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Dynamics of mitochondrial membrane potential and DNA damage during cryopreservation of cattle and buffalo bull spermatozoa

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ABSTRACT

Understanding the changes in the spermatozoa during cryopreservation is indispensable for tailoring and increasing the efficiency of cryopreservation process success. However, the dynamics of damage to sperm organelles during different stages of cryopreservation is underexplored. This study assessed the mitochondrial membrane potential (MMP) and DNA damage during different stages of cryopreservation, viz. immediately after ejaculation, after equilibration and after freezing and thawing in cattle and buffalo spermatozoa using flow cytometry. Proportion of spermatozoa with high MMP decreased significantly after equilibration (from 66.06±4.59 to 42.58±6.30 in Holstein bulls and from 60.32±5.51 to 39.98±7.58 in buffalo bulls). Sperm DNA integrity [DNA fragmentation index (DFI %)] in Holstein Friesian (HF) bulls did not differ significantly between fresh and equilibrated samples but a significantly higher % DFI was observed in frozen-thawed semen samples as compared to both fresh and equilibrated samples. In contrast, % DFI in buffalo spermatozoa did not differ among the three stages of cryopreservation. It was concluded that mitochondrial damages occur during equilibration while chromatin damages occur during freezing and thawing of cattle bull spermatozoa; whereas buffalo bull spermatozoa were lesser susceptible to DNA damage during cryopreservation as compared to cattle spermatozoa.

Keywords: Cryopreservation, DNA damage, Equilibration, Mitochondrial membrane potential

In spite of several developments in reproductive biotechnologies, the first-generation technology—artificial insemination with cryopreserved spermatozoa—remains a main stay of genetic improvement in dairy animals across the globe (Walters *et al*. 2009). Even though bull spermatozoa are considered as cryo-resistant, as compared to several other species, they also suffer from architectural and functional losses during cryopreservation (Bailey *et al*. 2003). Cryodamage to spermatozoa has been understood to certain extent; consistently, outer sperm organelles like plasma membrane and acrosome were damaged during cryopreservation (Bailey *et al*. 2003). However, the dynamics of internal micro-organelles of spermatozoa during the process of cryopreservation is hitherto underexplored.

Intactness of sperm chromatin, a highly condensed structure that encompasses DNA and its associated proteins, is important for delivering the paternal DNA (male haploid genome) to oocyte (Oleszczuk *et al*. 2016). The DNA fragmentation index (DFI %) has been assessed in cryopreserved spermatozoa from bulls with different fertility ratings and it was found that higher the DFI, lower was the bull fertility (Kumaresan *et al*. 2017). Mitochondria are the powerhouses of spermatozoa located in the mid piece and important for spermatozoa to retain motility, longevity and fertilising capacity (Luo *et al*. 2013). However, mitochondria are one of the most cryo-susceptible organelles during cryopreservation (Moraes and Mayers 2018). The proportion of spermatozoa, in given frozen-thawed semen, with high mitochondrial membrane potential (MMP) has been reported to be related to fertility (Agnihotri *et al*. 2016).

Although it is understood that the process of cryopreservation alters sperm chromatin structure and MMP, the dynamics of DNA integrity and MMP during the process of cryopreservation is less understood. Among the tools used by various authors for sperm quality assessment, flow cytometric evaluation is objective, repeatable, fast and more quantitative (Martínez Pastor *et al*. 2010). Therefore, we assessed the sperm chromatin integrity and MMP in cattle and buffalo spermatozoa by using flow cytometry at

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different stages of cryopreservation, viz. immediately after ejaculation, after equilibration and after freezing and thawing. Here, we aim to explore the effect of equilibration and ultra-low temperature (-196°C) preservation on DNA integrity and MMP in cattle and buffalo spermatozoa.

