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RESEARCH ARTICLE

ASSESSMENT OF DNA INTEGRITY AND *IN VITRO* FERTILIZING CAPACITY IN FROZEN THAWED BULL SEMEN

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ABSTRACT

Fresh semen ejaculates as well as frozen thawed semen of a fertile Jersey bull were subjected to certain physio-morphological and *in vitro* fertilization study in 10 replications. Sperm DNA integrity was assayed with Neutral Comet Assay using single cell gel electrophoresis technique. Mean progressive motility, live sperm and morphologically normal spermatozoa were recorded in fresh and frozen thawed and post capacitated frozen thawed semen of Jersey bull as 78.5 ± 2.11, 65.5 ± 1.89 and 82.5 ± 2.01 per cent; 85.5 ± 1.38, 76.1 ± 1.90 and 88.0 ± 0.82 per cent and 93.4 ± 0.68, 87.3 ± 1.07 and 95.8 ± 0.57 per cent respectively. Mean sperm concentration was found to be 1298.9 ± 69.02 and 83.0 ± 2.41 millions/ml respectively in fresh and frozen thawed semen. The percentage of acrosome reacted spermatozoa was reported to be 2.6 ± 0.34 and 51.8 ± 2.02 per cent respectively in incapacitated frozen thawed and post capacitated frozen thawed semen. Respective comet formation percentage in the present study was reported to be 8.1 ± 0.77, 10.3 ± 0.77 and 4.3 ± 0.30 in fresh, frozen thawed and post capacitated frozen thawed semen. ANOVA revealed that progressive motility, acrosome reaction and comet formation differed significantly ($P<0.05$) due to different status of semen. Relationship study between different attributes of fresh, frozen thawed and post capacitated frozen thawed semen showed positive and significant ($P<0.05$) correlation coefficient between motility and morphology in frozen thawed semen, however correlation coefficient of comet formation with motility and morphology in frozen thawed semen was observed to be negative. *In vitro* maturation and *in vitro* fertilization percentages were recorded in the present study as 92.31 and 50.81 respectively.

INTRODUCTION

Reproduction is an important consideration in the economics of livestock production. During the last decades the use of assisted reproductive techniques (ARTs) has increased substantially to enhance livestock productivity. Spermatozoa are nowadays subjected to for use in assisted reproductive technologies, such as artificial insemination or *in vitro* fertilization. Therefore, assessment of fertilizing ability of sperm is very important. Sperm DNA integrity is important for the success of natural or assisted fertilization, including normal development of embryo, foetus or offspring (Morris *et al*, 2002). The semen cryopreservation is an important technique for the conservation of genetic resources. The freezing-thawing procedures cause various damages to sperm cells including sperm DNA (Bilodeau *et al*, 2000). The DNA damage in spermatozoa induced by freezing-thawing is needed to be clarified for efficient fertility process. However, conventional methods based on microscopic examination and biochemical assays do not provide any information regarding sperm DNA damage. The present study was carried out to evaluate DNA integrity of frozen thawed spermatozoa by Neutral Comet Assay and fertilizing ability of frozen thawed semen of Jersey bull.

MATERIALS AND METHODS

Fresh semen ejaculates collected from a fertile Jersey bull maintained at Frozen Semen Centre, Intensive Cattle Development Project (ICDP), Govt. of Assam, located in the

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North Eastern part of India were used in the present study. Evaluation of physio-morphological parameters viz., progressive motility, sperm concentration, percentage of live spermatozoa, normal and abnormal spermatozoa count in fresh, frozen-thawed and post capacitated frozen-thawed semen and acrosome reaction in frozen-thawed and post capacitated frozen-thawed semen was done as per standard protocol. Ten observations were made for each parameter. Sperm DNA integrity was assessed by Neutral Comet Assay as described by Nandre (2007). Fragmented DNA with comet like structures indicated unintegrated DNA and those remained intact not forming comet like structures considered as spermatozoa with integrated sperm DNA.

