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Article

Motion characteristics and plasma integrity evaluation of Murrah buffalo semen

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Abstract: Buffalo semen collected from Murrah bull were cryopreserved and evaluated for different motility parameter, kinematics and plasma membrane integrity. Buffalo bulls were maintained uniform standard management and nutritional practices. Semen was collected regularly twice a week semen collection schedule from four (04) Murrah bull. Collected semen was immediately transported to laboratory and evaluated for different macroscopic parameter (color, volume and thickness). Fresh semen was then diluted with saline solution and evaluated for sperm concentration, motility, sperm kinematics and morphology. Semen samples that fill all the standard were selected for freezing and diluted with Tris-egg yolk citrate diluter. Diluted semen was equilibrated, cryopreserved and finally evaluated for post thaw sperm quality. Different motility parameter (total, progressive, static and slow motility) varied significantly ($p<0.01$) irrespective of different freezing stages. Significantly higher progressive sperm motility and viability of buffalo spermatozoa were observed at fresh semen whereas lower progressive sperm motility and viability was found at post thaw stage. Total and progressive motility reduced by 2.5 and 2.12% following equilibration, whereas following cryopreservation, total and progressive motility reduced by 35.7 and 28.51% and static motility increases accordingly (35.4%). Significantly higher plasma membrane integrity of sperm was observed at fresh semen followed by pre freeze and post thaw semen. Following freezing, integrity of plasma membrane reduces at the rate of 10.81% and 26.7% at pre freezing and post thaw stages. Significantly higher average path velocity (VAP), straight line velocity (VSL), curvilinear velocity (VCL), straightness (STR), amplitude of lateral head displacement (ALH) and beat cross frequency (BCF) were found for fresh semen followed by pre-freeze and post-thaw semen. Frozen buffalo semen with higher progressive motility and motion characteristics may be produced if motility losses can be reduced during freezing stage as this stage results higher motility losses.

Keywords: buffalo; semen; motility; cryopreservation; kinematics; CASA

1. Introduction

Artificial insemination (AI) is considered is one of the potential and widely used assisted reproductive technologies to disseminate and faster multiplication of high genetic potentiality and to produce more milk form cattle and buffalo species. Although AI in buffalo is not as popular as cattle in Bangladesh but its popularity and demand is increasing day by day. Government is also taken different initiatives in different level to make this technology more popular and viable among the farmers. But to make this technology more viable we have to ensure best quality frozen semen with acceptable conception rate since, AI allows semen from one bull to inseminate thousands of female. Furthermore, bulls have greater effects on herd genetics and production performances.

In order to produce quality frozen semen, the emphasis must be given on fresh semen production and evaluation technique. Established conventional semen analysis technique are effective to some extend but they are known to have some limitations as they are unable to detect different sperm functional impairments, which are responsible for a decreased fertility (Aitken, 2006).This conventional system is relatively inaccurate, imprecise and time consuming (Christensen *et al*., 2005). Whereas, computer assisted sperm analyzer (CASA) is a quite faster, precise and useful tool for identifying differences in sperm parameters, related to motility, velocity and morphology, and it avoids subjective errors (Johnson *et al*., 1996). Hence now-a-days, there is an increasing trend and interest in evaluating sperm motion characteristics by CASA (Amann and Waberski 2014; Rodriguez-Montaña and Roa-Guerrero, 2017). The kinematic values determined for each spermatozoon cover the velocity of movement, viz., average path velocity (VAP), curvilinear velocity (VCL) and straight line velocity (VSL), the width of the sperm head's trajectory and frequency of the change in direction of the sperm head (Mortimer *et al*., 1990) and thus provide quantitative assessment of sperms.

Furthermore, fresh spermatozoa undergoes a series of changes during cryopreservation. During this process the motility and different kinematic parameters of the spermatozoa losses in dilution, equilibration, freezing and storage stage. Plasma membrane integrity is another important parameter that determines whether the sperm is biochemically and functionally active during this freezing process. Understanding the losses in each stage might help to minimize the motility losses and to identify the major stages of quality reduction and thus to conduct research to minimize this loss and to produce good quality frozen semen. Hence, this study was planned to evaluate and compare the motion characteristic, morphology and plasma membrane integrity of buffalo bull semen at different stages of freezing using CASA.