MATERIALS AND METHODS

Semen collection and preservation: Holstein Friesian (HF) bulls (n=6) and Murrah buffalo bulls (n=6) maintained at Central Frozen Semen Production and Training Institute, Hesaraghatta, Karnataka, India under uniform feeding (Daily ration of 3 kg Concentrate, 2–4 kg hay, 100 g mineral mixture, *ad lib.* green fodder and fresh water) and housing conditions were used in this study. Ejaculates were collected from the bulls using artificial vagina (AV) as per the standard procedure in early morning. Briefly, the bulls were sexually prepared by giving false mounts and the sterile bull aprons were used to avoid the penis touching dummy's hindquarters. The penis of the bull was guided through AV to collect the ejaculate during thrust. Three ejaculates from a bull, which fulfilled the standard requirements (>600 million spermatozoa/mL; >70% progressive motility and <20% sperm abnormalities), were used for the experiment. The semen was cryopreserved as per the standard protocol (Layek *et al*. 2016, Grotter *et al*. 2019). Briefly, semen was diluted in Tris-citric acid extender (3.02% w/v Tris, 1.67% w/v citric acid, 1% w/v fructose, *p*H 6.8; osmotic pressure, about 300 mOsm/kg) containing egg yolk (20% v/v) and glycerol (7% v/v) and loaded in 0.25 mL plastic straws (IMV, France). The concentration of glycerol and egg yolk used in the study were as per the standard method recommended for cattle and buffaloes (Layek *et al*. 2016, Grotter *et al*. 2019). Semen was equilibrated in cold handling cabinet at 5°C for 4 h (IMV technologies, France) as per previous reports (Leite *et al*. 2010, Shahverdi *et al*. 2014) and transferred to the chamber of a programmable freezer. The freezing rate from 5°C to –10°C was 5°C/min, from –10°C to –100°C was 40°C/min and thereafter from –100°C to –140°C, it was 20°C/min. Once the temperature reached –140°C, semen straws were immediately plunged into liquid nitrogen (-196°C) for storage.

Sperm preparation for MMP and chromatin structure assay: Aliquots of spermatozoa were taken at three time periods (immediately after collection, after equilibration and after freeze thawing) and analyzed for MMP and chromatin structure by using flow cytometry. Cryopreserved spermatozoa were thawed at 37°C for 30 s before analysis. MMP of spermatozoa was assessed by using 5, 5′, 6, 6′-tetrachloro 1, 1′, 3, 3′-tetraethyl benzimidazolyl carbocyanine iodide (JC-1) as per the procedure given by Moce and Graham (2008) with slight modifications. The principle behind this test was that spermatozoa with actively functioning mitochondria (high MMP) takes more amounts/ forms aggregates of JC-1 as compared to poorly functioning mitochondria. At low concentrations, JC-1 existed as a monomer and fluoresces green, but at higher concentrations, it formed aggregates and fluoresces red-orange. Initially, JC-1 was diluted in Dimethyl sulfoxide (DMSO) to obtain a stock solution of 7 mM. From this, working solution of JC-1 (1.53 mM) was made by further diluting the stock solution with DMSO. Approximately 150 µL of JC-1 (1.53 mM) was taken into 1 mL of Tris diluent (200 mM Tris, 65 mM citric acid and 55 mM glucose) to obtain a final JC-1 concentration of 0.2 mM working solution. JC-1 working solution (28 µL) was added to 100 µL of semen suspension containing 10 million sperm, and incubated for 30 min at 39°C. After incubation, 50 µL of the stained spermatozoa were diluted in 1 mL of Tris diluent, and analysed in a flow cytometer.

Sperm chromatin structure assay (SCSA) was used to measure the susceptibility of sperm DNA to fragmentation after denaturation *in situ*, which was induced by low *p*H treatment. Acridine orange (AO) was the probe used in SCSA, which could bind to intact DNA to fluoresce green and could bind with the DNA strands at the sites of breaks to stain red. SCSA was carried out by using the protocol given by Evenson (2013) with little modifications. Spermatozoa (10 million) from fresh, equilibrated and cryopreserved semen were diluted in TNE (Tris, NaCl, EDTA) buffer [0.01 M of Tris–HCl, 0.15 M of NaCl, 1 mM of ethylene diamine tetra acetic acid (EDTA), *p*H 7.4] to make a final sperm concentration of approximately 1 million per mL. From this suspension, 200 µL was taken and mixed with 400 µL of low *p*H detergent solution for inducing partial DNA denaturation (0.1% of Triton X-100, 0.15 M of NaCl, and 0.08 N of HCl, *p*H 1.2). After 30 s, 1.2 mL of staining solution (0.2 M of Na_2HPO_4,1 mM of EDTA, 0.15 M of NaCl, 0.1 M of citric acid, 6 mg AO/mL staining buffer, *p*H 6.0) was added and analyzed within 3–5 min after AO staining in a flow cytometer.

