.2-54.9% (Table 1). It indicated low mean values of percent viability, acrosome integrity and distance covered by spermatozoa/30 min in cervical mucus in pure-bred as compared to cross-bred cattle bulls (Table 1). Percent viability was higher in frozen-thawed semen of cross-bred bulls (76.8±2.5%) than that of pure-bred cattle bulls (59.9±1.9%, Fig. 1). Permissible limit for conventional semen is <25% abnormalities. Abnormalities observed in frozen-thawed semen of pure-bred (19.3±1.1%) and

Table 1: Mean±SE and range of sperm parameters in frozen-thawed semen of pure-bred and cross-bred cattle bulls

								CMPT	
Breed	Post-thaw motility	Viability	Abnormalities	HOST	Sperm count (million mL^{-1})	Acrosome integrity	Acrosome reaction	Distance covered in 30 min	No. of sperms penetrated in peak 0.5 cm
Pure (HF, Jersy and Sahiwal)	41.5±1.1[a] (40-50)	59.9±1.9[a] (51.9-74.4)	19.3±1.1[a] (14.4-24.3)	32.6±0.8[a] (29.8-38.7)	848.5±75.2[a] (758-1178)	79.9±3.8[a] (66.2-94.6)	33.7±2.3[a] 23.3-50	18.9±2.8[a] (9.0-35)	378.9±42.7[a] (219-578)
Cross (HF, Red dane and Sahiwal)	40.3±1.1[a] (30-45)	76.8±2.5[b] (59.1-84.8)	17.0±1.1[b] (9.73-23.2)	48.4±3.5[b] (29.5-66)	932±33.3[b] (573-1301)	92.9±2.4[b] (61.3-98.2)	32.9±2.8[a] (26.2-54.9)	23.5±1.1[b] (17-31)	376.7±24.5[a] (261-528)

Values in parentheses represent range, values with different superscripts are significant ($p<0.05$)

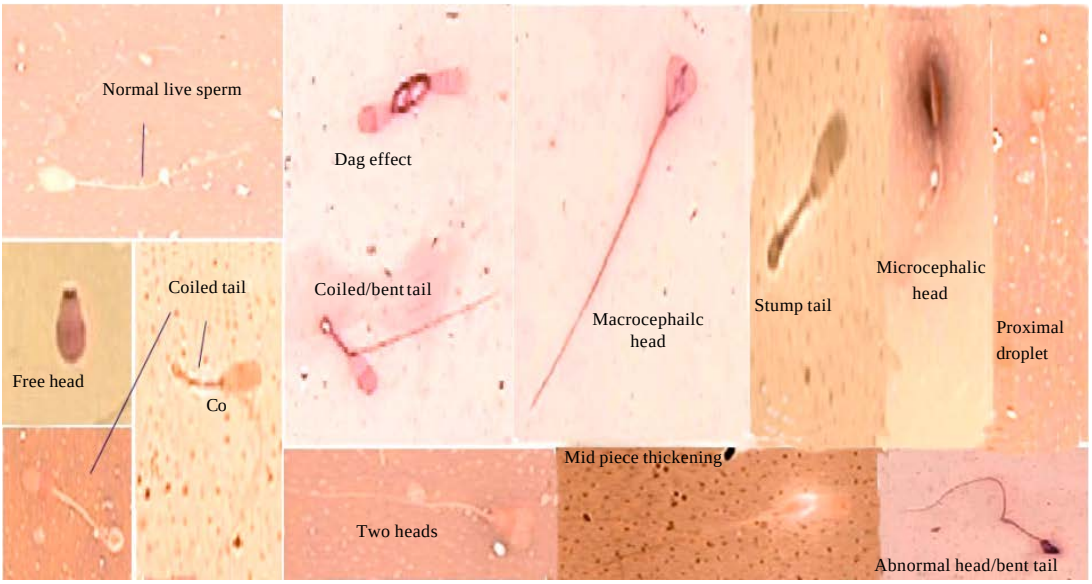

Fig. 1: Various types of sperm abnormalities in frozen-thawed semen of cattle bulls

