The Haryana Veterinarian (December, 2023) **62(2),** 129-132 ISSN 0033-4359 (P) **Research Article**

CORRELATIONS OF MITOCHONDRIAL MEMBRANE POTENTIAL AND OXIDATIVE STRESS LEVELS WITH POST-THAW SEMEN QUALITY IN BULLS

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Received: 22.08.2022; Accepted: 13.06.2023

ABSTRACT

The objective of this work was to assess the mitochondrial membrane potential (MMP) and oxidative stress levels in the spermatozoa of Frieswal Bulls and to correlate the results with the conventional semen quality parameters/tests.The MMP was higher in spermatozoa of freezable group as compared to the non-freezable group and also had a positive correlation with progressive motility and all the sperm parameters analysed. MDA levels were inversely correlated with progressive motility, viability, plasma membrane and acrosome integrity. Catalase activity, however, did not show any differences between the two groups, nor was it correlated with the sperm parameters examined. The study concludes that the post-thaw evaluation of MMP and MDA levels reflects quality of semen in bulls, which makes these tests highly recommendable to be applied as a complement to conventional sperm analyses in semen stations.

Keywords: Catalase, Frieswal bulls, Malondialdehyde (MDA), Mitochondrial membrane potential (MMP), Oxidative Stress, Semen

How to cite: Pande, M., Ghosh, S.K., Tyagi, S., Srivastava, N., Sirohi, A.S., Chand, N., Sarika, Katiyar, R., Kumar, A., Kumar, S. and Mahajan, S. (2023). Correlations of mitochondrial membrane potential and oxidative stress levels with post-thaw semen quality in bulls. *The Haryana Veterinarian* **62(2)**: 129-132.

Artificial insemination (AI) plays an imperative role in improving the productivity of bovines by upgrading their genetic potential as well as enhancing the milk production and productivity in the country. However, as per 2015-16 data available from the states, average three semen doses are required for attaining one successful conception (GOI, 2017). While conception rate is influenced by many factors, production of high quality cryopreserved semen, remains the target of male germ-plasm producing units of the country. However, this well-established technique of cryo-preservation is recognized to have an injurious effect on spermatozoal functions. Nearly 40 to 50% of spermatozoa are assumed to be decimated during the entire process. This post-thaw semen deterioration is even more evident in crossbred bulls (Tyagi *et al.*, 2000).

To check the quality of semen post-freezing, there arevarious assays and out of all, the most accepted one is the post-thaw progressive motility (PTM). Although, PTM is an essential criterion for judging fertilizing ability of spermatozoa, it is not the sole attribute responsible for fertility (Kumaresan *et al.*, 2020). To be more sure about the quality of semen following cryopreservation, the recent guidelines have given emphasis on regular post-thaw incubation tests and periodic acrosome and plasma membrane integrity test. Further, there exists a need for including more in vitro tests which can hastily determine the quality of cryopreserved semen in Bulls. Here, the objective of the work was to assess the sperm mitochondrial membrane potential (MMP) and oxidative stress levels in the spermatozoa of Frieswal Bulls and to correlate the results with the conventional semen quality parameters.

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MATERIALS AND METHODS

Sample collection, cryopreservation and grouping: The present research was undertaken at Cattle Physiology and Reproduction Division, ICAR-Central Institute for Research on Cattle, Meerut, India. Three healthy Frieswal (HF×Sahiwal) breeding bulls maintained under uniform feeding, housing and managerial conditions with an average weight of 550-650 Kg were utilized for the study. The ejaculates were collected early morning between 8 to 9 AM in graduated glass collection tubes, as per regular standard practice. A total of 24 ejaculates (8 ejaculates per bull) with Initial progressive motility (IPM) of ≥ 70% and a concentration of ≥ 500 × 10^6 sperm/mL were selected for the experiment. Concentration was calculated with bovine semen specific photometer (Accucell, IMV- France).

