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Time Dependent Impact of Reactive Oxidants on Seminal Attributes of Murrah Bull during Cryopreservation and Storage

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ABSTRACT

Background: Cryopreservation is an invaluable technique yet it is also known to be detrimental to sperm function and fertility due to cryo-injury and concomitant generation of reactive oxidants. During laboratory manipulation for the cryopreservation and freeze-thaw process, spermatozoa undergo osmotic stress, ionic imbalance, metabolic decoupling, membrane phase transition, destabilization of the cytoskeleton and antioxidant depletion which communally hampers the semen quality.

Methods: With the aim of determining implications of cryopreservation and storage, semen samples were collected by artificial vagina technique from 12 Murrah bulls and subsequently examined at 0 hour (before cryopreservation) and at 24 hour, 1 month and 2 month of storage for various seminal attributes. Simultaneously seminal plasma was separated and preserved at -20°C till the analysis of biochemical indicators of semen quality *viz*., nitric oxide (NO), total antioxidant quantity (TAC) and lipid peroxidation status (TBARS).

Result: A sharp reduction ($p<0.01$) in the semen quality was observed only at 24 h after cryopreservation except for viability. Significant reduction ($p<0.05$) in viable counts was observed up to 1 month interval. The capacitated sperm percentage was greater ($p<0.01$) in the cryopreserved semen as compared to fresh ejaculate. The mean±SE levels of NO (µmol/L), TAC and TBARS (Units/ml) was 2.31±0.27, 0.73±0.04 and 1.11±0.16 respectively in seminal plasma of neat semen stored at -20°C, while the values in the extended seminal plasma after cryopreservation was 2.37±0.31, 0.44±0.03 and 0.65±0.03 respectively. So it can be inferred that most of the damage encountered by spermatozoa is during the initial period of freezing, but the damage associated by various stressors cannot be ignored.

Key words: Cryopreservation, Murrah bulls, Nitric oxide, Seminal plasma, Viability.

INTRODUCTION

Cryo-injury and the associated generation of reactive oxidants during and after cryopreservation are responsible for inflicting stress on sperm cells (Kumar *et al*., 2019a). Excessive supercooling during the process of freezing results in a rapid ice formation, with the possibility of physical damage resulting in significant deteriorations in seminal parameters, exaggerated by the supra-physiological level of free radicals. Various studies have indicated that male germ cells at various stages of its differentiation (Agarwal and Said, 2003) and through interaction between spermatozoa and dissolved oxygen in extender (Amin *et al*., 2018; Lone, 2018) have the potential to generate reactive oxygen species (ROS) and reactive nitrogen species (RNS). The ROS include singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl radicals (OH^-) (Lone *et al.,* 2018), likewise, RNS include nitrogen dioxide (NO_2), peroxynitrite ($ONOO^-$) and nitric oxide (NO) (Nash *et al*., 2012), which have been recently implicated in inducing poor sperm function and sperm fertilizing ability in bovines. These ROS and concomitant RNS perform a bimodal role and roots into oxidative stress (OS) at higher concentrations when manipulated in vitro during the assisted reproductive technique (ART). Dead and debilitated spermatozoa and associated leukocytes release these free radicals in an excess amount which are highly unstable and reactive, causing a cascade of chain reactions resulting in damage of biomolecules (Agarwal *et al*., 2005; Nash *et al*., 2012; Ezzati *et al*., 2020). During laboratory manipulation for the cryopreservation process, spermatozoa undergo osmotic, oxidative and nitrosative stress since they are not adapted to endure low temperatures and associated stresses during the whole freezing-thawing process (Kumar *et al*., 2018; Ugur *et al*., 2019). Spermatozoal phospholipid configuration and mitochondrial membrane fluidity are highly vulnerable to

