RESEARCH ARTICLE

Intracytoplasmic Motile Sperm Selection in Fresh and Frozen Semen and its Correlation with Seminal Attributes of Kankrej Bull

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Abstract

Total 8 mature healthy Kankrej bulls (7 replicates) were selected for evaluation of seminal attributes at fresh and frozen stage of cryopreservation. Seminal attributes evaluated included sperm viability, acrosome integrity, HOST-EN test, intracytoplasmic motile sperm selection (IMSI) and sperm mucus penetration test (SMPT). Based on the nuclear abnormalities, sperms were classified in Grade I, II, III and IV. Per cent sperm viability, acrosome integrity, HOST- reacted sperm and Grade I sperm were found significantly lower in frozen semen as compared to fresh semen. Grade IV sperm as assessed by IMSI tool was found significantly higher in frozen semen as compared to fresh semen. Correlation study revealed significant and positive correlations of sperm viability with acrosome integrity and HOST, while significant negative correlation with grade IV sperm. Sperm viability and acrosome integrity were positively correlated with SMPT.

Key words: Cryopreservation, IMSI, Kankrej bull, Semen, SMPT

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Introduction

Standard sperm analysis including assessment of spermcount, motility, vitality, and morphology is often used to evaluate male fertility. It does not provide any information about abnormalities in the spermatozoa nuclei. In recent years, there has been more focus on genomic quality of the male gametes and its relationship with improvements in outcomes of assisted reproductive technology (ART) procedures and reduction in the risk of passing genetic aberrations to the embryo.

In human spermatozoa nuclear vacuoles were revealed with the introduction of Nomarski differential interference contrast microscope, which can examine the fine nuclear morphology of motile spermatozoa in real time at a magnification of up to 6600x. Motile sperm organelle morphology examination (MSOME) is a new concept for observing spermatozoa, which enables to examine the fine nuclear morphology of motile spermatozoa (Bartoov *et al.,* 2001). The origin of the vacuoles is subject to controversy. Several studies have found sperm head vacuoles, which might be of acrosome origin (Kacem *et al*., 2010), while others showed these of nuclear origin (Oliveira *et al.,* 2010).

Cryopreservation plays an important role in reproductive science, in particular for preservation of gametes, embryos, and reproductive tissues. Although, spermatozoa seem to be less sensitive to cryostorage than other cells, cryopreservation is associated with alterations of sperm structure (Watson, 2000). The aim of this study was to evaluate the impact of freezing-thawing on the sperm head vacuoles and to assess the potential value of MSOME for selection of frozen-thawed spermatozoa in clinical setting along with its correlation with seminal attributes.

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Materials and Methods

Bull Selection and Evaluation of Seminal Attributes

For this study on fresh and frozen semen parameters, total 8 healthy breeding bulls of Kankrej breed maintained identically at Livestock Research Station, Kamdhenu University, Sardarkrushinagar-Dantiwada, Gujarat (India) were selected. Semen was collected from these bulls in

artificial vaginal regularly at weekly interval. In all 7 ejaculates from each bull (total 7x8=56) were included in this study for evaluation of fresh seminal attributes, cryopreservation in liquid nitrogen and post-thaw evaluation. Spermatozoal attributes including sperm viability (Eosin-Nigrosin stain), acrosome integrity (Giemsa stain, Kutty *et al.,* 1996), HOST-EN test (Quintero-Moreno *et al.,* 2011) and sperm mucus penetration test (Matousek *et al.,* 1989) were performed in fresh and post-thawed semen using standard procedures.

Intracytoplasmic Morphological Sperm Selection

Evaluation of motile spermatozoa for nuclear ultrastructural defects was carried out at 8500 X magnification using an IMSI lens fitted into a microscope equipped with micromanipulator. A specially developed IMSI Petri dish (Willco-Dish®, Glass bottom dish, REF No. GWST-5040) was used for a real-time sperm examination. 7% ready to use Polyvinylpyrrolidone (PVP) medium was used to slow down the movement of the spermatozoa for better examination. A total of 100 spermatozoa from every ejaculate (n=7 each fresh and cryopreserved) from each of 8 bulls were examined for the presence of nuclear vacuoles and abnormalities using a high magnification inverted microscope.