Flow cytometric analysis of MMP and chromatin structure: Flow cytometer (BD Accuri C6; BD biosciences – Becton Dickinson) equipped with 14.7 mW 640 nm diode red laser and 20 mW 488 nm solid state blue laser was used to analyze spermatozoa labelled with JC-1 and AO. While existing as monomer, JC-1 produced green fluorescence (530 nm), which was detected by using fluorescence channel (FL) 1 (533/30 nm). On the other hand, when JC-1 existed in the form of polymer, it showed orange-red fluorescence (580 nm), which was detected by using FL2 (585/40 nm). Green florescence (dsDNA) produced by AO orange was detected by using FL1 (533/30 nm) and red (denaturated, ssDNA) colour produced by AO was detected by FL3 (>670 nm). In both assays, the spermatozoa population was first identified by a combination of forward-scatter and side-scatter information. Gatings were done to eliminate small particles and cell debris. In JC-1 assay, % of sperm having high MMP was identified by a combination of FL1 and FL2 detector. In SCSA, cells stained with AO were gated from the plot produced by the combination of both FL1 and FL3. Florescence compensation was performed for minimizing the fluorescence spill over.

Statistical analysis: Flow cytometry related data were handled by BD Accuri C6 software and the data were

exported to FCS Express 6 plus software (De Novo Software, Los Angeles, CA, USA) for further analysis and creating quality images. The values were expressed as mean±standard deviation (SD). Statistical analysis of the data was done by using SPSS software (version 22.0, IBM, USA). One-way analysis of variance with repeated measures was used to find out the differences in the values obtained at three different stages of cryopreservation. Mixed model analysis of variance was used to find the interaction effect between species and different stages of cryopreservation. DFI% was calculated from the SCSA results of flow cytometry based on the formula given by Evenson (2013). The difference between two parameters was considered significant when the P value was <0.05.

Ethical approval: All the experimental procedures were duly approved by the Institute Animal Ethics Committee (CPCSEA/IAEC/LA/SRS-ICAR-NDRI-2019/No.04 dated 3rd January 2019) and performed in accordance with relevant guidelines and regulations.

RESULTS AND DISCUSSION

The functional state of sperm mitochondria as an ATP producing site is important for reaching the site of fertilization, and maintenance of DNA integrity is crucial for fertility, health and well-being of the next generation. Since it is shown that cryopreservation affects sperm quality, understanding the dynamics of crucial sperm attributes during the process of cryopreservation is important to take a decision on tailoring cryopreservation procedure in future. In this regard, we reported the dynamics of MMP and DFI in cattle and buffalo spermatozoa during the process of cryopreservation. After freezing and thawing, all the samples had post-thaw sperm motility >50%, which was a pre-requisite for use in artificial breeding. Sperm MMP and DNA integrity were assessed during the process of cryopreservation at three time points and the results are represented in Tables 1 and 2.

Mitochondrial membrane potential (*MMP*): The flow cytometric plots of MMP analysis are shown in Fig. 1. The green outlined population (gate 4) indicates the sperm having high MMP, whereas the red outlined population (gate 6) indicates the sperm having low MMP. The proportion of spermatozoa with high MMP decreased gradually during the process of cryopreservation in both the species (cattle and buffalo) studied. The proportion of spermatozoa with high MMP decreased significantly (P<0.05) after equilibration (from 66.06±4.59 to 42.58±6.30 in HF bulls and from 60.32±5.51 to 39.98±7.58 in buffalo bulls). On the other hand, there were no significant differences in the proportion of spermatozoa with high MMP between equilibrated and frozen-thawed samples in both the species. Consistently, the proportion of spermatozoa with low MMP was significantly (P<0.05) higher in equilibrated and frozen-thawed semen samples as compared to fresh semen in both the species.