The semen was extended in Tris-fructose-egg yolk-glycerol extender to yield a desired final concentration of approximately 80 million progressive motile sperm cells/mL. The extended semen was filled and sealed in 0.25 mL French mini straws and kept for equilibration at 5° C for 4 h in the cold handling cabinet. The freezing of samples was done using a programmable automated freezing system (IMV, France). Following freezing, the straws were stored in cryo-containers plunged in liquid nitrogen till analyzed. The semen samples were rearranged in two groups according to the post-thaw motility (PTM)

analysis viz. freezable (n=15; PTM ≥ 50%) and non-freezable semen sample (n=9; PTM < 50%).

Semen quality assessment: Frozen samples were thawed in a thawing unit at 37° C for 30 seconds. The contents of semen straws were evacuated in a plain borosilicate glass test tube maintained in a water bath at 34° C. The semen samples underwent assessment for parameters related to semen quality, including sperm motility, viability, acrosome intactness, and plasma membrane integrity after the thawing process. The forward progressive motility of the sperm cells was assessed under a light microscope (Olympus BX40) at a magnification of ×200 and at least 10 different fields were observed.

The supra-vital stain Eosin was used to differentiate between viable and non-viable spermatozoa. A minimum of 200 sperm heads were counted across the slides in different fields. The sperm plasma membrane integrity was assessed using HOST. Aminimum of 200 spermatozoa were counted in different fields at 400× under a phase-contrast microscope for determining HOST reactive spermatozoa. Acrosomal integrity was assessed using the Giemsa stain, as per the standard protocol. The spermatozoa wereobserved under oil immersion (1000 ×). A minimum of 200 sperm cells were counted per slide for assessment.

The sperm MMP was assessed using a membrane-permeable fluorimetric cationic dye, 5,6-dichloro-2-[3-(5,6-dichloro-1,3-diethyl-1,3-dihydro-2H-benzimidazol-2-ylidene)-1-propen-1-yl]-1,3-diethyl-1H-benzimidazolium, monoiodide (JC-1 byCayman, USA; Cat. No. Item No. 15003). This satin selectively enters into mitochondria and changes fluorescence characteristics with alteration in MMP. In live and active sperm cells JC-1 forms complexes (multi-meres known as J-aggregates), which fluoresce red/orange (high-MMP). A decrease in MMP, which is a very early event in apoptosis, results in JC-1 monomers, which fluoresce green, low MMP (Fig. 1). The procedure followed was described by Srivastava and Pande (2017). Briefly, 1 mL of sperm suspension was mixed with 1 mL of working JC-1 solution. The mixture was kept in incubator (5% CO_2 for 20 min at 37° C) in humidified chamber. Thereafter, it was centrifuged, and the sperm pellet was re-suspended in1 mL ice-cold staining buffer. A drop was then placed on a slide and observed under a coverslip. The observations were performed immediately under an epifluorescence microscope (80i Nikon, Japan) under 400 × in green filter.

The levels of lipid peroxidation (LPO) were

Table 1. Semen quality assessment of fresh semen (Means± SEM)

Semen Attributes	
Volume (mL)	5.54±0.11
Progressive Motile Sperm (%)	74.58 ±1.03
Concentration (10^6/mL)	961.25±29.79
Viability (%)	78.21±1.05
Membrane Integrity (%)	73.29±1.35
Intact Acrosome (%)	75.53±1.67
Abnormality (%)	8.18±1.03

N, number of samples = 24

Table 2. Semen quality parameters of Frieswal bulls at post-thaw stage (Means ± SEM)

Groups n	Freezable 15	Non-Freezable 9
PTM (%)	54.67±1.33^b	34.44±1.76^a
Motility PI-120 min (%)	35.00±1.38^b	15.56±1.76^a
Viability (%)	58.73±1.46^b	38.78±1.87^a
Acrosome Intactness (%)	53.93±1.60^b	34.89±1.84^a
Membrane Integrity (%)	48.93±1.49^b	36.00±1.43^a
High-MMP (%)	50.05±1.15^b	36.78±1.01^a
Catalase (U/mg)	0.01±0.00	0.01±0.00
MDA (µmol/mL)	1.73±0.04^a	2.26±0.08^b

Values bearing different superscripts in a row differ significantly ($p<0.05$). n, number of samples, PTM, post-thaw motility, PI, post-incubation, MMP, Mitochondrial membrane potential, MDA, malondialdehyde.