To study the fertilizing ability of post capacitated frozen-thawed sperm *in vitro* fertilization was carried out. Ovaries were collected from abattoirs and oocytes were collected by aspiration technique. Only A and B graded oocytes were subjected to *in vitro* maturation (IVM). *In vitro* maturation (IVM) of bovine oocytes were done using modified tissue culture media (TCM-199) supplemented with 10% follicular fluid, foetal bovine serum (FBS), sodium pyruvate, L-glutamine, pFSH, 17 β-estradiol in CO_2 incubator maintaining at 38.5°C, 5% CO_2 in humidified air for 24 hours. Capacitation of post thawed frozen semen for fertilization *in vitro* as well as evaluation has been made by Swim up method using BO (Bracket and Oliphant) media. *In vitro* fertilization (IVF) of matured oocytes with capacitated spermatozoa was carried out in IVF droplet using BO media supplemented with bovine serum albumin (BSA) for 18 hours in CO_2 incubator at 38.5°C, 5% CO_2 in humidified air. *In vitro* fertilization was confirmed by the presence of cell division in the presumed zygotes.

Statistical analysis was done as per Snedecor and Cochran (1994).

RESULTS AND DISCUSSION

Mean progressive motility, live sperm and morphologically normal spermatozoa, sperm concentration, acrosome reacted sperm and comet formation percentage are presented in Table 1. Mean progressive motility, live sperm and morphologically normal spermatozoa were recorded in fresh, frozen thawed and post capacitated frozen thawed semen of Jersey bull as 78.5 ± 2.11, 65.5 ± 1.89 and 82.5 ± 2.01; 85.5 ± 1.38, 76.1 ± 1.90 and 88.0 ± 0.82; 93.4 ± 0.68, 87.3 ± 1.07 and 95.8 ± 0.57 per cent respectively. Analysis of variance revealed significant differences (P<0.05) in progressive motility, live sperm and morphologically normal spermatozoa due to different status of semen.

Sperm concentration was found to be 1298.9 ± 69.02 and 83.0 ± 2.41 millions/ml respectively in fresh and frozen thawed semen and was found to be significantly (P<0.01) higher in fresh semen. The acrosome reacted sperm was found to be 2.6 ± 0.34 and 51.8 ± 2.02 per cent respectively in incapacitated frozen thawed and post capacitated frozen thawed semen and value was significantly higher (P<0.01) in post capacitated frozen thawed semen.

Reported progressive motility in fresh semen and sperm concentration in frozen thawed semen of Kankrej bull by Patel and Siddiquee (2013) corroborates well with the present findings. However they reported higher percentage of live sperm and morphologically normal sperm both in fresh and frozen thawed semen than the present findings.

The percentage of spermatozoa showing hyper activated motility following capacitation was also reported by Kato and Nagao (2015) in bull semen. On the other hand, Rachamawati (2011) reported 21.28 to 26.31 per cent of acrosome reaction in post thawed bull semen. The variation observed in results of different workers might be attributed to differences in season of study, breed, age, feeding regime, extender, freezing process, evaluation procedure etc.

Sperm DNA integrity was assessed by Neutral Comet Assay where DNA fragmentation is marked by the formation of comet like structures (Fig. 1, 2 and 3). Respective comet formation percentage in the present study was reported to be 8.1 ± 0.77, 10.3 ± 0.77 and 4.3 ± 0.30 in fresh, frozen thawed and post capacitated frozen thawed semen and that comet formation differed significantly due to different status of semen (Table 1).

Nandre *et al*, (2011) also reported that comet percentage ranged from 3.0 to 7.6 in fresh and 4.6 to 13.6 in frozen thawed buffalo semen which agrees with the present findings. Swim up and selection for high density sperm nuclei reduced the frequency of sperm DNA damage by about one third as revealed by Van Kooij *et al*, 2004. The significantly lower percentage of comet formation in the post capacitated frozen thawed semen with higher percentage of acrosome reaction in the present study might be the result of swim up sperm washing and subsequent capacitation with BO media.

Relationship between different attributes of fresh, frozen thawed and post capacitated frozen thawed semen was also studied in the present study and presented in Table 2.

Table 1 Physio-morphological characteristics and per cent comet formation in fresh, frozen thawed and post capacitated frozen thawed semen of Jersey bull.

