ASSESSMENT OF POST-THAW SEMEN QUALITY OF BUFFALO AND SAHIWAL BULLS USING NEW SEMEN ASSAYS

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ABSTRACT

Different semen assays were employed to determine the post-thaw fitness of buffalo and Sahiwal bull spermatozoa. Semen (two ejaculates) was collected from buffalo and Sahiwal bulls (six each) once a week for three consecutive weeks. Semen was diluted in lactose-fructose-egg yolk glycerol extender, equilibrated at 4°C for 4 hr and frozen in liquid nitrogen vapors. A highly significant difference between fresh and post-thaw motility of both buffalo and Sahiwal bulls was observed ($P < 0.001$). Post-thaw percentage of swollen spermatozoa and normal acrosomes were 24 ± 9.4 and 27.3 ± 12.2 in buffalo bulls and 23.4 ± 9.5 and 26.4 ± 10.4 in Sahiwal bulls, respectively. The respective percentages of buffalo and Sahiwal bull spermatozoa involved in head to head agglutination were 12.1 ± 6.7 and 12.4 ± 6.7 at 0 hr and 25.6 ± 10.8 and 28.5 ± 7.2 at 2 hr. Fluorescent microscopy revealed that buffalo and Sahiwal bulls had 22.8 ± 9.1 and 20.1 ± 8.3 per cent spermatozoa with intact, 8.2 ± 1.6 and 11.6 ± 4.4 per cent partially damaged, and 68.8 ± 9.6 and 68.5 ± 10.4 per cent completely damaged plasma membrane. After thawing, there was no significant difference between buffalo and Sahiwal bulls in all semen characteristics. The significant relationships between various semen assays indicate their validity in evaluation of buffalo and Sahiwal bull semen.

INTRODUCTION

Laboratory assessment of frozen spermatozoa is one of the major concerns in semen research and artificial insemination. Inadequate accuracy in measuring the semen quality in the laboratory is one of the major problems (Graham and Crabo, 1978). The probability of fertilization is governed by the total concentration, the progressive motility, the morphology and half-life of spermatozoa (VanDuijn, 1964). The increase of abnormal spermatozoa in semen samples has been associated with infertility (Hancock, 1959; Saacke *et al.*, 1980).

The common semen assays include motility, velocity, penetration of cervical mucus, metabolic activity, cell contents, ability to agglutinate (head to head) in the presence of blood serum, structural integrity (cell membrane and acrosomes) and the ability to pass through Sephadex glass wool filter (Saacke, 1984). Unfortunately, no single physical or biochemical measurement can represent all the semen characteristics necessary for successful fertility. Therefore, it is recommended to employ more than one semen assays, instead of a single test, to predict the quality of frozen semen (Hirao, 1975; Linford *et al.*, 1976).

Main objective of this study was to assess the quality of frozen semen of buffalo and Sahiwal bulls using new assays.

MATERIALS AND METHODS

Semen from Nili-Ravi buffalo and Sahiwal cow bulls (six each) maintained under uniform conditions of nutrition and management was collected once a week for three consecutive weeks. Prior to semen collection, animals were sexually stimulated by 10 min restraint in the collection yard and one false mount. Two ejaculates were collected from each bull by means of prewarmed artificial vagina (42°C) with an interval of 15 min. Each ejaculate was evaluated for volume, concentration and motility as per routine. The ejaculates from same bull were pooled and diluted in semen extender containing lactose (5 % w/v), fructose (3% w/v), egg yolk (20 per cent v/v), glycerol (7 per cent v/v), benzyl penicillin 1000 i.u./ml) and streptomycin sulfate (100 μg/ml) such that each ml contained 60×10^6 spermatozoa.

Extended semen was packaged in 0.5 ml French straws and cooled at the rate of 0.1°C/min to 4°C for at least 4 hr. After equilibration, the straws were placed 4 cm above the level of liquid nitrogen (LN2) for 10 min. The straws were then stored in LN2 (-196°C) for two weeks. Frozen straws from each bull were thawed in a water bath at 37°C for 15 sec. Post-thaw quality of

frozen semen was assessed using the following assays:

Motility (%)

A drop of semen was placed on a prewarmed glass slide (37°C) and covered with a coverslip. The sample was viewed using a phase contrast microscope. Motility of spermatozoa was obtained by observing four to six fields per slide.