Table 3: Correlation* coefficients and the P values between MMP, LPO, Catalase activity and semen quality parameters in Frieswal Bulls

	MMP (%)		MDA (µmol/mL)		Catalase (U/mg)	
	r	P	r	P	r	P
PTM (%)	0.725	<0.0001	-0.642	<0.001	0.071	NS
Motility PI-120 min (%)	0.585	<0.003	-0.720	<0.0001	0.194	NS
Viability (%)	0.702	<0.0001	-0.627	<0.001	0.060	NS
Acrosome Intactness (%)	0.664	<0.0001	-0.547	<0.006	-0.141	NS
Membrane Integrity (%)	0.694	<0.0001	-0.597	<0.002	0.007	NS

NS, not statistically significant; *Pearson's correlation; n = 24, PTM, post-thaw motility, PI, post-incubation, MMP, Mitochondrial membrane potential, MDA, malondialdehyde

determined by measuring the levels of malondialdehyde (MDA) which is an end product of lipid peroxidation. MDA was estimated using thiobarbituric acid (TBA) assay (Sanocka and Kurpisz, 2004). Catalase activity was determined in sample using Aebi's method (1984) and estimated using the formula by Cuellar-Cruz *et al.* (2009).

Statistical analyses: The data underwent statistical analysis using standard procedures. The results obtained are presented as mean ± SEM. Data were analyzed by using SPSS 20.0 (SPSS Inc., Chicago, USA) statistical software. The regression analysis graph was designed using GraphPad Prism software version 5.0 (GraphPad Software, San Diego, CA, USA).

RESULTS AND DISCUSSION

Assessment of neat semen

The neat semen characteristics of Frieswal bulls are presented in Table 1.

Assessment of frozen-thawed semen

The percentage of progressively motile spermatozoa, post-thaw incubation motility (120 min), viability, acrosome intactness and plasma membrane functionality in both the groups are presented in Table 2. The forward progressive motility (%) exhibited a significant decrease ($p < 0.001$) in all cryopreserved semen samples, regardless of the group, as compared to neat semen. However, in the non-freezable semen samples there was a huge reduction in all the semen quality parameters. Further, the levels of MDA in freezable group was significantly lower than that in the non-freezable group. However, the results of catalase test did not show statistically significant differences between the groups.

The results of this study also showed a significant correlation between post-thaw MMP and lipid per-oxidation levels with all the routine semen analysis parameters assayed (Table 3).

In the present study, a positive correlation between MMP levels was observed with sperm motility, which substantiates previous reports in crossbred bulls (Bollwein *et al.*, 2008) and confirms the importance of mitochondrial potential to maintain sperm functionality. We also confirmed the significant correlation of MMP levels with post-incubation motility after two hrs, viability, acrosome intactness and plasma membrane integrity (Table 3). These observations demonstrated that the assessment of early apoptosis marker of sperm cell i.e. MMP may reflect sperm quality. Likewise, the importance of mitochondrial potential and its positive correlation with the conception rate in Frieswal cattle (Srivastava *et al.*, 2018) was earlier observed. In the present study, the linear regression

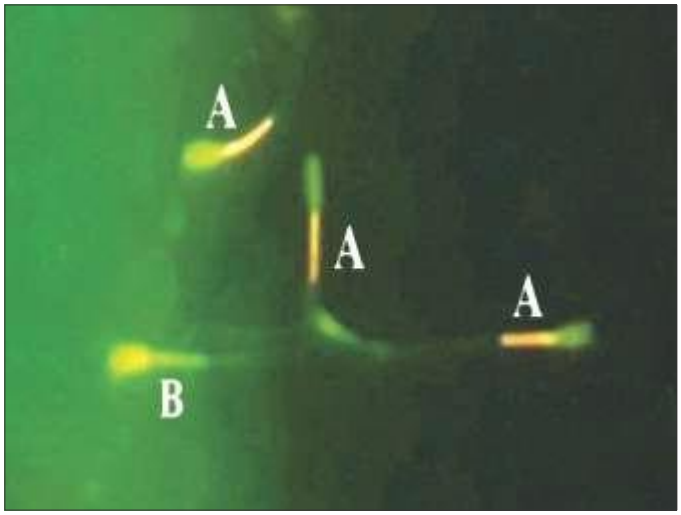

Fig. 1. Spermatozoa with High (red/orange mid-piece; A) and low (green mid-piece; B) mitochondrial membrane potential (400×)