Status of semen	Progressive motility (%)	Live sperm (%)	Morphology (%)		Sperm concentration (million/ml)	Acrosome reaction (%)	Comet formation (%)
			Normal	Abnormal			
Fresh	78.5±2.11[a]	85.5±1.38[a]	93.4±0.68[a]	6.6±0.68[a]	1298.9±69.02[a]	-	8.1±0.77[a]
Frozen thawed	65.5±1.89[b]	76.1±1.90[b]	87.3±1.07[b]	12.7±1.07[b]	83.0±2.41[b]	2.6±0.34[a]	10.3±0.77[b]
Post capacitated frozen thawed	82.5±2.01[c]	88.0±0.82[c]	95.8±0.57[c]	4.2±0.57[c]	-	51.8±2.02[b]	4.3±0.30[c]

Different superscript in a column differ significantly (P<0.01)

Positive and significant (P<0.05) correlation coefficient was observed between motility and morphology in frozen thawed semen, however correlation coefficient of comet formation with motility and morphology in frozen thawed semen was observed to be negative.

Comet formation had positive correlation with motility and morphology in post capacitated frozen thawed semen, though not significant. Trisini *et al*, (2004) reported that the comet extent and percent DNA in the comet tail were associated with a decline in sperm motility.

In vitro maturation and *in vitro* fertilization percentages were recorded in the present study as 92.31 and 50.81 respectively.

Table 2 Relationship between different attributes of fresh, frozen thawed and post capacitated frozen thawed semen of Jersey bull.

Status of semen	Relation between	Coefficient of correlation
Fresh	Motility-Morphology	0.313
	Motility-Comet formation	0.113
	Morphology-Comet formation	0.047
Frozen thawed	Motility-Morphology	0.987*
	Motility-acrosome reaction	0.207
	Motility-Comet formation	− 0.352
	Morphology-Acrosome reaction	0.248
	Morphology-Comet formation	− 0.409
Post capacitated frozen thawed	Motility-Acrosome reaction	0.055
	Motility-Comet formation	0.313
	Morphology-Acrosome reaction	0.094
	Morphology-Comet formation	0.045

* P <0.05

Assessment of DNA integrity in frozen thawed bull semen by Neutral Comet Assay

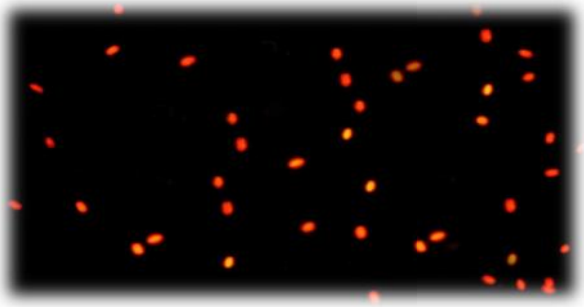

Figure 1 (40X) Non fragmented DNA of spermatozoa

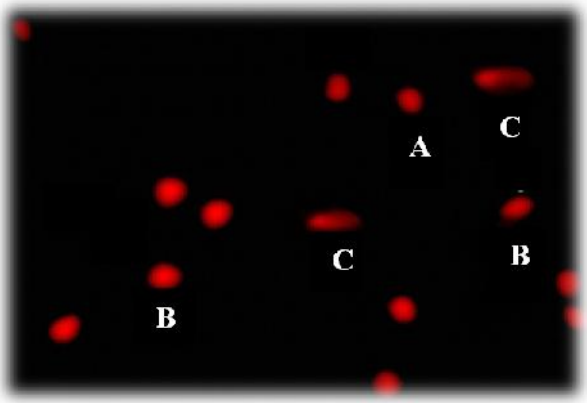

Figure 2 (40X) Non fragmented (A), Partially fragmented (B) and Fragmented (C) DNA of spermatozoa

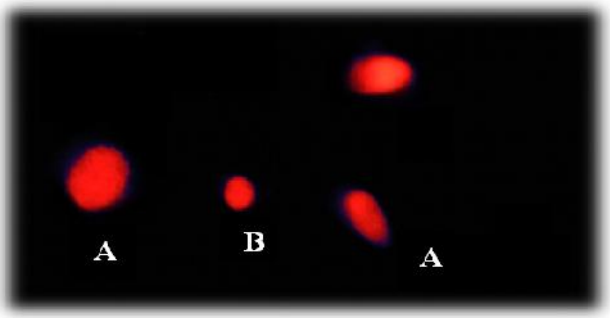

Figure 3 (100X) Fragmented (A) and non Fragmented (B) DNA of spermatozoa

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