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these oxidative attacks since the extended semen lack significant antioxidant protection and appropriate armory of defensive enzymes (Said *et al*., 2010; Lone *et al.,* 2017). These all stressors ultimately roots in inevitable damages on acrosome (Balamurugan *et al*., 2018), head membrane (Hsieh *et al*., 2006; Gadea *et al*., 2013), tail and also aggravates premature capacitation (Shah *et al*., 2017; Yadav *et al*., 2017), lipid peroxidation (Khodaei *et al*., 2016; Kumar *et al*., 2019b), which unduly compromise the quality and fertility of frozen-thawed sperm. The reduction in quality and concomitant fertility arises due to spermatozoa disintegration as a consequence of anatomical and biochemical destruction of subcellular organelles and alterations in epigenetic profile (Chatterjee *et al*., 2017). The concept of the susceptibility of semen to injury during the freezing and thawing process has been detailed previously by various researchers but the arrival of certain stressors during the storage period is least meticulated. The present study aimed to investigate the concept of time dependent susceptibility of Murrah bull semen to cryo-injury on various seminal attributes and biomarkers during different cryopreservation intervals.

MATERIALS AND METHODS

Location of study and experimental animals

The present investigation was carried out on 12 Murrah breeding bulls maintained at Artificial Breeding Research Centre, ICAR- National Dairy Research Institute, Karnal, Haryana, India during the year 2018-2019. Bulls selected as experimental animals were healthy with an av. body weight of 500 kg, free from diseases, sexually mature (av. 6 years), in good libido, clinically normal and vaccinated as per the standard schedule of the Institute farm. Semen samples were collected during the morning hours at 7:00 AM by artificial vagina techniques twice a week. For all sampling groups, a sperm concentration of 20 million/ml of diluted semen sample was maintained. Bulls were maintained on a diet which contains 21% CP, 70% TDN and green fodder 5-7 kg daily. The animal experiments performed were acceptable to the ethical standards of the National Dairy Research Institute, Karnal, India vide order number 44-IAEC-19-10.

Semen evaluation pre and post freezing

Immediately after collection, the ejaculates were brought to the laboratory and kept in a water bath at 32°C. The semen samples were classified based on mass activity and colour. The semen sample was subsequently examined at 0 hour (before cryopreservation) for volume, colour, mass-activity, individual motility, sperm concentration, non-eosinophilic (live), hypo-osmotic swelling test, acrosome reaction and subsequent experimentation. From remaining sample, straws were prepared for further assessing the above mentioned seminal attributes at 24 hour, 1 month and 2 month intervals and capacitation status. Simultaneously from the same bull semen, seminal plasma was separated and aliquoted in different vials and preserved at -20°C till analysis of biochemical indicators of semen quality *viz* TAC, NO concentration and lipid peroxidation status (TBARS).

Assessment of seminal parameters

The individual motility was recorded as a percentage of progressively motile spermatozoa after the extension of semen. This was assessed by placing a drop of diluted semen (diluted with TRIS egg yolk extender) on a clean, grease-free glass slide mounted on a stage maintained at 37°C and observed under 20X objective after covering with a coverslip. The percentage of progressively motile sperm was estimated by observing five representative areas of the slide. Sperm viability as assessed as per the method described by Blom (1950) and Hancock (1951) and evaluated the non-eosinophilic or live sperm count. Functional membrane integrity of spermatozoa was evaluated by the HOST. The HOST was performed as per the method described (Jeyendran *et al*., 1984) with slight modifications to assess the functional integrity of the sperm tail membrane which gives an idea of the fertilizing capacity of spermatozoa *in vitro*. Acrosome integrity was judged by the Giemsa staining technique as per the methodology described by Hancock (1952). Smear prepared for non-eosinophilic sperm count was also used for enumerating sperm abnormalities. The slides were observed under an oil immersion lens. About 200 spermatozoa were counted in different fields and the percentage of different types of abnormal spermatozoa and total abnormal spermatozoa were calculated.

Capacitation status

The chlortetracycline (CTC) fluorescent staining was used for assessment of the capacitation status of fresh and frozen-thawed sperm as per the method described by Fraser *et al*. (1995). CTC staining solution was made by (750-µM CTC, 5-mM cysteine in 130-mM NaCl and 20-mM Tris HCl, pH 7.4).