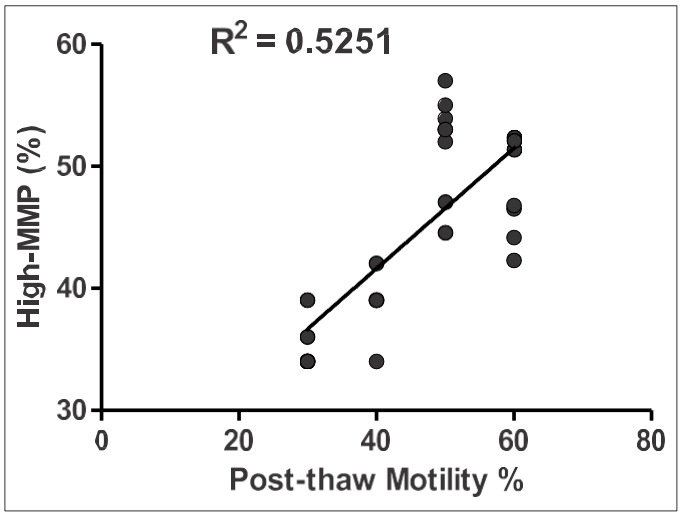

Fig. 2. Linear regression analysis between mitochondrial membrane potential (MMP) and post-thaw motility in Frieswal Bulls

analysis between MMP and post-thaw motility, along with the regression equation, is illustrated in Fig. 2.

A significant negative correlation of MDA levels with the routine semen analysis variables was observed (Table 3). LPO, a second messenger of oxidative stress (OS), was inversely correlated with post-thaw progressive motile spermatozoa (%) at 0 min, post-thaw 120 min incubation, acrosome intactness and plasma-membrane functionality of cryopreserved semen samples in Frieswal bulls. Spermatozoa are susceptible to OS as the sperm plasma membrane is composed of substantial amount of polyunsaturated fatty acids which are the target sites of reactive oxygen species (Agarwal *et al.*, 2014). High levels of LPO are detrimental to sperm functions and decreases membrane fluidity, which in turn affects sperm motility (Atiken, 2020). In our study, the correlation between MDA levels and semen parameters are in congruence with the other research reports (Kadirvel *et al.*, 2014; Soni *et al.*,

2019; Gallo *et al.*, 2021).

Catalases are antioxidant enzymes that catalyse the conversion of hydrogen peroxide to water and oxygen thus, combatting the OS in most of the body systems/fluids including semen.In the present study, no correlation was observed among catalase concentration and other semen quality parameters and the activity was lower in Frieswal bulls as compared to other crossbred bulls (Sharma *et al.*, 2016; Singh *et al.*, 2020). However, it has been reported that exogenous addition of catalase in bull semen, improves post-thaw semen quality and a significant correlation can be seen between catalase activity in freezable and non-freezable semen samples following catalase supplementation (Singh *et al.*, 2020).

The sperm mitochondria produce energy for the movement of the sperm (Hirata *et al.*, 2002). If the mitochondria are intact, then the sperm's fertilisation ability is likely to be good. The cryopreserved semen used for AI undergoes a series of tests to ensure the best semen quality for end-users. However, still the conception rate through AI is relatively low (GOI, 2017). In humans, recent researches have concluded that threshold levels of MMP should be established and included for semen examination (Barbagallo *et al.*, 2020). This study also validates the use of fluorescence microscope in combination with LPO levels as a simple and powerful methodology for supplementary evaluation of sperm quality. In conclusion, this study states that, the evaluation of MMP and LPO levels can reflect sperm quality making its application as a complement to routine semen analyses in Frieswal breeding bulls. For the validation of the MMP and LPO to act as compliment test for semen analysis in the breeding bulls needs a state wide study throughout the semen stations across the country.

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