cross-bred bulls (17±0.1%) were within permissible limit. Sperm abnormalities like coiled tail, bent tail, free heads, short tail, stump tail, dag defect, proximal/distal droplets and microcephalic/macrocephalic heads were common in the tested samples, whereas sperms with two heads/tails, mid piece thickening were rarely seen in the tested samples (Fig. 1). HOS-positive spermatozoa were higher (48.4±3.5%) in frozen-thawed semen of cross-bred bulls than pure-bred cattle bulls (32.6±0.8%), whereas, spermatozoa with intact acrosomes were less (61.3-98.2%) in frozen-thawed semen of cross-bred bulls than that in pure-bred bulls (79.9±3.8%). Mean values of capacitated and acrosome reacted spermatozoa observed during the present study were almost same (33.7±2.3 and 32.9±2.8%) in frozen-thawed semen of pure-bred and cross-bred cattle bulls (Fig. 2). Distance covered by the spermatozoa in 30 min number of spermatozoa penetrated in peak 0.5 cm ranged from 9-35 mm 219-578 and 17-31 mm 261-528, respectively in pure-bred and cross-bred cattle bulls, whereas percent mean values were 18.9±2.8/378.7±42.7 and 23.5±1.1/376.7±24.5.

Since, fertilizability of semen can be predicted from rate of capacitation/acrosome reaction and CMPT, therefore, Pearson's correlations were calculated between routine sperm parameters (percent motility/viability), sperm function tests (HOS-positive spermatozoa/acrosome integrity) and fertility tests (rate of capacitation/acrosome reaction and CMPT). There was an average positive correlation between distance covered by spermatozoa/30 min in cervical mucus and viability (+0.58)/HOST(+0.47). But weak positive correlation was obtained between number of spermatozoa penetrated in peak 0.5 cm and viability (+0.12)/HOST (+0.14)/motility (+0.07). However, CMPT was negatively correlated with abnormalities and acrosome integrity. There was very weak correlation between rate of acrosome reaction and viability (+0.06)/HOST (+0.11)/acrosome integrity (+0.02). But rate of acrosome reaction was negatively correlated with sperm abnormalities and motility.

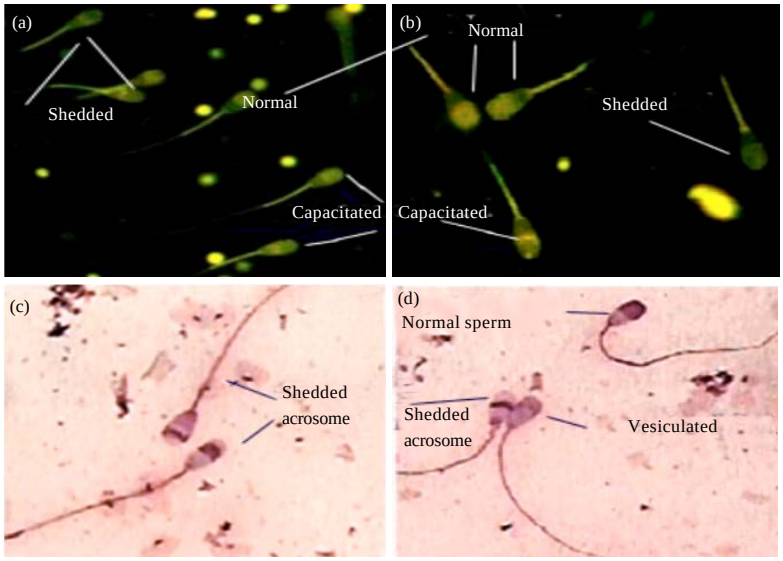

Fig. 2(a-d): Various stages of capacitation/acrosome reaction in cattle bull spermatozone, (a, b) CTC stained and (c, d) Giemsa stained

DISCUSSION

Individual motility of frozen-thawed semen in pure-bred and cross-bred cattle bulls observed during the present study was less than observed in bovine (Correa *et al*., 1997), Sahiwal and Jersy bulls (Dhami *et al*., 1998), HF bulls (Patel *et al*., 2000; Singh *et al*., 2000), Estonian Holstein bulls (Padrik *et al*., 2012), Kankrej bulls (Patel and Siddiquee, 2013) but higher than frozen-thawed semen of Sahiwal bulls (Forero-Gonzalez *et al*., 2012). Percent viability, evaluated by Singh *et al*. (2000), Perumal *et al*. (2009) and Patel and Siddiquee (2013) in Holstein Friesian, Jersey/cross-bred and Kankrej bulls was similar to that observed in pure-bred bulls during the present studies. Sperm abnormalities observed in the frozen-thawed semen of cross-and pure-bred bulls was higher than observed by Babu Rao *et al*. (1999), Fiaz *et al*. (2010) and Patel and Siddiquee (2013) in Punganur, Holstein Friesian and Jersey, Kankrej bulls, respectively.