Acrosome Morphology

A few drops of semen sample were added to 0.5 ml of a buffered saline solution (Na citrate dihydrate, 0.3675 gm; fructose, 0.6755 gm; distilled water, 50 ml) containing 1 % (v/v) formalin (Hancock, 1959). One drop of sample was taken on a clean slide and covered with a coverslip. The suspended fluid was allowed to settle for 1-2 min. to avoid the sperm movement. At least 200 spermatozoa were examined for normal acrosomes, using phase contrast microscopy. The percentage of spermatozoa with coiled tails were also recorded as a control in hypo-osmotic swelling assay.

Hypo-osmotic Swelling (HOS) Assay

An aliquot (100 μl) from each frozen semen sample was diluted in 100 μl of hypo-osmotic (OP = 150 mOsm/kg) sodium citrate solution. The mixture was incubated at 37°C for at least 30 min. A drop from the mixture was placed on prewarmed glass slide (37°C) and coverslipped. At least 200 spermatozoa were examined for percentage of swollen spermatozoa characterized by the typical coiled tail. The percentage of swollen spermatozoa was calculated after substracting the spermatozoa with coiled tails in control sample from the treated sample.

Head to Head Agglutination (HHA)

Immediately after thawing, cow or buffalo serum (20%, v/v) was added to semen of the respective species. Each sample was evaluated immediately after serum addition (0 hr) and after 2 hr incubation at 37°C. For HHA, a drop from mixture was placed on the prewarmed glass slide and covered with a coverslip. The percentage of spermatozoa engaged in head to head agglutination was recorded under oil immersion using a phase contrast microscope (Senger and Saacke, 1976).

Plasma Membrane Integrity

This assay was performed following procedures of Garner *et al*. (1986) and Harrison and Vickers (1990) with slight modifications. A stock solution of 6-carboxy-fluorescein-diacetate (CFDA; Calbiochem Boehring Corp., La Jolla, CA) was prepared by dissolving 4 mg/ml of dimethyl sulfoxide (DMSO). Propidium iodide (PI; Sigma Chemical Co., St. Louis, MO) was dissolved (0.27 mg/ml) in potassium phosphate buffer (Na_2HPO_4, 1.615 gm and K_2HPO_4, 5.75 gm in 100 ml of water; pH 7.0). Both stock solutions were kept in dark and stored at -18°C. Another stock solution was prepared by dissolving 2.5 mg formaldehyde per ml in iso-osmotic sodium citrate (3.4 gm/100 ml of water) and stored at 4°C. The ratio of commercial formaldehyde (37%) and sodium citrate was 1:148.

Semen was carefully collected in a glass vial containing 1 ml iso-osmotic (OP=300 mOsm/kg) sodium citrate solution and 50μl stock solution of formaldehyde, and centrifuged at 1000 rpm to remove seminal plasma and extender from thawed semen. The supernatant was discarded and the pellet was resuspended in 1 ml iso-osmotic sodium citrate solution. The staining solution was prepared in a glass tube by adding stock solutions of formaldehyde, CFDA (20 μl each) and PI (50 μl) in 1 ml iso-osmotic sodium citrate. The mixture was kept in dark at 37°C for at least 10 mins. A 5 μl aliquot of the stained suspension was placed on a clean slide and coverslipped. Random fields were observed under 100 magnification with epifluorescence illumination using the standard fluorescein isothiocyanate (FITC) filter set. For quantitative assessment of plasma membrane integrity, at least 200 spermatozoa were counted from each stained sample. The spermatozoa were classified as: intact (spermatozoa that fluoresced green throughout their length and excluded PI), partially damaged (similar to intact except that post-acrosomal region fluoresced red due to damaged plasma membrane allowing PI infiltration) or completely damaged (the whole head of spermatozoa was red due to infiltration of PI).

Statistical Analysis

This study was repeated six times on semen samples of buffalo and Sahiwal bulls to investigate the effect of bull and species. The data were subjected to analysis of variance using randomized complete block desigh (RCBD). Paired t-test was used to study the difference between fresh and post-thaw motility in both species and un-paired t-test was used to analyze the percent decline in motility after thawing.