Seminal biomarkers

Nitric Oxide was estimated in seminal plasma samples using “Bovine Nitric Oxide ELISA Kit” (Cat. No. E0249Bo) supplied by Bioassay Technology. Total Antioxidant Capacity was estimated in seminal plasma samples using the “Bovine TAC-ELISA Kit” Cat. No. E0384Bo. For lipid peroxidation, TBARS was estimated in seminal plasma using Quanti Chrom TMTBARS Assay Kit (DTBA-100), Lot, BI04A18.

Statistical analysis

The variations in semen parameters were quantified using one-way ANOVA. Unpaired t-test was used to assess levels of NO, TAC and TBARS in deep freeze and extended semen. Graph Pad prism (version 7) and SPSS software were used for statistical analysis. The *P* value≤0.05 was considered statistically significant. Results were expressed as mean±SE.

RESULTS AND DISCUSSION

In the present study it was observed that progressive motility, viability, membrane integrity (HOST +ve), acrosome integrity (Table 1) and total abnormalities (Table 2) declined significantly (P<0.01) at 24 h of cryopreservation as compared to fresh semen. These results are in conformity to the observations of various researchers who concluded that cryopreservation causes adverse effects on the spermatozoa manifested as a depression in viability rate, structural integrity, depressed motility and conception rates (Chen *et al*., 2015; Amidi *et al*., 2016; Ahmed *et al*., 2019; Dewry *et al*., 2020). This sharp reduction could be due to cryopreservation induced extensive biophysical damage as a consequence of supercooling, extracellular ice crystals formation, concentration of solutes such as sugars, salts and proteins (Lemma, 2011) and ultimately dehydration (Andrabi, 2007) causing the ultrastructural damage to membranes and destabilizing them; predisposing sperms to gross morphologic defects, such as missing and abnormal acrosomes. The higher level of ROS generation during the process of freezing was also reported to damage normal spermatozoa by inducing lipid peroxidation and DNA damage (Moustafa *et al*., 2004; Aitken *et al.,* 2016) and also deteriorate its quality by compromising membrane integrity, chromatin integrity and blocking oxidative metabolism (Lone *et al*., 2016). ROS attack reduces intracellular ATP concentration that leads to decreased sperm motility and viability; axonemal damage and increased sperm morphological abnormalities (Bansal and Bilaspuri, 2011). Moreover, in the present study, there was a non-significant reduction in the above mentioned sperm parameters observed at 1 and 2 month intervals. However, sperm viability reduced significantly up to 1 month of cryopreservation. This minute reduction in quality during the storage period could be due to the excess generation of ROS and RNS. Actually, neither of these reactive oxidants is toxic *in vivo* as these are regulated to a physiological concentration by the homeostasis process or by the complex antioxidant system. While during *in vitro* conditions the sperm competency is challenged due to higher production levels of ROS as compared to their detoxification (Du Plessis *et al*., 2008; Balamurugan *et al*., 2018). This hypothesis was in agreement with Pacher *et al*. (2007) and Radi (2013) where they reported that the detrimental effects of NO and O_2^- is mediated by formation of more noxious peroxynitrite. In consonance with the present study, Bansal and Bilaspuri (2011) detailed the excessive production of these reactive oxidants in semen can cause insufficient axonemal phosphorylation and ATP depletion which ultimately decreases sperm motility and viability. Moreover, polyunsaturated fatty acids (PUFAs) rich sperm plasma membrane is very susceptible to the attack of ROS due to binding of PUFAs to oxygen (Gadea *et al*., 2013) and RNS due to abstraction of hydrogen by NO, that causes a cascade of reactions, resulting in the production of free radicles triggering lipid peroxidation (Dhindsa *et al*., 2004; Semenova *et al*., 2005; Makker *et al*., 2009). The scrambling in phospholipid configuration is associated with misconfiguration of protein and lipids; morphological changes in the organization, fluidity, permeability of the sperm membranes and disruption of protein-lipid interactions (Gadella and Harrison, 2002) which disrupt sperm morphology and physiological activity (Casas *et al*., 2010; Flores *et al*., 2010; Sood *et al*., 2020).

