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Characteristics of frozen thawed semen in predicting the fertility of buffalo bulls

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ABSTRACT

Information regarding the fertility index in relation to sperm attributes, which helps in the selection of future breeding bulls are meager in buffaloes. The present study was conducted to measure the differences in motility characteristics, head biometry, acrosome, plasma membrane and DNA of cryopreserved semen of fertile and sub-fertile buffalo bulls. The fertility of bulls was classified on the basis of conception rates (CR), where bulls having CR 28–35% and >55% were considered as sub-fertile and fertile bulls respectively. Computer assisted semen analyzer was used for motility and viability studies. Total motility, average path velocity (VAP), straight linear velocity (VSL) and curvilinear velocity (VCL) of sperm for fertile bulls were significantly higher than sub-fertile bulls. Significant differences were found in the length and width of sperm head between the 2 groups. The percentage of intactness of sperm acrosome of fertile bulls was significantly higher than sub-fertile bulls. The percentage of apoptotic sperm differed significantly between fertile and sub-fertile bulls. The sperm DNA integrity of fertile and sub-fertile bulls was not significantly different. In conclusion, the total motility, VAP, VCL, VSL, length and width of sperm head, acrosome integrity and percentage of apoptotic sperm, are useful for evaluating bulls' semen quality to reduce the risk of using semen of poor-fertility bulls in AI programme.

Key words: Acrosome integrity, Apoptosis, CASA, DNA integrity, Fertility, Plasma membrane integrity, Sperm motility

The fertility assessment of frozen-thawed bull semen is essential for the effective use of cryopreserved semen in buffalo breeding. The labouratory evaluation of semen from healthy bulls, before and after freezing for AI, is largely based on subjective scoring of sperm mortility and concentration. Hence, we need to have an accurate, simple and efficient *in vitro* method to predict fertility of buffalo serum. In the last decade, methods were adopted for semen analysis like assessment of sperm kinetics by computer assisted sperm analyzer (CASA), evaluation of plasma membrane integrity with fluorescent dyes, evaluation of acrosomal status with fluorescein–isothiocyanate-conjugated lectins, investigation of DNA integrity using the sperm chromatin structure assay, or assessment of membrane architectural status (Gillan *et al*. 2005, Silva and Gadella 2006). Some of these parameters help explain the sources of individual variation in animal fertility (Hallap *et al*. 2006). The semen analyzed through CASA are able to objectively determine sperm kinetics and biometric parameters and allow us to compare results with standard values to predict fertility (Verstegen *et al*. 2002). In addition, the combinations of sperm motility parameters with sperm kinetics as assessed by CASA were significantly correlated with bull fertility (Farrell *et al*. 1998). Furthermore, semen evaluation on a single parameter is not effective predictors of fertility. A combination of several assays may provide better prediction of fertility.

There is meager information available regarding the fertility index in relation to sperm attributes, so that future breeding bull could be selected at the age of maturity. The factors associated with low fertility in buffalo bulls make better strategy for improving the field fertility of bulls following insemination with frozen-thawed semen. Therefore, to predict fertility of buffalo breeding bulls, the present study was undertaken to find out differences in motility characteristics, head biometry, acrosome, plasma membrane and DNA of cryopreserved semen of fertile and sub-fertile buffalo bulls.

MATERIALS AND METHODS

Semen was collected with artificial vagina and conventionally assessed for volume, color and sperm concentration. Sperm motility was subjectively assessed under phase contrast microscope equipped with a warm

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stage (37°C) at 400 × magnification and only ejaculates with >70% sperm motility were used for cryopreservation. The fresh semen was diluted and filled in 0.25 ml straws, each containing approximately 80 × 10^6 spermatozoa/ml and then frozen with standard procedure (Singh *et al*. 2013). The frozen semen straws were used for field progeny testing programme of the institute. In the present study, bull fertility was classified on the basis of conception rate (CR) obtained from field progeny testing programme, where buffalo bulls having CR 28–35% and >55% were considered as sub-fertile and fertile bulls respectively. Six bulls (3 in each group), were selected, and randomly 3 frozen straws were taken from each bull; and pooled semen were used for different *in vitro* quality analysis. Each experiment was repeated thrice and at least 200 sperm were counted for each analysis from each bull.

