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Post-thawed and fresh spermatozoa motion characteristics of Sahiwal bulls under computer-assisted semen analyser (CASA)

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In this study, motion characteristics of spermatozoa was assessed by computer assisted semen analyser (CASA) for evaluating fertility potential of Sahiwal bulls. Twelve bulls were selected and grouped into two on the basis of age (AGI < 50 months old; AGII > 50 months old) and scrotal circumferences (SCI < 33 cm; SCII > 33 cm). The following CASA parameters i.e., velocity average path (VAP, µm/s), velocity straight line (VSL, µm/s), velocity curvilinear (VCL, µm/s), amplitude of lateral head displacement (ALH, µm), motility (%)(the percentage motile cells of the total) and straightness (STR) were recorded. Results of the study revealed that there is no significant different (p>0.05) in progressive motility either in age or Sc groups of bulls. However, significantly (p<0.05) higher mean post thaw motility was observed after 24 h cryopreservation for the younger (76.40±3.07) than the older (65.00±3.50) bulls and for larger SC than smaller SC bulls (65.56±3.78 vs. 56.56±3.78, p<0.05). Similar trends observed at 0 h after freezing were not significantly different (p>0.05) for both age and SC groups. In most motion characteristics especially in motility and linearity of the motion, younger bulls and bulls with larger SC performed better than older bulls and bulls with smaller SC indicating the possibility of selecting bulls at an early age on the basis of testis size to save the money, space and time which otherwise spent on rearing such inferior bulls. This study also clearly indicated that CASA is a good supplementation to aid for selection of breeding bulls.

Key words: Sahiwal bull, computer-assisted semen analysis (CASA) parameters, spermatozoa motion characteristics.

INTRODUCTION

Sahiwal is one of the indigenous breeds of South Asia, and has its origin in Montgomery district of Pakistan, and is distributed in certain herds of Punjab and Rajasthan in India. The importance of this breed is evident from the fact that Sahiwal animals were imported by other countries (like Kenya, Tanzania, Australia, West Indies and Bangladesh etc.), either for crossbreeding with their local breeds or for incorporating some zebu genes in crossbred animals for developing synthetic strains like Jamaica Hope, Australian milking zebu and Australian

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Friesian Sahiwal (Joshi et al., 2001). Because of indiscriminate crossbreeding with exotic breeds, the pure breed of Sahiwal is considerably declining in number (Dahlin et al., 1998). However, it has also been recognized that crossbred animals have poor adaptability to the local environment (Rehman et al., 2006; Garcia et al., 2003; Joshi et al., 2001). Hence, it is extremely important to focus on further evaluation, selection and propagation of superior germplasm of Sahiwal cattle. Conventionally, sperm motility estimation is done by visual approximation of progressively moving spermatozoa using phase contrast microscope. The progressive motility estimation is only an assessment of quantity of moving spermatozoa. Even though an accurate and objective laboratory test for assessing the potential fertility of a bull only based on some specific semen characteristics have still not been successfully achieved (Raja and Rao, 1983), the assessment of quality of motility in terms of velocity, swimming pattern, sperm head behaviour etc., may help in better understanding of the possible function.

In this respect, the advent of Computer-assisted semen analysis (CASA) has brought a new dimension to semen evaluation. CASA is a recent laboratory tool for evaluating semen samples objectively and provides an opportunity to assess sperm kinetics more precisely, rapidly and accurately. The CASA technique yields repeatable and highly reliable results on kinematics of ejaculates based on measurements of individual sperm cells.

Adoption of CASA technique has been reported as the potential tool for improvements in evaluation of semen to enhance fertility (Sundararaman et al., 2012). Hence, it is of great interest to use a combination of semen motion characteristics which can predict bull of high fertility performance more accurately than a single test. Moore and Akhondi (1996) indicated that CASA provided significant information for determining sperm fertilizing capacity and will be a useful technique for reproductive toxicology. Methodologies for application of this technique in clinical evaluation have been described for spermatozoa of human, bull and stallion (Mohaney et al., 1989; Gokcen et al 1991; Broekhuijse et al., 2012). Therefore, the current study was carried out to assess sperm motility coupled with kinetic measurements during various stages of cryopreservation that would help in better evaluation of semen quality.