2. Materials and Methods

2.1. Animal selection and management

Four breeding Murrah buffalo (*Bubalus bubalis*) bulls (age, 3-4 yr) were used for semen collection. Total forty ejaculates (10 ejaculates/bull) were collected in the present study.

The bulls were maintained uniform standard nutritional and managerial practices at the Buffalo research farm of Bangladesh Livestock Research Institute, Savar, Dhaka, Bangladesh. The bulls were regularly dewormed and vaccinated against the common diseases.

The bulls were kept in intensive housing system and were fed green fodder (Napier, german and maize) concentrates, mineral mixture and ad libitum drinking water.

2.2. Semen collection and evaluation

Semen was collected regularly twice a week semen collection schedule using an artificial vagina (Walton, 1945). Semen collection were made in the early morning between 6.0 h to 6.30 h. Collected ejaculates were immediately transferred in to a water bath at 37°C and evaluated for volume, color and consistency.

2.3. Motility assessment

Motility, morphology, concentration and kinematics were measured using a Computer Assisted Sperm Analyzer (CASA) (Hamilton Throne, IVOS II). Briefly, the semen sample was diluted with pre-warmed tris buffer to give a sperm concentration of 2 - 6 x 10^6 spermatozoa/ml. The CASA software settings for recording sperm motility were set as; Frame rate 60Hz, Frames acquired 30, Minimum contrast 35, Minimum cell size, 5 pixels, Cell size, 9 pixels, Cell intensity 110 pixels, Path velocity (VAP) 50 μm/s, Straightness (STR) 70%, VAP cut-off, 30 μ/s and VSL cut-off 15 μ/s. In a prewarmed (38°C) Leja® 4 chamber slide (depth 20 μm), 1 μl prepared semen sample was loaded and analyzed for sperm motility characteristics. For each sample, five optical fields around the central reticulum of the chamber were used to count spermatozoa. The motion characteristics recorded were total motility (%), progressive motility (%), static motility (%), slow 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, %), linearity (LIN, %) of spermatozoa.

2.4. Dilution and equilibration

Fresh semen was diluted in tris-egg yolk citrate extender containing tris (3.03% w/v), citric acid (1.68 %w/v), fructose (1.2% w/v), egg yolk (20% v/v), glycerol (6.4% v/v), and streptomycin (660 μg/ml) to make a final concentration of 80x10^6 spermatozoa/ml.

2.5. Freezing protocol

After mixing with the extenders, diluted semen was placed in a cold handling cabinet (Minitube, Germany) for 4hr at 4°C for equilibration. The semen was diluted with the extender to give a sperm concentration of 20 million/dose. The semen samples were filled and sealed in standard printed straws (0.25 ml) using an automated filling sealing machine (MPP Uno, Minitube, Germany). After equilibration, freezing of straws was carried out in liquid nitrogen (LN_2) vapor using a programmable bio-freezer (Turbo Freezer M, Minitube, Germany). The straws were then plunged in LN_2 (−196°C) for overnight storage.

2.6. Post Thaw evaluation of semen

Semen straws were thawed after 24 hr of storage in a water bath at 37°C for 30 sec. The post thawed semen was evaluated for sperm motility, kinetics and membrane integrity. For analysis, semen samples were washed twice in tris buffer to remove seminal plasma and extender. About 1 µl prepared semen sample was loaded on Leja® 4 chamber slide (depth 20 µm) and analyzed for sperm motility characteristics. For each sample, five optical fields around the central reticulum of the chamber were used to count spermatozoa.

2.7. Hypoosmotic swelling (HOS) test

The plasma membrane integrity of spermatozoa was assessed using a HOS test employing 150 mOs/L solution of sodium citrate and fructose with 30 min of incubation at 37°C. At the end of incubation, 5 µl of formalized eosin solution (10%) was added in order to stop the reaction, and stain and fix the sperm cells. The wet preparations of semen were then evaluated using a phase contrast microscope (×40). Nearly 200 spermatozoa were assessed from different fields for each trait and the spermatozoa with various forms of morphological defects, acrosome defects, and swollen coiled tail were counted and expressed as percentage.