Evaluation of sperm motility, viability and biometry under CASA: Sperm motility was assessed by using CASA system. Before analysis under CASA, the semen sample was diluted with pre-warmed Tris buffer to give a sperm concentration of about 2–6 × 10^6 spermatozoa/ml. The CASA software settings for recording sperm motility are summarized in Table 1. A 1 µl prepared semen sample was loaded in a pre-warmed (38°C) 8 chamber slide (depth 20 µm) and analyzed for sperm motility characteristics. For each sample, 5 optical fields around the central reticulum of the chamber were used to count spermatozoa. The motion characteristics recorded were total motility (%), progressive motility (%), rapid motility (%), straight linear velocity (VSL, µm/s), average path velocity (VAP, (µm/s), curvilinear velocity (VCL, µm/s), average lateral head displacement (ALH, µm/s), beat cross frequency (BCF, Hz), straightness (STR,%) and linearity (LIN,%) of the spermatozoa. Bis benzimide trihydrochloride (5 µg/ml) dye was used to stain the sperm cells for viability testing. The stained samples were incubated at 37°C for 2 min and analyzed with blue light and Viadent filter block under CASA. The biometry of sperm head was performed by rapid papanicolaou staining method (Boersma *et al.* 2001).

Evaluation of acrosome integrity: The acrosomal integrity was evaluated using fluorescing conjugated lectin *Pisum sativum* agglutinin (FITC-PSA) staining method (Mendoza *et al*. 1992), with minor modifications. Briefly, 20 µl of diluted semen was re-suspended in 500 µl PBS and centrifuged at 1,500 rpm for 10 min and supernatant was discarded and re-suspended in 250 µl PBS. One drop of the solution was taken to make a smear onto a pre-cleaned microscope slide. The smear was dried and fixed with paraformaldehyde at room temperature for 45 min. The slides were covered with FITC-PSA (50 µg/ml in PBS solution) in dark place for 20 min at room temperature. Excess stain was removed by washing with water, the slide was air dried and a cover slip was applied with glycerol and examined under fluorescent microscope. Sperm with intact acrosome showed more intense fluorescence in the acrosome region with a distinct ring whereas damaged sperm head had less intense fluorescence.

Table 1. Set-up of CASA for motility assessment of frozen-thawed buffalo sperm

Parameters	Value set at
Frame rate (Hz)	60
Frames acquired	30
Minimum contrast	35
Minimum cell size (pixels)	5
Cell size (pixels)	9
Cell intensity (pixels)	110
Path velocity (VAP) (µm/s)	50
Straightness (STR) (%)	70
VAP cut-off (µ/s)	30
VSL cut-off (µ/s)	15

Detection of translocation of phosphatidylserine from plasma membrane: A commercial kit was used to detect the translocation of phosphatidylserine (PS) from the inner to the outer leaflet of the sperm plasma membrane as recommended by the manufacturer with slight modifications. The spermatozoa were washed (1,500 rpm for 10 min) twice in Tris-buffered saline (pH 7.0). The sperm pellet was resuspended in Annexin-V binding buffer (10 mM Hepes/NaOH, 140 mM NaCl, and 2.5 mM $CaCl_2$) at room temperature to a final concentration of 1–2 × 10^5 spermatozoa/ml. The cell suspension (97.5 µl) transferred to a clean tube, 2.5 µl Annexin V-FITC was added, mixed and incubated for 15 min at room temperature. After incubation, sample was washed with binding buffer and centrifuged at 1,000 rpm to pellet the cells and the supernatant was discarded. The cell pellet was re-suspended in 95 µl binding buffer and 5 µl PI (50 mg/ml) was added to it, gently mixed, incubated for 15 min at room temperature in the dark, and then analyzed under fluorescence microscope. This assay is able to differentiate the apoptotic, early necrotic, necrotic and viable cells. Apoptotic sperm labeled with Annexin-V only, gave green fluorescence, necrotic sperm labeled only PI, showed red fluorescence, early necrotic sperm labeled with both Annexin-V and PI, appeared green and red fluorescence, whereas viable sperm did not get label with any one, hence showed no fluorescence.