In brief, 10 µL of semen (fresh or frozen-thawed) was transferred to the Eppendorf tube. 990 µL of sterile phosphate-buffered saline (PBS) (HIMEDIA, Catalog No. TS1101-1X1L) was mixed to make a dilution of 1:100 followed by centrifugation at 300 g for 5 min. Supernatant was removed and pellet was transferred in another Eppendorf tube and 900 µL of PBS was added and mixed well. 4 µL of this diluted semen was added into the 7 % PVP media drop in the IMSI plate to slow down the sperm motion and evaluated under the inverted microscope.

Using motile sperm organelle morphology examination (MSOME) criteria, grading of spermatozoa into four groups according to the presence or size of the vacuoles as classified by Vanderzwalmen *et al.* (2008) was performed (Table 1).

Table 1: Grading of the sperms based on vacuoles (Vanderzwalmen *et al.*, 2008)

Grade I	:	Normal form and no vacuoles
Grade II	:	Normal form and ≤ 2 small vacuoles covering less than 4% of head area
Grade III	:	Normal form, >2 small vacuoles or at least one large vacuole (covering >4 % of head area)
Grade IV	:	Large vacuole (>4 % of head area) and other head abnormalities

Statistical Analysis

The data obtained on various fresh and frozen-thawed sperm parameters (sperm viability, acrosome integrity, HOST-EN test, SMPT and IMSI) were statistically analysed using two-way Analysis of Variance using SPSS v.17 software. Duncan's post hoc multiple range test was used to determine significant differences between means at $p<0.05$. Pearson's correlation coefficients were determined between various semen parameters in fresh and frozen semen (Snedecor and Cochran, 1994).

Results and Discussion

Sperm Quality Attributes of Fresh and Frozen Semen

Overall mean sperm concentration in Kankrej bulls under study was 1224.36±1.89 million/mL, which varied between 720 and 1630 million/mL. The overall mean (±SE) values of sperm viability, acrosome integrity, hypoosmotic swelling test, sperm mucus penetration test and different grades of intracytoplasmic morphological sperm selection in fresh and frozen-thawed semen of same ejaculates/bulls are presented in Table 2. The mean per cent sperm viability, acrosome integrity, HOST-reacted sperm and Grade I sperm were found highly significantly ($p<0.01$) lower in frozen semen as compared to fresh semen, whereas Grade II and Grade IV sperm as assessed by IMSI tool were found significantly higher in frozen semen as compared to fresh semen, suggesting that the process of cryopreservation definitely damages the sperm structure and function with increase in nuclear vacuoles and other abnormalities, which hamper the sperm fertility.

Table 2: Mean (±SE) values of sperm quality parameters of fresh and frozen-thawed semen of Kankrej bulls (n=58).

Parameters	Fresh semen		Frozen semen	
	Mean ± SE	Range	Mean ± SE	Range
Sperm viability (%)	84.38 ± 0.33	68 - 95	62.63 ± 0.33**	52 - 78
Acrosome integrity (%)	83.95 ± 0.33	65 - 97	67.55 ± 0.33**	65 - 97
HOS reactivity (%)	78.30 ± 0.34	68 - 92	58.88 ± 0.23**	47 - 75
SMP test (mm/h)	16.50 ± 0.27	12.00-22.43	22.14 ± 0.28**	17.14-29.14
IMSI Grade I (%)	86.05 ± 0.27	79-93	78.80 ± 0.31**	69-92
IMSI Grade II (%)	6.04 ± 0.51	2-12	6.64 ± 0.21	2-13
IMSI Grade III (%)	3.73 ± 0.18	0-9	4.95 ± 0.20**	1-11
IMSI Grade IV (%)	4.18 ± 0.24	0-13	9.61 ± 0.29**	1-22