MATERIALS AND METHODS

Description of the study site

The study was carried out at Artificial Breeding Research Centre, National Dairy Research Institute, Karnal Hariana - India. The farm is situated at an altitude of 250 m above the mean sea level on 29.42°N latitude and 77.42°E longitude. The climate of the farm is sub-tropical nature. The range of atmospheric temperature varies from near freezing point (0°C) in winter months to about 45°C in summer months. The average annual rainfall is approximately 760 to 960 mm, which is received mostly during months of July to August. Relative humidity varies from as low as 41% to as high as 85%.

Animals and semen collection

Twelve breeding Sahiwal bulls were selected and were grouped into two on the basis of age groups (AGI < 50 months old; AGII > 50 months old) and scrotal circumferences groups (SCI < 33 cm; SCII > 33 cm). Semen samples (180) were collected in the morning hours between 8:00 to 9:00 am using dummy bull. The bulls were thoroughly washed, cleaned and dried at least 15 to 30 min before collection. Two consecutive ejaculates were taken weekly, using Danish Model standard Artificial Vagina (AV) (14 inches). The temperature of AV was maintained at 45°C with sufficient pressure and lubrication. The semen was kept in water bath maintained at 31°C and was evaluated for physical attributes and fertility parameters immediately after collection and the important seminal attributes for all the bulls were recorded.

After the subjective assessment of sperm for progressive motility, the fresh diluted semen samples were subjected to CASA ("Cell track/s", Automated Sperm Analysis, Santa Rosa, Ca., 1994). An aliquot of diluted semen was placed on the clean grease-free slide maintained at 37°C and covered slip. The slide was observed at 20X magnification and phases 1 (P1) combination under Olympus phase contrast microscope attached to CASA system. For post-thaw examination of frozen semen, cryopreservation of semen was performed using the semen samples having mass activity 3.5 and above.

Glycerolization, equilibration time and storage

Tris-citric egg yolk cryodiluter was used for cryopreservation. The diluent was divided into two parts (Parts A and B). Part A was mixed with semen and Part B was mixed with glycerol at the rate of 7% of total diluent. Both the Parts A and B were cooled from 30 to 5°C. When both parts reached 5°C, they were mixed together and 0.25 ml of French straws was used for storage. After packing on average 30 million progressive motile spermatozoa in each dose, the straws were sealed with polyvinyl alcohol powder. The sealed straws were kept in cold handling cabinet at 5°C for 4 h for equilibration to avoid cold shock. After completion of equilibration, the straws were placed horizontally in freezing rack. The rack along with straws was kept in the liquid nitrogen vapour for 10 min for cryopreservation and were transferred to goblets and immersed into liquid nitrogen.

Thawing and examination of frozen semen

Immediately after removing from the liquid nitrogen, the straws were placed in water bath at 37°C for 15 to 30 s. Post-thaw sperm motility was examined using subjective sperm motility assessment and objectively by CASA, at interval of 0 and 24 h after freezing. The normal range for the CASA setup parameters were: VSL > 25.8 Microns/s, VCL > 40.8 Microns/s, LIN > 40.0 Microns/s, ALH > 3.0 Micron and VAP > 40.0 Microns/s. The CSA calibration setup used in this study is given as follows:

CASA calibration setup

Frame rate (Frames/ sec) – 30; Duration of data capture – 15; Minimum motile speed (microns/s) – 28; Maximum burst speed (microns/s) – 600; Distance scale factor (microns/s) - 7.5071; Cent. Cell size minimum (Pixiles) – 6; Cent. Cell size maximum (Pixles) - 13; Number of cell to be find per well - 100; Minimum number of fields per sample – 3.

Table 1. Least square means ± SE of CASA parameters in fresh semen of Sahiwal bulls by age and SC group.

Motion characteristics	Age group		Scrotal circumference group	
	AG I	AG II	SC I	SC II
MOT (%)	77.12 ± 2.85	79.49 ± 2.83	77.12 ± 2.85	82.10 ± 2.10
VSL (µ/s)	29.40 ± 3.68	27.85 ± 3.66	28.31 ± 1.70	28.73 ± 1.54
VCL(µ/s)	107.94 ± 4.92	104.88 ± 4.89	112.26 ± 6.86	110.0 1 ±2.96
LIN (%)	27.78 ± 1.68^{b}	37.04 ± 3.27^{a}	27.76 ± 1.67^{b}	37.04 ± 3.22^{a}
ALH (µ)	6.36 ± 0.37	6.56 ± 0.37	6.34 ± 0.37	6.58 ± 0.35
VAP (µ/s)	60.59 ± 2.80	62.76 ± 2.76	62.91 ± 2.79	60.44 ± 2.68

Within age or SC group row values bearing different superscripts are statistically significant.