The correlation coefficients were computed between various semen characteristics like fresh motility, post-thaw motility, acrosome morphology, hypo-osmotic swelling, head to head agglutination and intact plasma membrane in buffalo and Sahiwal bulls. All statistical

procedures were conducted following Steel and Torrie (1980).

RESULTS

Sperm Motility

Sperm motility in fresh ejaculates of buffalo and Sahiwal bulls averaged 68.6±7.8 and 65.5±5.6 per cent, respectively (Table 1). The relationship between fresh motility and other semen assays was non significant (Table 2).

Post-thaw motility was 24.4±10.5 per cent in buffalo and 23.9±8.8 per cent in Sahiwal bulls (Table 1). Paired t-test revealed a significant decline ($P<0.001$) in sperm motility during freezing and thawing processes in both species. In buffalo bulls, post-thaw motility was significantly correlated ($P<0.05$) with normal acrosomes, swollen spermatozoa, agglutinated spermtozoa (0 and 2 hr incubation) and spermatozoa with intact plasma membrane (Table 2). In Sahiwal bulls, post-thaw motility was significantly correlated ($P<0.05$) with the percentages of normal acrosomes, swollen spermatozoa, agglutinated spermatozoa (2 hr incubation) and spermtozoa with intact plasma membrane (Table 2).

Acrosome Morphology

The normal acrosomes averaged 27.3±12.2 per cent in buffalo bulls (Fig. 1) and 26.4±10.4 per cent in Sahiwal bulls (Table 1). In buffalo bulls, the percentage of normal acrosomes was significantly correlated with all semen assays ($P<0.05$; Table 2) except fresh motility. In Sahiwal bulls, the acrosome morphology was significantly correlated with percentages of swollen spermatozoa, agglutinated spermatozoa (2 hr incubation) and spermatozoa with intact plasma membrane ($P<0.05$; Table 2).

Hypo-osmotic Swelling Assay

The swollen spermatozoa in hypo-osmotic swelling solution averaged 24.0±9.4 per cnet in buffalo (Fig. 2) and 23.4±9.5 per cent in Sahiwal bulls (Table 1). In buffalo bulls, the relationships of swollen spermatozoa were significant with post-thaw motility and acrosome morphology ($P<0.05$) and non significant with other semen assays (Table 2). In Sahiwal bulls, the percentage of swollen spermatozoa was significantly correlated with post-thaw motility, acrosome morphology, agglutinated spermatozoa (2 hr incubation) and spermatozoa with intact plasma membrane ($P<0.05$; Table 2).

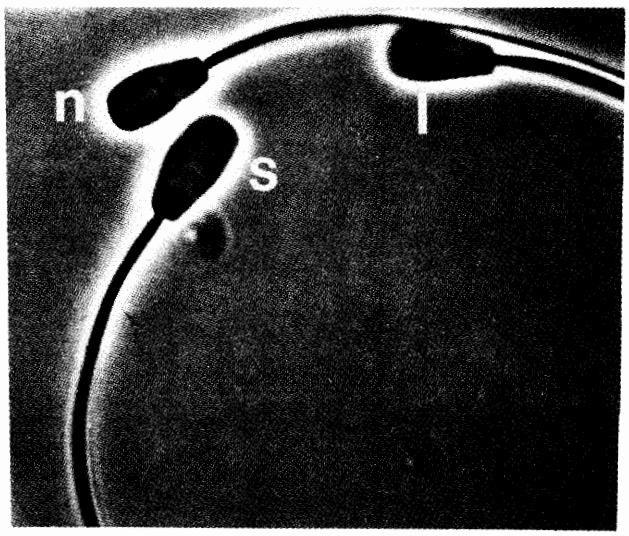

Fig. 1: Normal (n), swollen (s) and lifted (l) acrosomes of buffalo spermatozoa (x1000).