Cooling/equilibration process lowers down the metabolic activity of spermatozoa; on the other hand, at ultra-low temperature, freezing of spermatozoa almost arrests the sperm metabolism. Contrary reports exist on the effect of different stages of cryopreservation process on sperm MMP. The proportion of spermatozoa with high MMP observed in our study is in agreement with those reported by Gurler *et al*. (2016). The finding that the proportion of spermatozoa with high MMP decreased during equilibration is in agreement with Shah *et al*. (2016) who also observed a detrimental effect of equilibration on sperm MMP in buffaloes. Similarly, a significant reduction in the proportion of spermatozoa with high MMP during cryopreservation

Table 1. High MMP % in HF and Murrah buffalo bulls during different stages of cryopreservation

Species/breed	Stage of cryopreservation	Mean± Standard Deviation	Relationship between stages	P-value
Cattle/Holstein Friesian	Fresh	66.06^{a}±4.59	Fresh vs Equilibrated	0.001
	Equilibrated	42.58^{b}±6.30	Equilibrated vs Frozen	0.074
	Frozen	39.10^{b}±7.52	Fresh vs Frozen	0.001
Buffalo/Murrah	Fresh	60.32^{y}±5.51	Fresh vs Equilibrated	0.005
	Equilibrated	39.98^{z}±7.58	Equilibrated vs Frozen	1.000
	Frozen	37.36^{z}±4.20	Fresh vs Frozen	0.001

Mean values having different superscripts within species are significantly different (P<0.05).

Table 2. DFI% in HF and Murrah buffalo bulls during different stages of cryopreservation

Species/breed	Stage of cryopreservation	Mean± Standard Deviation	Relationship between stages	P-value
Holstein Friesian	Fresh	5.04^{a}±0.26	Fresh vs Equilibrated	1.000
	Equilibrated	4.81^{a}±0.57	Equilibrated vs Frozen	0.000
	Frozen	11.04^{b}±0.64	Fresh vs Frozen	0.000
Murrah	Fresh	4.91^{z}±0.10	Fresh vs Equilibrated	0.618
	Equilibrated	5.08^{z}±0.27	Equilibrated vs Frozen	0.929
	Frozen	4.94^{z}±0.23	Fresh vs Frozen	1.000

Mean values having different superscripts within species are significantly different (P<0.05).

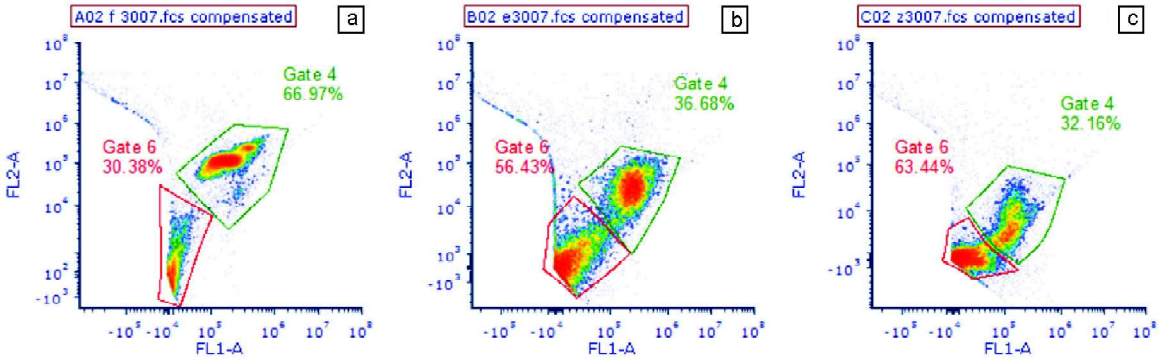

Fig. 1. Flow cytometric plots of sperm mitochondrial membrane potential during different stages of cryopreservation (A, fresh semen; B, equilibrated semen; C, frozen semen).