A brief description of CASA motion parameters

Percent motility (MOT %)

The number of motile cells divided by the number of cells analyzed, expressed as a percent. Here, for every analysis, a total of 200 cells were analyzed. A cell was considered motile if its average straight-line speed (VSL) met or exceeded the minimum motile speed parameter.

Straight line speed (VSL)

This is defined as the average velocity measured in a straight line from the beginning to the end of the track. It is a measure of the cell's foreword progression and is computed by multiplying the curvilinear velocity (VCL) times the mean linearity (divided by 100). This measure is computed as the average for all motile cells. This has been adapted from the manual method of calculating the speed of a cell or group of cells.

Curvilinear velocity (VCL)

This is computed as the average scalar velocity (or speed) for all motile paths. It is calculated by computing the total distance travelled along each path and dividing by the time interval. The population VCL is computed only for motile cells (these with an average VSL > threshold speed), and is achieved by averaging the mean values from each individual cell.

Mean linearity (LIN)

The distance a cell travels along its normal (or un-smoothed) path is referred to as its gross displacement. The straight-line distance from its straight point to its current X-Y position (as the crow flies) is referred to as net displacement. The ratio of these two measures (time 100) is the linearity measure. It is evaluated at the end of each of the motile paths, and all of the motile path values are averaged to form the single number for the report. A cell that swam in a straight line has a value of 100; a cell that had just completed a circle had an instantaneous value of zero.

Lateral head displacement (ALH)

For each cell, the distance between the actual curvilinear path and the smoothed (or average) path is computed. These values are sometimes referred to as RISERS. This measure computed twice the maximum value of the RISER for each motile path, and then computed as the average value of all of the individual maxima as the single value to include in the report.

Velocity of the average path (VAP)

This is defined as the average velocity over the smoothed cell path. This parameter is used to characterize the overall trajectory of the sperm cell.

Statistical analysis

Data on motility of spermatozoa using subjective judgement as well as objective evaluation of CASA motion parameters were subjected to analysis of variance (ANOVA). All data from the experiment were analysed using the General linear model (GLM) procedure of SAS (SAS Institute, 2000) with the following model:

$$Y_{ijk} = \mu + A_i + S_j + e_{ijk}$$

Where, µ = overall mean, A_i = fixed effect of age groups; S_j = fixed effect of Scrotal circumference groups, e_{ijk} = random error effect.
The average scrotal circumference, 33 cm and the average age, 50 months of the experimental bulls were used to divide the group into two SC groups and two age groups, respectively. Significance was declared at $P \leq 0.05$ and a trend at $0.05 < 0.10$, unless otherwise stated. When a significant F-test was detected, multiple comparisons were done using a Turkey's adjustment for the probability.

RESULTS AND DISCUSSION

CASA motion parameters in frozen semen

The overall mean percent motility of fresh semen from Sahiwal bulls in different ages and scrotal circumferences (SC) was 78.49 ± 17.27% (Table 1). Though there is a trend which indicated higher percent motility in older (79.49 ± 2.83) than younger bulls (77.12 ± 2.85) and in larger SC (82.10 ± 2.10) than smaller SC (77.12 ± 2.85) group the difference was not significant ($p > 0.05$). In contrary, Ulfina et al. (2005) reported significantly higher mass motility for younger than the older age groups for indigenous Ethiopian Horro cattle breed. However,

Table 2. Least square means ± SE of CASA parameters in Frozen-thawed Sahiwal bulls semen in different freezing time and age group.