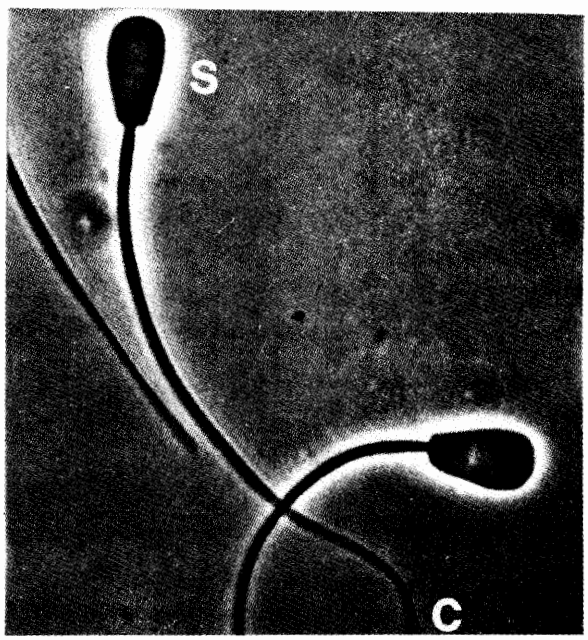

Fig. 2: Swollen spermatozoa (s) of buffalo bull characterized by coiling of tail (c) (x1000).

Head to Head Agglutination (HHA)

The agglutinated spermatozoa after treatment with buffalo/cow serum in buffalo and Sahiwal bulls were 12.1±6.7 and 12.4±6.7 per cent at 0 hr and 25.6±10.8 and 28.5±7.2 per cent at 2 hr incubation, respectively (Table 1). The difference in agglutinated spermatozoa due to species was non significant. In both species, the spermatozoa mostly agglutinated in doublets

and triplets. In buffalo bulls, HHA of spermatozoa at 0 hr and 2 hr incubation was significantly correlated with post-thaw motility and acrosome morphology ($P<0.05$; Table 2). In Sahiwal bulls, the HHA of spermatozoa (0 hr incubation) was significantly correlated with the percentage of spermatozoa with intact plasma membrane ($P<0.05$). The HHA of spermatozoa after 2 hr incubation was significantly correlated with post-thaw motility, acrosome morphology, swollen spermatozoa and spermatozoa with intact plasma membrane ($P<0.05$; Table 2).

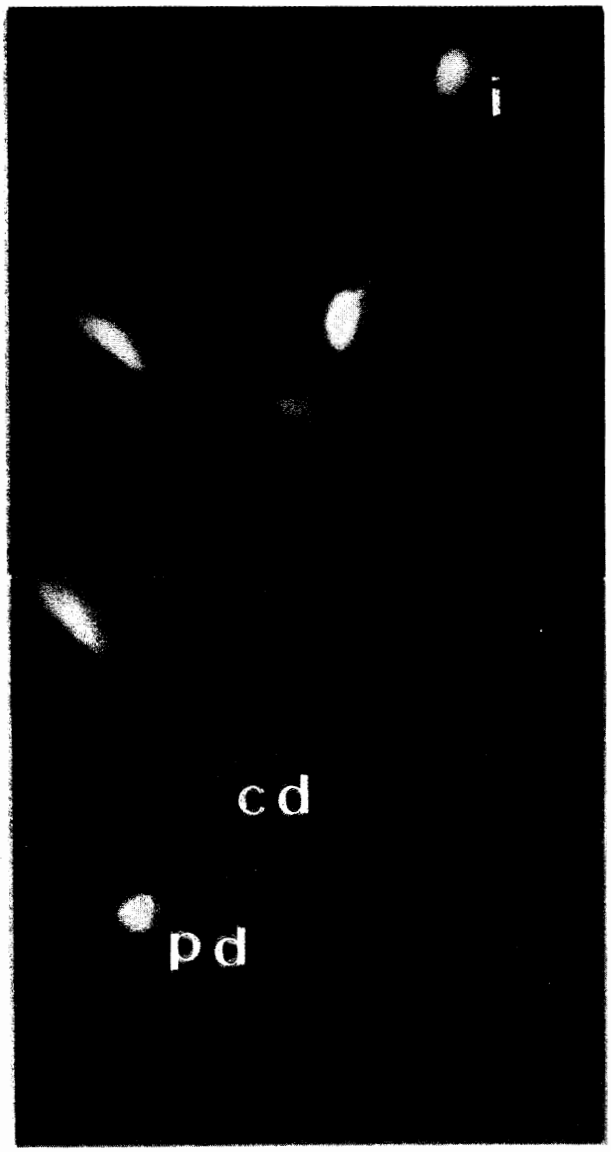

Fig. 3: Intact (i), partially damaged (pd) and completely damaged (cd) plasma membrane of buffalo spermatozoa (x400).