2.8. Statistical analysis

Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) (20.0). One-way ANOVA followed by Duncan multiple range post-test was used to assess differences among mean of different stages of cryopreservation on sperm motion characteristics and plasma membrane integrity. P value ($p<0.05$) was considered as statistically significant.

3. Results and Discussion

3.1. Macroscopic evaluation

Buffalo bull (Murrah) produced semen of creamy color with thick creamy consistency. The mean volume and concentration of semen was found 3.09±0.10 ml and 2787.84 ± 139.19 million/ml respectively.

3.2. Motility evaluation

Buffalo semen cryopreservation was carried out with Tris-egg yolk citrate extender. The mean percentage of total, progressive, static and slow motility of fresh, pre- freeze and post thawed buffalo semen are presented in Table 1. Different motility parameter (total, progressive, static and slow motility) varied significantly ($p<0.01$) irrespective of different freezing stages. In general, significantly higher progressive sperm motility of buffalo spermatozoa (Figure 1) were observed in fresh semen (68.99%) whereas lower progressive sperm motility was found at post thaw stage (40.48%). Following 4 hrs of equilibration total and progressive motility reduced by 2.5 and 2.12% respectively and static motility increased simultaneously. Whereas following cryopreservation, total and progressive motility reduced by 35.7 and 28.51% from fresh semen and static motility increases accordingly (35.4%). With the progression of freezing slow motility at both pre freezing and freezing stages increases by 1.35 and 2.69% respectively. After freezing and thawing of buffalo semen total motility reduction was found 49% (Rasul *et al.*, 2001).

Table 1. Motility parameters (Mean± SE) of buffalo spermatozoa based on freezing stage.

Motility Parameter (%)	Fresh	Pre freeze	Post thaw
Total motility	86.40^{a}±1.26	83.90^{a}±1.26	50.70^{b}±1.08
Progressive motility	68.99^{a}±1.77	66.87^{a}±1.78	40.48^{b}±1.06
Static motility	13.90^{a}±1.28	16.10^{a}±11.26	49.30^{b}±1.06
Slow motility	1.71^{a}±0.34	3.06^{b}±0.38	4.40^{c}±0.41

Mean values in the same row with different superscripts (a, b, c) differ significantly ($P<0.01$)

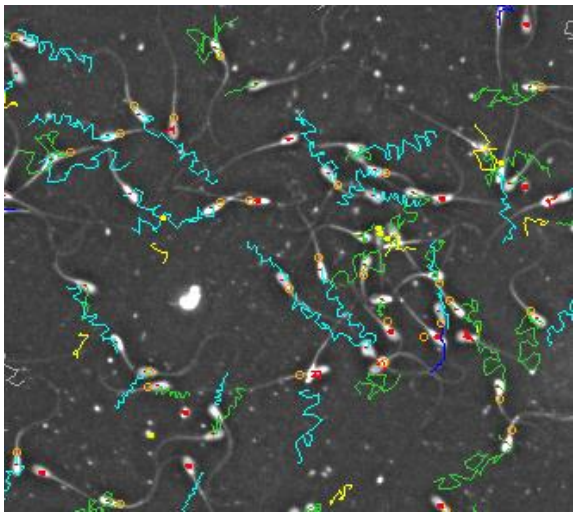

Figure 1. Motility of Murrrah bull sperm measured with CASA (Aqua color: Progressive sperm, Green color: Motile sperm, Red color: Static sperm).

In cattle, the reduction in total motility was about 9% after cryopreservation (Thomas *et al.*, 1998). The reduction of total and progressive motility in frozen thawed semen may be due to adverse effect of cryopreservation. Others reports also observed significantly higher motile, viable and morphologically normal sperms in fresh buffalo semen than cryopreserved semen diluted with conventional tris-egg yolk citrate (Singh *et al.* 2012, 2013). Pre-freeze total (86.40 ± 1.26) and progressive motility (68.99 ± 1.77) and post-thaw total (50.70 ± 1.08) and progressive motility (40.48 ± 1.06) in case of Tris-egg yolk citrate extender are comparable with the findings of Kumar *et al.* (2016), Chaudhari *et al.* (2015), Akhter *et al.* (2011) and Sing *et al.* (2013).