Parameter	Thawing time interval			
	After freezing at 0 h		After freezing at 24 h	
	AG I	AG II	AG I	AG II
MOT (%)	70.83 ± 3.36	66.70 ± 3.94	76.40 ± 3.07[a]	65.00 ± 3.50[b]
VSL (µ/s)	24.60 ± 3.68	30.41 ± 3.34	25.20 ± 4.36	32.18 ± 2.91
VCL(µ/s)	94.45 ± 5.66	90.72 ± 3.07	94.90 ± 15.31	97.06 ± 2.74
LIN (%)	26.98 ± 6.4[b]	40.64 ± 5.40[a]	28.86 ± 6.38[b]	41.20 ± 4.67[a]
ALH (µ)	5.28 ± 0.47	5.83 ± 0.38	4.94 ± 0.70	6.24 ± 0.33
VAP (µ/s)	58.26 ± 6.00	58.30 ± 3.64	53.22 ± 6.67	54.86 ± 1.75

Within age or SC group row values bearing different superscripts are statistically significant

Keshava (1996) reported significantly lower (65.22%) mean motility than the current study for the same breed of bulls but with high variability within the range of 39.4 for Frieswal to 86.2% in Karan Fries (KF) crossbred dairy bulls. Table 1 depicts the least square means ± SE of VSL, VCL, LIN, ALH and VAP for the two ages and SC groups of Sahiwal bulls. The mean straight-line velocity (VSL) as measured by CASA was 28.54 ± 8.28 µ/s. There was no significant variation in straight-line speed between age and SC groups. The trend shows higher VSL with the advancement of age and larger scrotal circumference. Keshava (1996) also observed similar trends in KF crossbred bulls and slightly higher values for Sahiwal. The higher mean values of curvilinear velocity (µ/s) for younger bulls (AGI, 107.94 ± 4.92 vs AGII, 104.88 ± 4.89) and for smaller SC (SCI, 112.26 ± 6.86 vs SCII, 110.01 ± 2.96) were not different ($p > 0.05$). But the mean linearity between bulls of different age (AGI, 27.78±1.68 vs. AGII, 37.04 ± 3.27) and SC(SCI, 27.76 ± 1.67 vs SCII, 37.04 ± 3.22) groups were significantly different ($p < 0.05$). Linearity of spermatozoa, which was reported (Christensen et al., 2005) to have strong correlation with non-return rate, is one of the main interests of this study. In agreement to this finding, Farrell et al. (1998) reported highly significant correlations (0.99) between bull fertility, 59 day non-return rate to first service, and CASA motility parameters. The slight increase with older age groups and larger SC in amplitude of Lateral Head Displacement (ALH µ) were not significant ($p > 0.05$). The overall mean value of VAP was 61.62 ± 15.07 µ/s. The difference between bulls of different age and SC were not significant. Similarly, Keshava (1996) reported mean value of 62.28 µ/s for the same breed of bulls, but lower mean values in Karan Fries crossbred bulls (50.22 µ/s).

CASA motion parameters in frozen semen

The mean post-thaw motility of Sahiwal bull spermatozoa was 69.62 ± 14.23 and 65.61 ± 11.13 at 0 and 24 h after freezing, respectively. Similar reports were available (Keshava, 1996) for different breeds of cattle. In contrary, Muhammad et al. (2010) reported lower post thaw percentage of Sperm motility of Sahiwal bull epididymal spermatozoa at 0 (50.6 ± 1.5), 2 (33.8 ± 0.9) and 4 (18.1 ± 1.3) h post-thaw, which might be attributed to less matured epididymal spermatozoa in the latter. Raina (1999) also reported lower values (ranging from 43.00 ± 6.25 to 62.57 ± 4.59) than the current results using different freezing rates in buffalo semen. Similar to fresh semen percent motility discussed above, there was no significant difference in post thaw percent motility of spermatozoa either between age or SC groups after 0 h freezing. But after 24 h freezing, the percent post-thaw motility of spermatozoa in younger bulls (AGI, 76.40 ± 3.07) and in larger SC (56.56 ± 3.78) were significantly ($P < 0.01$) higher than in older bulls (AGII, 65.00 ± 3.50) and smaller SC (56.56 ± 3.78) group. This could probably indicate the significance of age in freezability of spermatozoa. However, further study which accommodate more number of bulls with more age variation as well as in longer freezing periods than the current study are warranted.