**indicates highly significant ($p<0.01$) differences between fresh and frozen semen.

In fresh semen, lower per cent of live sperm than the present observation has been reported in Hariana bulls by Gupta *et al.* (2022), whereas higher mean per cent live sperm has been recorded by others in Kankrej bulls (Kapadiya *et al.,* 2018; Chaudhary, 2021), while in case of frozen semen, lower percentages of viable sperm than the present findings were

found in `Hariana bull (Anand *et al.,* 2017) and Jaffarabadi buffalo (Vijyeta *et al.,* 2024). However, higher sperm viability was found by others in Kankrej bull (Kapadiya *et al.,* 2018, Chaudhary, 2021) and Piedmontese bull (Alkhawagah *et al.*, 2022).

A higher per cent acrosomal integrity than the present finding in fresh semen has been reported by some workers in Kankrej (Kapadiya *et al.,* 2018; Chaudhary, 2021), and Gir bulls (Chaudhary *et al.,* 2017). In contrast to present study, higher per cent acrosomal integrity in frozen semen was reported in Kankrej bull and Gir bull by several workers (Kapadiya *et al.,* 2018; Chaudhary *et al.,* 2017).

Comparatively higher mean HOST reactive sperm in fresh semen than the present finding has been reported in Kankrej bulls (Kapadiya *et al.,* 2018), while others noted quite lower mean HOST reactive sperms than that of the present one in Kankrej (Chaudhary, 2021), Gir (Chaudhary *et al.,* 2017), and Hariana bull (Gupta *et al.,* 2022) semen.

In case of frozen semen, similar results were found in Kankrej bull (Kapadiya *et al.,* 2018; Chaudhary, 2021). In contrast to present study, lower percentage of HOST reactive sperm was found in Gir bulls by Chaudhary *et al.* (2017). Moreover, higher per cent HOST reactive sperm was found in Hariana (Gupta *et al.,* 2022) and Piedmontese bulls (Alkhawagah, *et al.*, 2022).

Significant reduction in the sperm viability, acrosome integrity and plasma membrane integrity in the cryopreserved semen compared to fresh one could be attributed to oxidative stress (Doshi *et al.,* 2012; Husna *et al.,* 2017).

The present finding on SMPT concurred with the reported results ranging from 28-65 mm in fresh and/or frozen-thawed bull semen (Singh *et al.,* 2016; Chaudhary, 2021). Compared to present study, higher sperm penetration distance in fresh and frozen semen was however observed in Gir bulls (Sonar *et al.,* 2016). Significantly ($p<0.01$) higher sperm penetration distance in frozen semen as compared to fresh semen found might be due to the fact that frozen thawed spermatozoa having hyperactivated motility which might be due to cryopreservation as cryopreserved spermatozoa have poor calcium efflux mechanisms and are less efficient in extruding calcium ions resulting in rapid accumulation of cytosolic calcium ion (Cormier and Bailey, 2003). Some of the proteins that become tyrosine phosphorylated during capacitation have been localized to the flagellum, and therefore it has been proposed that they are involved in hyperactivation of sperm (Soren, 2011).

In human spermatozoa, Yari *et al.* (2017) studied the effect of cryopreservation on sperm head morphology and found significantly reduced vacuoles-free sperm in frozen semen (9.07 ± 1.48 %) as compared to fresh semen (10.23 ± 1.79 %). Moreover, they found that following cryopreservation non-significant ($p = 0.296$) increase in the small vacuoles sperm was found (56.90 ± 5.04 %) as compared to fresh semen (58.30 ± 5.50 %), which also supports the current findings. In a study of Boitrelle *et al.* (2011) cryopreservation promoted sperm nucleus vacuolization, reduced the incidence of grades I+II spermatozoa, and increased the incidence of sperm with non-condensed chromatin, which is in consistence with the present findings. The significant increase in the Grade IV spermatozoa in frozen semen might be due to hazardous effect of cryopreservation as it induces alteration in lipid-protein complexes in plasma membrane, chromatin destabilsation results in loss of DNA integrity (Gliozzi *et al.,* 2011) and excessive generation of reactive oxygen species (Johnston *et al.,* 2012).