The capacitation status and level of seminal biomarkers have been presented in Table 3 and Fig 1 respectively. The present study revealed significant loss of non-capacitated, acrosome intact (F pattern) sperms during cryopreservation in comparison to fresh semen. Percent capacitated sperms (B pattern) were significantly (p<0.01) greater in cryopreserved semen (37.13±0.64) as compared to fresh ejaculate (26.02±0.60) which is in consonance with

Table 1: Effect of storage intervals on seminal attributes.

Attributes	Fresh semen (0 h)	Cryopreserved semen		
		24 hours	1 month	2 months
Progressive motility	75.42^{x}±1.47	47.92^{y}±1.83	44.38^{y}±1.57	43.96^{y}±1.56
Viability	87.08^{x}±1.46	53.42^{y}±2.08	48.08^{z}±1.63	47.58^{z}±1.69
HOST+ve	61.25^{x}±2.48	30.21^{y}±1.93	27.46^{y}±1.36	27.04^{y}±1.22
Acrosome integrity	82.29^{x}±1.43	39.50^{y}±2.41	36.04^{y}±2.05	35.17^{y}±1.77

Values with different superscripts x, y and z within the rows differ significantly (P<0.05).

Table 2: Effect of storage intervals on percent head, mid-piece, tail and total abnormality.

Abnormality	Fresh semen (0 h)	Cryopreserved semen		
		24 hours	1 month	2 months
Head	2.75^{x}±0.31	3.83^{y}±0.30	3.79^{y}±0.25	4.46^{y}±0.28
Mid piece	2.08^{x}±0.29	3.00^{y}±0.21	2.83^{xy}±0.21	2.83^{xy}±0.21
Tail	7.21^{x}±0.38	9.79^{y}±0.57	10.04^{y}±0.61	9.75^{y}±0.52
Total	12.04^{x}±0.77	16.46^{y}±0.80	16.67^{y}±0.79	17.04^{y}±0.77

Values with different superscripts x and y within the rows differ significantly (P<0.05).

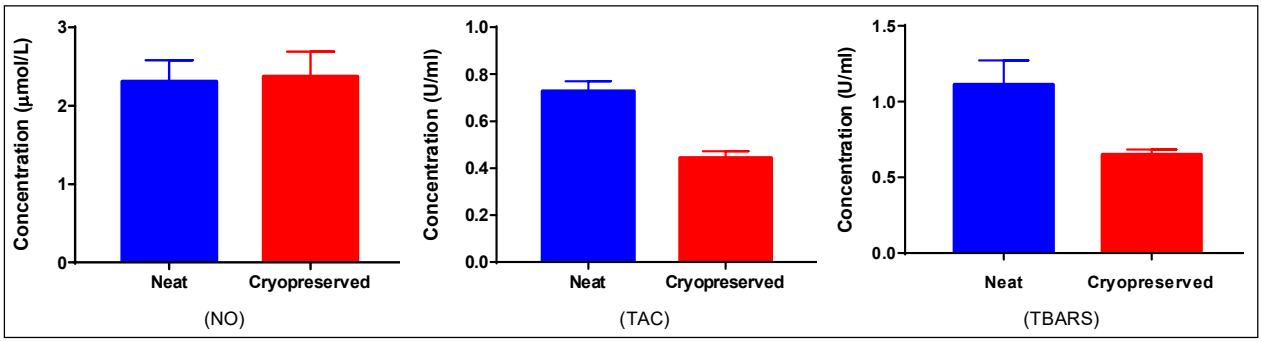

Fig 1: NO, TAC and TBARS levels in neat seminal plasma stored at -20°C and cryopreserved extended seminal plasma.

Table 3: Capacitation status in fresh and cryopreserved semen.