Least square means ± SE of VSL, VCL, LIN, ALH and AVP for the two age and SC groups are presented in Tables 2 and 3. The overall mean of VSL was 28.75 ± 12.04 at 0 h after freezing and 30.24 ± 10.52 at 24 h after freezing. There was increasing trends as age advances and for larger SC bulls at the two test hours. Similar reports were available for KF crossbred bulls (25.95 ± 0.68 µ/s) (Keshava, 1996) and Murrah buffalo spermatozoa (26.76 ± 1.58 to 33.74 ± 2.10) (Raina, 1999). The mean curvilinear Velocity (VCL µ/s) of post-thaw spermatozoa at 0 h after freezing was higher for younger bulls (AGI, 94.45 ± 5.66 vs AGII, 90.72 ± 3.07) and for smaller SC (SCI, 94.48 ± 6.86 vs SCII, 91.32 ± 2.94). After 24 h freezing it was higher for older bulls (AGI, 94.90 ± 15.31 vs AGII, 97.06 ± 2.74) and for larger SC bulls (SCI, 91.32 ± 2.94 vs SCII, 95.02 ± 2.73). But there was no significant difference $P > 0.05$) after either of the two freezing time. The current results are at par with Raina (1999) who reported a mean VCL ranging

Table 3. Least square means ± SE of CASA parameters in frozen thawed Sahiwal bulls semen in different freezing time and SC group.

Parameter	Thawing time interval			
	After freezing at 0 h		After freezing at 24 h	
	SC I	SC II	SC I	SC II
MOT (%)	66.56 ± 4.04	67.56 ± 3.78	56.56 ± 3.78[b]	65.56 ± 3.78[a]
VSL (µ/s)	30.18 ± 3.13	30.18 ± 3.13	25.20 ± 4.36	31.17 ± 2.91
VCL(µ/s)	94.48 ± 6.86	91.32 ± 2.94	91.32 ± 2.94	95.02 ± 2.73
LIN (%)	28.40 ± 7.65[b]	39.34 ± 5.22[a]	28.86 ± 6.38[b]	40.20 ± 4.65[a]
ALH (µ)	5.22 ± 0.57	5.81 ± 0.36	4.85 ± 0.71	6.24 ± 0.33
VAP (µ/s)	54.72 ± 5.93	59.40 ± 3.56	53.22 ± 6.67	57.86 ± 1.77

Within age or SC group row values bearing different superscripts are statistically significant

from 92.90 ± 8.59 to 126.67± 9.21 for post-thawed buffalo spermatozoa frozen at various freezing rates. Keshava (1996) reported also similar result (87.10 ± 4.08) in KF bulls. The overall mean linearity was 33.74 ± 2.22 percent at 0 h after freezing and 34.82 ± 1.52% after 24 h of freezing in Sahiwal bulls. The higher percent linearity for older age group as well as larger scrotal circumference was significantly different ($P < 0.05$). Similar values within the range of 23.87 ± 2.12 to 34.74 ± 3.31 were reported (Raina, 1999) in linearity of post-thawed buffalo spermatozoa frozen at various freezing rates. Keshava (1996) also reported similar result (34.48 ± 2.48) in KF crossbred bulls. The mean average of ALH (µ) for post-thawed frozen semen were 5.68 ± 0.40 at 0 hr after freezing and 5.88 ± 0.92 at 24 h after freezing in Sahiwal bulls. Higher values than in the current study have been reported (Keshava, 1996; Raina, 1999). The difference in ALH either in age or SC groups of post-thaw frozen semen did not reach statistically significant level ($p > 0.05$). Overall mean of VAP (µ/s) for post-thawed frozen Sahiwal bull spermatozoa were 58.29 ± 13.85 at 0 h after freezing and 56.58 ± 9.26 at 24 h after freezing. The present result is in agreement with Keshava (1996) who reported 52.03 ± 1.93 for KF bulls and Raina (1999) who reported a VAP values ranging from 58.82 ± 3.03 to 73.32 ± 5.12 for Murrah buffalo bulls.

Conclusions

Based on the results, it is concluded that significantly high sperm kinetic characteristics of CASA, especially higher sperm linearity is recorded for bulls with larger SC which may indicate the possibility of including this sperm parameter in routine evaluation of bulls for better judgement of bulls for fertility and also the probability of culling bulls based on testicular size, especially at an early age without spending money, space and time on rearing of such inferior bulls. Moreover, this study also clearly indicated that CASA is a good supplementation to aid genetic selection in breeding bulls. Nonetheless, further study which could encompass different age groups as well as longer freezing periods than the current study is worth to mention.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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