Evaluation of DNA damages by TUNEL assay: The TUNEL assay was used for the detection of nicked DNA in spermatozoa as per Anzar *et al*. (2002). Briefly, the semen samples were diluted to 2 × 10^6 cells/ml in PBS then centrifuged twice at 800 ×g for 10 min. The diluted spermatozoa were fixed by adding 10 ml of paraformaldehyde (1% w/v) in PBS (pH 7.4) on ice for 15 min. After fixation, sperm were washed twice in PBS, and the pellet was resuspended in 0.5 ml of PBS followed by 5.0 ml of ice-cold ethanol (70% v/v). The fixed sperm suspension was stored at –20°C until analyzed for nicked DNA. On the day of analysis, the fixed sperm were centrifuged to remove ethanol. The sperm suspension was washed twice in 1 ml of wash buffer (provided with kit) and the supernatant was removed by aspiration. The pellet

was resuspended in 51 µl of freshly prepared DNA-labeling solution containing TdT enzyme (0.75 µl), BrdUTP (8.0 µl), reaction buffer (10.0 µl), and distilled water (32.25 µl). The sperm were incubated in the DNA labeling solution at 37°C for 2 h. The suspension was gently shaken at every 30 min. At the end of the incubation time, the sperm were washed twice in 1 ml of rinse buffer, and the pellet was resuspended in 100 µl of freshly prepared antibody solution containing 5 µl monoclonal antibody (fluorescein-labeled PRB-1) and 95 µl of rinse buffer. The sperm suspension was again incubated in the dark for 30 min at room temperature. Spermatozoa were analyzed under fluorescence microscope after addition of 300 µl of PI/RNase A solution.

Statistical analysis: The data were analyzed by one-way ANOVA followed by Duncan multiple range post-test to assess differences among mean of fertile and sub-fertile bull sperm motion characteristics, acrosome integrity, plasma membrane integrity and DNA integrity using SAS 9.2 for window. The level of significance was observed at $P < 0.05$, and data were reported as mean ± standard error (SE).

RESULTS AND DISCUSSION

The mean values of total motility, VAP, VSL and VCL of sperm for fertile bulls were significantly higher than sub-fertile bulls (Table 2). In the present study, spermatozoa from bulls with high field fertility displayed an increased total motility, VAP, VSL and VCL and these results are in agreement with Gillan *et al*. (2008) in dairy bulls in which spermatozoa from bulls with superior fertility exhibited high motility, viability and VSL. The progressive and rapid motility and ALH, BCF, STR, LIN and viability did not differ in both groups. The sperm motility was considered as the most important characteristic parameters for evaluating the fertility potential of ejaculated spermatozoa because it is essential for transport of sperm through female reproductive tract to reach at the site of fertilization.

Table 2. Mean of buffalo sperm motility, kinetic parameters and viability of fertile and sub-fertile bulls

Parameters	Fertile	Sub-fertile
Total motility	56.88±3.2[a]	43.82±1.7[b]
Progressive motility	16.59±1.1	16.57±2.1
Rapid motility	20.25±2.5	20.35±2.8
Slow motility	30.62±2.6[a]	18.25±1.4[b]
VAP	87.22±2.4 [a]	79.02±1.6 [b]
VSL	68.93±1.9[a]	63.42±1.2[b]
VCL	156.52±4.3[a]	142.37±2.8[b]
ALH	6.80±0.1	6.57±0.07
BCF	33.98±0.4	33.43±0.5
STR	79.21±0.7	78.74±0.6
LIN	45.59±0.4	45.50±0.7
Size	8.15±0.2	7.99±0.1
Viability	71.24±0.83	68.91±0.8

Values with different superscripts within row differ significantly ($P<0.05$).