Plasma Membrane Integrity

Fluorescent staining revealed 22.8 ± 9.1 per cent intact, 8.2 ± 1.6 per cent partially damaged and 68.8 ± 9.6 per cent completely damaged plasma membrane in buffalo bull spermatozoa (Fig. 3) and 20.1 ± 8.3 per cent intact, 11.6 ± 4.4 per cent partially damaged and 68.5 ± 10.4 per cent completely damaged plasma membrane of Sahiwal bull spermatozoa. In buffalo bulls, the intact plasma membrane of spermatozoa was significantly correlated with post-thaw motility and normal acrosomes ($P<0.05$; Table 2). In Sahiwal bulls, the intact plasma membrane of spermatozoa was significantly correlated with post-thaw motility, normal acrosomes, swollen spermtozoa and agglutinated spermatozoa (0 hr and 2 hr incubation)($P<0.05$; Table 2).

In all semen assays, the differences due to species were non significant.

DISCUSSION

The aim of this study was to assess the frozen semen quality of buffalo and Sahiwal bulls using new assays. It is recommended that quality of post-thaw semen samples should be determined using multiple assays for their future usefulness and selection of a breeding bull.

Motility facilitates sperm transport through cervix (Wood, 1966) and sperm head penetration into ovum (Bedford, 1970). Several reports suggest a strong correlation between sperm motility and fertility (Linford *et al.*, 1976; Saacke *et al.*, 1980). In this study, sperm motility before freezing in both species ranged from 60 to 85 per cent and the decline in motility during freezing and thawing processes ranged from 36 to 90 per cent. The decline in motility is mainly due to freezing and thawing shocks that should be minimized to ensure the use of high quality frozen semen in the field. During feezing, the enzymes responsible for sperm motility leak from intracellular compartment into seminal plasma. This leakage is mainly due to weak plasma membrane of spermatozoa which is sensitive to dehydration and ice-crystal formation during freezing. This weakness of plasma membrane was confirmed when spermatozoa were stained with fluorescent markers used for membrane integrity.

The acrosome morphology is important in penetration and fertilization of the ovum (Bedford, 1970). A strong relationship between percentage of normal acrosomes and fertility has been documented (Saacke and White, 1972; Berndtson *et al.*, 1981). Saacke (1984) reported that acrosomal aberrations were

Table 1: Post-thaw semen characteristics (Mean±SD) of buffalo and Sahiwal bulls.

Semen characteristics (%)	Buffalo bulls	Sahiwal bulls
Fresh motility	68.6±7.8	65.5±5.6
Post thaw motility	24.4±10.5	23.9±8.8
Normal acrosomes	27.3±12.2	26.4±10.4
Swollen spermatozoa	24.0±9.4	23.4±9.5
Head to head agglutination 0 hr	12.1±6.7	12.4±6.7
2 hr	25.6±10.8	28.5±7.2
Intact membrane	22.8±9.1	20.1±8.3
Partially damaged membrane	8.2±1.6	11.6±4.4
Completely damaged membrane	68.8±9.6	68.5±10.4

In all semen characteristics, the difference due to species was non significant.

Table 2: Relationships between various post-thaw semen characteristics of buffalo and sahiwal bulls.

	1	2	3	4	5	6
2	0.204					
	-0.281					
3	0.087	0.978*				
	-0.346	**0.914***				
4	0.248	0.895*	0.889*			
	-0.329	**0.893***	**0.894***			
5	0.139	0.848*	0.794*	0.851*		
	-0.076	**0.434**	**0.283**	**0.27**		
6	0.293	0.928*	0.921*	0.884*	0.740*	
	-0.199	**0.733***	**0.821***	**0.684***	**0.405***	
7	0.149	0.989*	0.882*	0.750	0.728	0.903*
	-0.315	**0.864***	**0.718***	**0.680***	**0.566***	**0.623***

*P<0.05. Values in bold and italic face belong to Sahiwal bulls

1. Fresh motility; 2. Post-thaw motility; 3. Normal acrosomes; 4. Swollen spermatozoa;
5. Agglutinated spermatozoa, 0 hr; 6. Agglutinated spermatozoa, 2 hr; 7. Spermatozoa with intact plasma membrane

associated with decreased fertility or sterility in bulls. In this study, the acrosomal cap was found to be absent in most of spermatozoa. During freezing of spermatozoa, pronounced changes occur in the acrosomal region characterized by swelling and corrugation of the anterior part of the acrosome (Pursel *et al.*, 1972).