Correlation Findings

The correlation coefficients observed among various sperm quality parameters studied in fresh and frozen-thawed semen are presented in Table 3. In fresh semen the correlation study revealed significant and positive correlations of sperm viability with acrosome integrity, HOST, and significant negative correlation with Grade I and Grade IV sperm. Acrosome integrity and HOS reactive sperm were positively interrelated. SMPT showed significant positive correlation with Grade I sperm, and HOS reactive sperm with Grade III sperm. Grade I and Grade IV sperm were negatively ($p<0.01$) interrelated. All other correlations were low and statistically non-significant (Table 3).

In frozen-thawed semen correlation study revealed that sperm viability had significant ($p<0.01$) positive correlations with HOST, SMPT and Grade I sperm. Acrosome integrity was positively correlated with SMPT, HOST showed positive correlations with SMPT and Grade I sperm, and later two were also positively correlated. Grade I sperm showed significant negative correlations with grade II and III sperm, and Grade IV sperm revealed significant ($p<0.01$) negative correlations with all the other sperm quality parameters studied (Table 3).

Similar to present study, significant and positive correlations of SMPD with sperm viability and acrosome integrity in frozen semen were reported in Murrah buffalo (Singh *et al.*, 2016). Similarly, Patel *et al.* (2022) evaluated fresh and frozen semen of Kankrej bull with IMSI and other seminal characteristics and found positive correlation of Grade I sperm with sperm viability and acrosome integrity in frozen semen. Moreover, Grade IV sperm were found negatively correlated with sperm viability and acrosome integrity which is in consistence with the present findings. In human, Fekonja *et al.* (2014) performed IMSI in fresh semen and found significant and negative correlation of Grade IV sperm with sperm morphology.

Table 3: Correlations among the various sperm quality attributes of fresh and frozen-thawed semen of Kankrej bull

Sperm parameters	Sperm viability	Acrosome integrity	HOS reactivity	Sperm-mucus PT	IMSI Grade I	IMSI Grade II	IMSI Grade III	IMSI Grade IV
				Correlations: Frozen-thawed semen				
Sperm viability	1	0.044	0.480**	0.384**	0.351**	-0.146	0.158	-0.400**
Acrosome integrity	0.639**	1	0.159	0.463**	0.159	0.085	0.180	-0.309**
HOS reactivity	0.534**	0.328*	1	0.447**	0.347**	-0.133	0.167	-0.404**
SM Penetration Test	0.127	0.072	0.111	1	0.280*	-0.023	0.169	-0.386**
IMSI Grade I	- 0.150**	0.116	0.027	0.361**	1	-0.430**	-0.347**	-0.738**
IMSI Grade II	0.012	0.171	-0.008	-0.271*	-0.210	1	0.223	-0.132
IMSI Grade III	0.117	0.045	0.298*	-0.145	-0.241	0.015	1	-0.210
IMSI Grade IV	-0.338**	-0.240	-0.187	-0.166	-0.393**	-0.044	-0.073	1
	Correlations: Fresh semen							

** Significant at the 0.01 level (p<0.01). *Significant at the 0.05 level (p<0.05).

Conclusion

Seminal attributes in frozen semen of Kankrej bull were found significantly lower than the fresh semen, except sperm mucus penetration test due to detrimental effect cryopreservation associated with oxidative stress. Grade IV spermatozoa was found significantly higher in frozen semen as compared to fresh semen insisting potential hazard of cryogenic shock to spermatozoa.

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