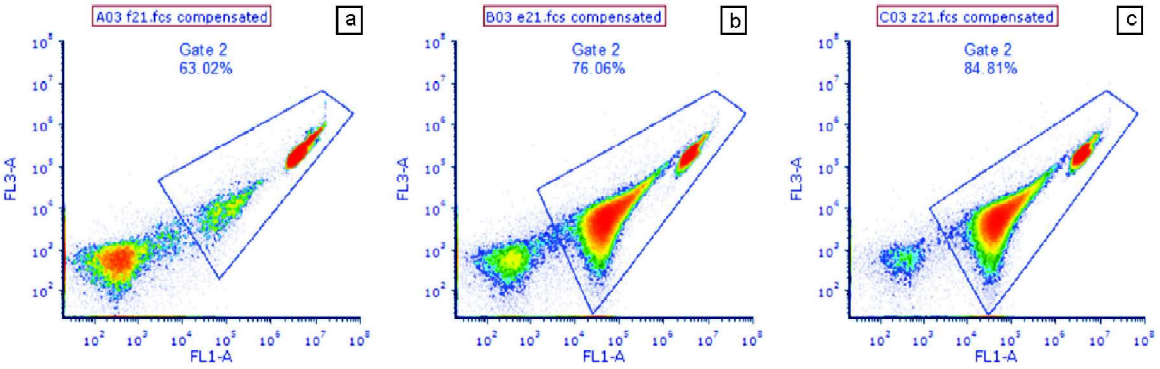

Fig. 2. Flow cytometric plots of sperm chromatin structure assay during different stages of cryopreservation (A-fresh semen; B-equilibrated semen; C-frozen semen). Gating is done for eliminating the cell debris. Gated population is indicating the cells that were stained by Acridine Orange.

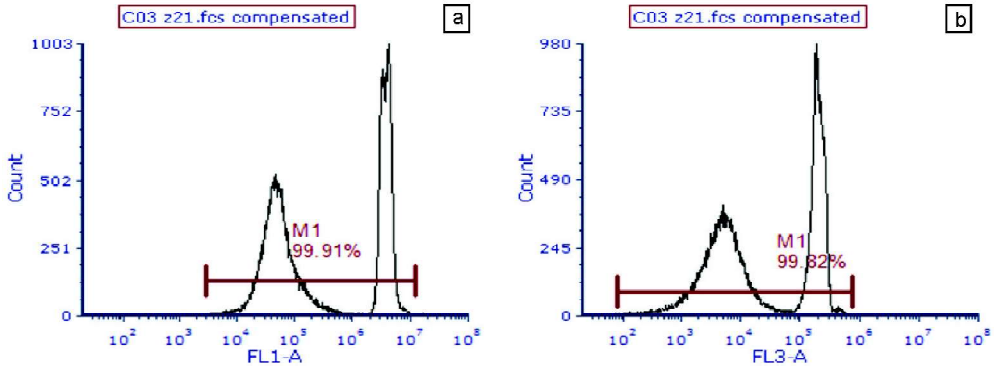

Fig. 3. Flow cytometric panels of green (A) and red (B) fluorescence detected during SCSA.

was also reported by Kadirvel *et al*. (2012) and Kumar *et al*. (2016). Sperm motility is one of the parameters most seriously affected by freezing (Raad *et al*. 2018). Despite its importance, the mechanism by which motility is reduced has not been elucidated. It is well understood that sperm motility is partially dependent on mitochondrial function and mitochondrial damage is an important factor associated with reduced motility (Kadirvel *et al*. 2012). The main function of mitochondria is to generate ATP through the oxidative phosphorylation, and ATP is the energy source for sperm tail swing. Therefore, loss of MMP may lead to obstacles of energy synthesis, thereby reducing sperm motility (Paoli *et al*. 2011), which might be the reason for reduced sperm motility after cryopreservation. Further, it was suggested that lipid peroxidation occurring during cryopreservation is the major reason for the damage of phospholipids, which is essential for the integrity of mitochondrial sheath and compromised motility (Ortega-Ferrusola *et al*. 2009, Ourique *et al*. 2013). Several reports are available about the mechanical, chemical and osmotic stress that is happening to spermatozoa during freezing and thawing. However, the information on the effect of equilibration on sperm MMP is limited. Our findings suggest that, the stress encountered by the spermatozoa

during equilibration itself is sufficient to induce damage to mitochondria leading to reduction in the proportion of spermatozoa with high MMP.