Semen Type	Capacitation status		
	Non-capacitated, acrosome intact (F pattern)	Percent capacitated sperms (B pattern)	Acrosome reacted (AR)
Fresh	66.17^{a}±0.67	28.40^{a}±0.60	5.43^{a}±0.45
Cryopreserved	55.15^{b}±0.54	37.13^{b}±0.64	7.52^{b}±0.47

Values with different superscripts a and b within the same columns differ significantly (P<0.05).

previous findings. After cryopreservation, surviving bull and human sperm contain more intracellular calcium than earlier, reûecting impaired membrane selective permeability mechanisms (Longobardi *et al*., 2017; Vignesh *et al*., 2020). The increased sperm calcium levels and reactive oxidants levels are thought to trigger an intracellular signaling cascade that has recently been associated with capacitation (Rodriguez and Beconi, 2009). This cryo-capacitation like change in sperms is induced due to the loss of membrane cholesterol during cryopreservation (Neild *et al*., 2003; Rajoriya *et al*., 2016). Thus it can be concluded that sperm membrane structural changes like efflux of cholesterol trigger the capacitation events, leading to an increase in the fluidity of plasma membrane, bicarbonate ($HCO3^{-}$), Ca^{2+}, intracellular pH and cAMP levels (Therien, 1995). A significant ($p<0.05$) increase in acrosome reacted spermatozoa was observed in cryopreserved semen (7.52±0.47) as compared to fresh samples in the present study. A body of evidence indicates that seminal plasma ameliorates cryo-injuries and possibly extends sperm longevity by somewhat delaying the undesired onset of capacitation and acrosome responsiveness (Vadnais *et al*., 2005; Vadnais and Roberts, 2007). In the present study, the mean levels of NO (µmol/L) in seminal plasma of neat semen stored at -20°C was 2.31±0.27, while the value in the extended seminal plasma after cryopreservation was 2.37±0.31. These results suggest that there is a non-significant increase in the production of reactive species, particularly NO during cryopreservation, which is in consonance with Ugur *et al*. (2019). Cryopreservation decreases the antioxidant defenses of whole semen characterized by the loss of superoxide dismutase activity in human and bull semen (Dewry *et al*., 2015; Aliakbari *et al*., 2016; Najafi *et al*., 2018) when compared to fresh semen. The total antioxidant capacity (Units/ml) in seminal plasma of neat semen (0.73±0.04) stored at -20°C was found to be higher in comparison to extended seminal plasma (0.44±0.03) obtained after cryopreservation and the possible reason behind this is the presence of oxygen and nitrogen free radicals which enhances its utilization and further reducing its quantity. Besides this, the reduction in the seminal antioxidant profile during cryopreservation further lowers the natural antioxidant capacity in semen (Lone *et al*., 2018). Thus seminal antioxidant defense becomes incompetent to maintain equilibrium between the production and detoxification of ROS, leading to oxidative stress during the freezing process (Dowling and Simmons, 2008) causing a reduction in the semen quality. The lipid peroxidation status (Units/ml) in seminal plasma of neat semen (1.11±0.16) stored at -20°C was found to be higher in comparison to extended seminal plasma (0.65±0.03) of the cryopreserved semen. Most of the lipid peroxidation occurs during phase transition of cryopreservation, however, due to the presence of antioxidants in egg yolk extender, reduction in lipid peroxidation during freezing in comparison to the storage of seminal plasma at -20°C was observed in our study. An earlier study indicated two fold increase in MDA production in frozen as compared to freshly ejaculated spermatozoa (Karan *et al*., 2018).

CONCLUSION

The findings of the present study suggest that most of the damages encountered by spermatozoa were during the initial period of freezing. This was evident by significant reduction in motility, viability, acrosome integrity, membrane integrity and there was an increment in the number of abnormal and capacitated spermatozoa. Even during the storage period, there were alterations observed which were marked by deteriorations in various seminal attributes, NO level and antioxidant status. Therefore, it is concluded that deteriorations in seminal parameters could be related to cryo-injury and free radicals induced stress perceived during the process of cryopreservation and also due to variations in seminal biomarkers during the storage period. We suggest

further investigation to analyze the levels of ROS and RNS at different intervals of storage and after thawing so that damages occurring during storage period by these reactive oxidants can be minimized.

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Compliance with ethical standards

The authors declare that they have no conflict of interest regarding authorship of this article. Authors also certify that neither article nor its any data send elsewhere for publication.

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