Similar to these observations Correa *et al*. (1997), Pons-Rejraji *et al*. (2009) and Padrik *et al*. (2012) also reported 34.3±6.6, 32.6±3.4 and 39.4±2.8% HOS-positive spermatozoa in frozen-thawed semen of cattle, pure-bred and Estonian Holstein bulls. Presence of acrosomal cap is very important in fertilization process and is related to fertility. Spermatozoa with higher motility cannot be fertile due to acrosomal loss (Raval and Dhami, 2010). Acrosomal cap undergoes biochemical and ultra structural changes and acrosomal enzymes play an important role during fertilization. Percentage of spermatozoa with intact acrosomes in frozen-thawed semen of pure-and cross-bred frozen-thawed semen was in accordance with that observed by Pons-Rejraji *et al*. (2009) and Forero-Gonzalez *et al*. (2012) in frozen-thawed semen of pure-bred (80%) and Sahiwal bulls (85.2%) respectively. Nagy *et al*. (2003) also observed spermatozoa with 63.57±12.4% mean intact acrosomes in frozen-thawed bovine semen using dual staining method, whereas, Patel and Siddiquee (2013) observed only 52.6±0.6% spermatozoa with intact acrosomes in frozen-thawed semen of Kankrej bull semen.

After ejaculation, mammalian spermatozoa undergo a maturational process called capacitation either *in vivo* during transit through the female genital tract or *in vitro* in a defined medium. Fozen-thawed spermatozoa of pure-and cross bred bulls were successfully capacitated *in vitro* and on an average 33.7±2.3 and 32.9±2.8% spermatozoa showed acrosome reaction. Capacitation and acrosome reaction of frozen-thawed semen has been successfully induced *in vitro* in cattle bulls (Cormier and Bailey, 2003; Felipe-Perez *et al*., 2008).

An average positive correlation was found between distance covered by spermatozoa/30 min in cervical mucus and viability; a weak positive correlation between number of spermatozoa penetrated in peak 0.5 cm and viability/HOST/motility and a very weak between rate of acrosome reaction and viability/HOST/acrosome integrity. The results of a study done on cross-bred bulls (Sahiwal X local hill), Sharma *et al.* (2012) revealed a highly significant ($p<0.01$) correlation between the various semen evaluation parameters. Zubair *et al.* (2013) found a significant ($p<0.05$) positive correlation between progressive motility, morphologically normal spermatozoa, sperm viability and percentage of HOS-positive spermatozoa in HF, Sahiwal and cross-bred (HF X Sahiwal) bulls. Galli *et al.* (1991) also demonstrated that sperm penetration in mucus was significantly correlated with total motility, sperm morphology and progressive motility, acrosome integrity and concentration. But sperm abnormalities were negatively correlated with CMPT in tested bulls during this study. Ola *et al.* (2003) were of the opinion that distance covered by spermatozoa in CMPT has a low accuracy in the evaluation of semen as compared to sperm count. Tas *et al.* (2007) also suggested CMPT as a successful method for estimation of potential fertility in bulls. Present study in pure-bred/cross-bred cattle bulls and work done on other breeds by various workers revealed a variation in sperm parameters among the bulls and different breeds. The variation in motility, viability, abnormalities, HOS-positive spermatozoa, sperm membrane and acrosome integrity of frozen-thawed semen depends upon many factors, viz; age of bull, season, quality of fresh semen, frequency of semen collection, handling during cryopreservation and composition of dilutor, etc. Therefore, this study reveals differences in all tested sperm parameters at bull level but statistical analysis revealed significant differences ($p<0.05$) only in viability, abnormalities, HOS-positive spermatozoa, spermatozoa with intact acrosomes and distance covered/30 min in CMPT among the two breeds.

During the present study, 23, 96, 53.8% of tested bulls exhibited >40% post-thaw motility, >55% viability and >35% HOST, whereas, 46.1, 65.8 and 50% bulls were with >35% acrosome reacted spermatozoa, >25 mm distance covered 30 min and >350 sperms penetrated in peak 0.5 cm in CMPT, respectively. Since motility and viability are not the only criteria for fertilizability of semen, therefore, on the basis of functional tests (acrosome integrity, HOST) and fertility tests (*in vitro* capacitation/acrosome reaction and CMPT), it can be predicted that semen of about 50% tested bulls was of good quality. Motility of sperm is required to reach the site of fertilization and sperm membrane-/acrosome-integrity are important for adhesion, penetration and fertilization of sperm, therefore, it is concluded that sperm function and fertility tests like HOST, CMPT, acrosome integrity and *in vitro* capacitation/acrosome reaction are better indicators for assessment of quality of frozen-thawed semen and should also be performed before selecting breeding bulls.

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