DNA fragmentation index (*DFI*): Sperm DNA damage was assessed by SCSA and the data were represented as DFI%. The flow cytometric plots of SCSA are shown in Fig. 2 (Gated population indicates sperm, while the remaining are cell debris). Flow cytometric plots of FL1 (green fluorescence – Fig. 3A) and FL3 (red fluorescence – Fig. 3B) detectors are shown in Fig. 3. DNA fragmentation index (%) was calculated by the formula:

$$[\text{DFI \%} = (\text{red}/\text{red} + \text{green fluorescence}) \times 100]$$

The effect of cryopreservation on sperm DFI (%) was species dependant. In other words, DFI (%) of HF bull sperm increased after freezing and thawing, but such changes were not observed in case of Murrah buffalo spermatozoa. In HF bulls, the DFI (%) did not differ significantly between fresh (5.04±0.26) and equilibrated (4.80±0.57) samples but a significantly ($P<0.05$) higher DFI (%) was observed in frozen-thawed semen (11.04±0.64) samples as compared to both fresh and equilibrated samples. There were no significant differences in the DFI (%) among the fresh, equilibrated and frozen-thawed semen samples in Murrah buffaloes. The interaction was not significant between species and stage of cryopreservation for MMP ($F=0.848$, $P=0.38$), whereas a significant interaction effect was observed for DNA damage ($F=640.63$, $P<0.01$).

Unlike MMP where the detrimental effect of equilibration was observed in both the species, a clear species variation was observed in terms of sperm DNA damage in frozen thawed spermatozoa. Our observations on HF bulls are in agreement with Gurler *et al*. (2016), who also reported an increase in DNA damage after freezing. However, he observed a marginal increase in the DNA damage after cryopreservation but we observed a significant variation. This might be due to individual bull variations and the extenders used for cryopreservation; earlier workers used soybean-based extender, while we used egg-yolk extender. Several studies indicated that cryopreservation process induced sperm DNA damage in human (Zribi *et al*. 2010), bull (Waterhouse *et al*. 2010), ram (López-Fernández *et al*. 2010) and boar (Fraser *et al*. 2011). Earlier studies opined that the oxidative stress induced during freezing–thawing process is associated with damages mediated by free radicals to sperm nuclear DNA. We observed that the DNA damage did not increase during equilibration, but increased only after freezing and thawing. This may be due to more oxidative stress that happens during freezing and thawing. Further, it was reported that oxidative stress due to endogenous and various exogenous factors is one of the leading causes of DNA damage and is the major cause of loss of integrity of both mitochondrial and nuclear genome (Ferramosca *et al*. 2013, Bui *et al*. 2018). The findings observed with buffalo spermatozoa that no change in the DNA fragmentation index during the process of cryopreservation was surprising. However, earlier reports also indicated that buffalo spermatozoa were less susceptible to DNA damage during cryopreservation. For instance, Kadirvel *et al*. (2009, 2012) also found no significant differences in DNA damage between fresh and frozen Murrah buffalo semen. Kumar *et al*. (2016) also assessed the DNA damages in the fresh, equilibrated and frozen semen of Murrah buffalo semen and reported that DNA integrity of buffalo bull semen was not affected during any stage of cryopreservation. Our results, along with earlier reports, indicate that buffalo sperm DNA is less affected during cryopreservation. This may be due to less susceptibility of sperm DNA to endonucleases during cryopreservation due to compact nucleus and DNA (Kumar *et al*. 2016).

Based on the above findings, it may be hypothesised that the quantum of stress encountered by cattle and buffalo spermatozoa during equilibration was sufficient to induce mitochondrial damage but not DNA damage in spermatozoa. During freezing, spermatozoa undergo huge thermal insults and while thawing, they undergo substantial oxidative stress, both might lead to sperm DNA damage (Kumaresan *et al*. 2020). Yet another reason for the differential susceptibility of mitochondria and DNA to cryopreservation associated damages could be due to differences in their location in spermatozoa, which is a possibility that needs to be tested.

In conclusion, our results indicate that mitochondrial damages occur during equilibration while chromatin damages occur during freeze thawing of cattle bull spermatozoa. Previously, sperm organelles damage was thought to be the effect of freeze-thaw procedures but the findings of our study indicate that damage to sperm mitochondria occur during the process of equilibration itself. Further, buffalo spermatozoa are comparatively less susceptible to DNA damage as compared to cattle spermatozoa.

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