3.3. Morphology of Murrah bull semen

Different morphological deformities of buffalo sperm are represented in Table 2. Most common deformities were bent tail, coiled tail, Distal Midpiece Reflex (DMR), proximal droplet and distal droplet. Among tail deformities, Murrah buffalo sperm has higher bent tail percentage (2.59%). Overall spermatozoa abnormality in the Murrah buffalo bulls was 39.68 percent (Table 2). Basically two types of droplet deformities are found, one is proximal deformities and another is distal deformities. In the present study, proximal deformities was found higher than distal deformities that indicates that the studied bull was comparatively young, as proximal deformities is more prominent in younger bull. Anilkumar *et al*. (2017) found 30.75% overall spermatozoal abnormality percentage in the Toda buffalo bulls. Several studies (Nordin *et al*., 1990 and Koonjaenak *et al*., 2007) has shown that spermatozoal abnormality in buffaloes used for AI should not exceed 15 percent and a healthy buffalo should not have >10% of tail defects. In the present study the spermatozoal abnormalities were higher but tail deformities was within the range. Moreover, the age of buffalo bull had a significant effect on the incidence of total pathological head shapes, acrosome defects, proximal cytoplasmic droplets, and total tail defects that similar to early reported in buffalo bull (Pant 2000).

Table 2. Morphological parameters (Mean± SE) of fresh buffalo spermatozoa.

Morphology	Fresh (Mean± SE)
Bent tail (%)	2.59± 0.41
Coiled tail (%)	0.22 ±0.58
DMR (%)	1.19± 0.17
Distal droplet (%)	1.94± 0.26
Proximal droplet (%)	33.74± 6.57
Normal fraction (%)	60.32± 6.27

3.4. Plasma Membrane integrity

Sperm plasma membrane integrity was measured by hypoosmotic swelling (HOS) test and presented in Figure 2. The HOS reactive sperm is a clear indication of plasma membrane integrity of sperm as well an indication that sperm is biochemically and functionally active. Significantly higher plasma membrane integrity of sperm was observed at fresh semen. Following freezing, integrity of plasma membrane reduces at the rate of 10.81% and 26.7% at pre freezing and post thaw stages (Figure 2). At pre-freeze and post-thaw stages, sperm plasma membrane integrity was lower compared to fresh stage. The higher plasma membrane integrity indicates the higher functionally active sperm. Plasma membrane integrity observed this study are comparable with the results obtained by Akhter *et al*. (2011), Chaudhari *et al.* (2015) and Nayan *et al.* (2020).

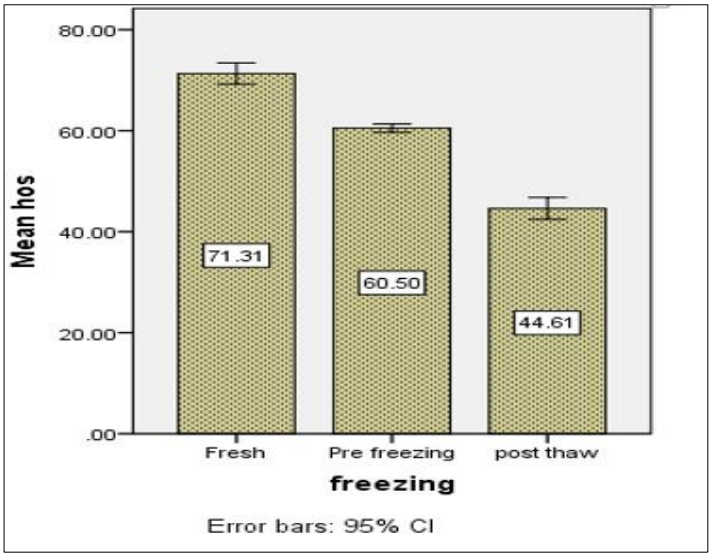

Figure 2. Plasma membrane integrity of Murrah semen at different stages of freezing.