Table 3. Mean value of biometry of the sperm head of fertile and sub-fertile buffalo bulls

Parameters	Fertile	Sub-fertile
Length (µm)	8.47[a]±0.11	7.98[b]±0.07
Width (µm)	5.12[a]±0.09	4.86[b]±0.06
Elongation (%)	58.92±0.43	58.51±0.38
Area (µm^2)	31.23±0.42	29.13±0.35
Perimeter (µm)	21.38±0.18	21.37±0.17
Acrosome (%)	51.65±0.93	49.83±0.66

Values with different superscripts within row differ significantly ($P<0.05$).

Presence of intact acrosome is pre-requisite for fertilization process and it is considered as a sperm quality parameter highly related with fertility of semen (Takahashi *et al*. 1992). In the present study percentage of intactness of sperm acrosome of fertile bulls was significantly higher (81.82±0.87%) than sub-fertile (76.86±0.87%) bulls. Apart from these parameters, length and width of sperm head of fertile bulls were also significantly more than sub-fertile bulls, indicating that bigger size of head is associated with fertility in buffalo, and whereas other parameters were remain unchanged between groups (Table 3). Our result was similar to the earlier finding of Gravance *et al*. (2009) except elongation (width/length). The normal structure of sperm plasma membrane is having negatively charged PS on the inner leaflet of the membrane (Hammerstedt *et al*. 1990). When the sperm membrane disturbed, the PS is translocated and exposed on the outer surface. This is one of the earliest detectable plasma membrane changes that indicate initiation of apoptosis-like structural change in sperm, which could be differentiated from necrosis (Martin *et al*. 1995, Kadirvel *et al*. 2012). In the current study, the percentage of apoptotic sperm differed significantly between fertile (15.59±0.75%) and sub-fertile (25.94±0.5%) bulls. The percentages of early necrotic, necrotic and viable sperm did not differ in the 2 groups (Table 4). Shen *et al*. (2002) found that the apoptotic alterations in sperm were positively associated with various forms of abnormal sperm morphology including the sperm head, mid piece and tail defects. They demonstrated that the adverse functional impact of sperm apoptosis on sperm quality. Hence, sperm membrane integrity appears to be more closely related to fertility than other conventional semen characteristics (Larson and Rodriguez-Martinez 2000).

Sperm DNA integrity of fertile (90.24±0.94%) and sub-fertile (88.37±0.91%) bulls does not differ significantly. However, 10–12% of DNA fragmentation was observed in frozen-thawed buffalo sperms, which indicate that the apoptotic alterations including DNA fragmentation and translocation of PS are likely to have originated from the apoptotic process during the spermatogenesis. In the human testis, it was estimated that a spermatogonia is able to produce about 100 spermatids, which is far lower than theoretical value of 4,096 spermatids (Woolveridge and Morris 2000), indicating the involvement of physiological

Table 4. Population of spermatozoa of fertile and sub-fertile buffalo bulls evaluated by Annexin-V/PI under fluorescence microscope (% ± SE)

Parameters	Fertile	Sub-fertile
Apoptotic	15.59±0.75[a]	25.94±0.51[b]
Early necrotic	14.53±0.51	14.07±0.60
Necrotic	13.08±0.53	14.53±0.46
Viable	56.78±0.90	54.44±0.93

Values with different superscripts within row differ significantly (P<0.05).

cell death in this process. Hence, it is understood that the germinal cell apoptosis is an underlying mechanism of normal spermatogenesis. The high percentage of apoptotic sperm observed in sub-fertile bulls imply that more number of spermatozoa may undergo apoptosis process during spermatogenesis. Furthermore, Seli *et al*. (2004) found that the degree of DNA damage cause male infertility and they also correlated it with the impairment of embryo development.

Understanding the basis for differences in fertility of individual bulls is an important objective towards developing a predictive *in vitro* test for male field fertility. According to the results obtained in this study, it is concluded that the total motility, VAP, VCL, VSL, length and width of sperm head, acrosome integrity and percentage of apoptotic sperm are useful for evaluating bull's semen quality to reduce the risk of using poor-fertility bulls in AI programme.

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