The intactness of outer plasma membrane is essential for the maintenance of sperm motility, acrosome reaction and possibly other events related to fertilization. The percentages of swollen spermatozoa in buffalo and Sahiwal bulls were lower than the findings of Bredderman and Foote (1969) and Anzar *et al.* (1997) who reported more than 40 per cent swollen spermatozoa in frozen thawed bull semen. In hypo-osmotic swelling assay, water enters the spermatozoa due to solute gradients and establishes an equilibrium between the fluid compartment of spermatozoa and the external surroundings. The total sperm volume increases as the plasma membrane bulges causing coiling of tail of spermatozoa. Capacitation, acrosome reaction and fusion with oocyte involves many changes in the plasma membrane of sperm head region. Jeyendran *et al.* (1984) reported a good correlation between these events and the swelling ability and spermatozoa in a hypo-osmotic solution. A significant relationship has been reported between percentage of swelling ability and fertility (Deibel *et al.*, 1978).

In HHA test, the addition of homologous cow/buffalo serum in frozen-thawed semen resulted in

head to head agglutination of spermatozoa. Gupta et al. (1985) reported a head to head and tail to tail agglutination of buffalo spermatozoa on storage at 37°C for a few hours without any addition of serum. The percentage of agglutinated spermatozoa increased with the passage of time. In the present study, most of the spermatozoa were motile and spermatozoa involved in head to head agglutination were less in number characterized by slow motility with vibrating tail. In contrast to the findings of Brown and Senger (1980), the increase in sperm motility after the addition of serum was not observed. The percentage of agglutinated spermatozoa increased, with complete lack of motility, after 2 hr incubation at 37°C. In preliminary studies, it was observed that the addition of heterologous serum caused less sperm agglutination compared with homologous serum. Therefore, the incubation of semen with homologous serum at 37°C for 2 hr is recommended for this assay.

In this study, CFDA and PI were used as fluorescent probes to assess the plasma membrane integrity of buffalo and Sahiwal bull spermatozoa. CFDA can penetrate into the spermatozoa and is de-esterified inside the cell by non-specific esterases. The resultant free carboxyfluoresceine cannot cross the membrane and builds up in the cytoplasm of intact cells causing them to fluoresce green throughout their length (Fig. 3). PI, which binds to and stains DNA red, cannot penetrate the intact plasma membrane. However, it can easily enter through damaged plasma membrane and stains the nucleus red (Fig. 3). In partially damaged spermatozoa, there was accumulation of CFDA on the acrosomal region as well as within the mid-piece (mitochondria) indicating their intactness, but the post-acrosomal region of the sperm head was damaged and fluoresced red due to PI penetration (Fig. 3). Phase contrast microscopy does not indicate the membrane integrity of the post-acrosomal region of spermatozoa. In post-thaw semen samples, majority of the spermatozoa had completely damaged plasma membrane. After freezing and thawing, the percentage of spermatozoa with intact plasma membrane was lower in buffalo and Sahiwal bull than in Friesian bull (Anzar, *et al.*, 1997), and ram and boar semen (Harrison and Vickers, 1990). In preliminary trials, the background of microscopic field was green which made the evaluation difficult. It indicated the extensive release of esterases from sperm cytoplasm into extracellular medium during freezing and thawing. Therefore, the seminal plasma and extender parts of semen were removed by centrifugation to get rid of non specific esterases.

The present data provided a baseline information on the post-thaw semen quality of the two species using multidimensional assays to increase the objectivity of the semen quality assessment. Post-thaw semen quality was poor in all assays regardless the quality of fresh semen. It may be due to the species or breed differences, management practices, semen handling procedures, extenders, freezing techniques etc. and requires more extensive research for better preservation of buffalo and Sahiwal germ plasm. The present information can be successfully used for grading of semen collected from individual bulls. Attempts should be made to develop the most suitable buffering system and efficient freezing technique for the maximum harvesting of viable spermatozoa after cryopreservation.

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