3.5. Sperm kinematics

The CASA analysis of fresh, pre-freeze and post thaw semen samples depicted various velocity and kinematics parameters. The mean ± SE values of these traits are presented in Table 3. There is a significant variation among different kinematic properties of Murrah bull irrespective of freezing stages. Significantly higher average path velocity (VAP), straight line velocity (VSL), curvilinear velocity (VCL), straightness (STR), amplitude of lateral head displacement (ALH) and beat cross frequency (BCF) were found for fresh semen followed by pre-freeze and post-thaw semen. Our results in terms of values were in accordance with the reports of Mandal *et al*. (2003), Patel *et al*. (2012) and Patel and Dhami (2016). However, the ratings of velocity and kinematics traits varied among different studies due to variation in the breed/species of bulls, initial semen quality, and software and model of CASA machines used with or without Leja slides in the assessment. Similar results due to freezing-thawing were reported in buffalo (Rasul *et al*., 2001). The reduction in VCL in frozen-thawed semen could be due to cryoinjuries to mitochondrial apparatus and axoneme of spermatozoa (Jones and Stewart, 1979 and Courtens *et al*., 1989). This suggested that buffalo sperm mitochondria and axoneme are more sensitive to cryopreservation. Goovaerts *et al*. (2006) studied quality of fresh bull sperm conventionally and by HamiltonThorne CASA and reported the values of motility (total & progressive), straight line velocity, curvilinear velocity, straightness and lateral head displacement as 79.9 & 58.4 per cent, 98.3 µm/ sec, 156.4 ìm/s, 84.5 per cent, and 5.0 µ, respectively. Hoûack *et al*. (2007) using CASA found lower quality of Belgian Blue bull sperms as compared to those of HF bulls. Patel *et al*. (2012) reported significantly ($p<0.05$) higher mean values of motile and progressively motile sperm, average path velocity and BCF in fresh semen of Jafarabadi and Mehsana buffalo bulls than in crossbred bulls, but the other Hamilton CASA traits did not differ between them. Our findings in terms of all motion characteristics of freshly diluted buffalo sperms concurred well with the recent report of Kumar *et al*. (2018) using similar Biovis CASA, suggesting that this simple CASA can give reliable and useful information more accurately than visual assessment and may serve the purpose of economically assessing the sperm kinematics on bovine semen stations.

Table 3. Kinematic parameters (Mean±SE) of Murrah bull semen at different freezing stages.

Freezing stage	Fresh	Pre-freeze	Post thaw
VAP (μm/s)	113.89^{a}±6.40	110.15^{a}±7.46	81.62^{b}±6.37
VSL (μm/s)	100.49^{a}±3.67	85.57^{b}±5.36	68.58^{c}±5.78
VCL (μm/s)	167.16^{a}±4.92	154.12^{a}±6.93	127.87^{b}±7.05
STR (%)	87.48^{a}±5.09	76.42^{ab}±6.78	64.57^{b}±7.59
LIN (%)	49.54±11.56	42.75±11.07	45.89±10.78
ALH (Mm)	7.71^{a}±0.25	7.14^{a}±0.31	6.26^{b}±0.36
BCF (Hz)	32.29^{a}±0.65	29.28^{b}±0.71	23.53^{c}±0.73

VAP: average path velocity, VSL: straight line velocity, VCL: curvilinear velocity, STR: straightness, LIN: linearity ALH: Amplitude of lateral head displacement and BCF: beat cross frequency.
Mean values in the same row with different superscripts (a, b, c) differ significantly (P<0.01 or P<0.05)

4. Conclusions

Semen evaluation with CASA may provide more precise information of semen quality and plasma membrane integrity of buffalo semen irrespective of different freezing stages. Significant percentage of motility, kinematics and plasma membrane integrity reduced at freezing stages than equilibration stages. Research needs to carry out to optimize the motility through reducing motility losses during freezing stages.

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Data availability

All relevant data are within the manuscript.

Conflict of interest

None to declare.

Authors' contributions

Md. Faizul Hossain Miraz and Gautam Kumar Deb were involved in conceptualization, design, experiment, formal analysis, writing - original draft, review and editing and project administration. SM Jahangir Hossain and Shahrina Akter contributed to writing - original draft, review and editing. All authors have read and approved the final manuscript.

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