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Nagendra Singh
Ph D scholar, Department of Veterinary Gynaecology and Obstetrics, College of veterinary science and animal science, Sardar Vallabhbhai Patel University of Agriculture and Technology, Modipuram, Meerut, Uttar Pradesh, India

Akhil Patel
Assistant professor, Department of Veterinary Gynaecology and Obstetrics, College of veterinary science and animal science, Sardar Vallabhbhai Patel University of Agriculture and Technology, Modipuram, Meerut, Uttar Pradesh, India

Suresh Kumar
Principal Scientist, ICAR-Central Institute for Research on Cattle, Meerut, Uttar Pradesh, India

Megha Pande
Scientist, ICAR-Central Institute for Research on Cattle, Meerut, Uttar Pradesh, India

Vijay Singh
Professor, Department of Veterinary Gynaecology and Obstetrics, College of veterinary science and animal science, Sardar Vallabhbhai Patel University of Agriculture and Technology, Modipuram, Meerut, Uttar Pradesh, India

Corresponding Author:
Nagendra Singh
Ph D scholar, Department of Veterinary Gynaecology and Obstetrics, College of veterinary science and animal science, Sardar Vallabhbhai Patel University of Agriculture and Technology, Modipuram, Meerut, Uttar Pradesh, India

Studies on detrimental effect of cryopreservation on sperm progressive motility, viability, plasma membrane integrity and oxidative stress in Frieswal bull semen

Nagendra Singh, Akhil Patel, Suresh Kumar, Megha Pande and Vijay Singh

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Abstract
This experiment was designed to study the effect of cryopreservation on post-thaw sperm quality and oxidative stress in Frieswal bull semen. For this purpose, 18 ejaculates were collected from three Frieswal bulls using artificial vagina at biweekly intervals. The semen sample which possesses more than 70% progressive motility and above 600 million/ml spermatozoa concentration was subsequently subjected to a process for LN¬2 vapour freezing. Semen samples were extended in the GEYT extender. At the fresh stage, semen was evaluated for percent live spermatozoa, percent progressive motility, percent intact acrosome, and percent HOST reactive spermatozoa. At the post-thaw stage, semen was evaluated for percent progressive motility, percent live spermatozoa, percent intact acrosome, percent HOST reactive spermatozoa. Post-thaw semen, showed a significant (p<0.05) decrease in percent progressive motility, percent live spermatozoa, percent intact acrosome, and percent HOST reactive spermatozoa, while level of ROS was elevated. It was concluded that cryopreservation leads to the detrimental effect on spermatozoa, this detrimental effect was due to OS.

Keywords: Cryopreservation, Post thaw, Oxidative stress, Spermatozoa

Introduction
Overall production of developing nations is quite low, despite having abundant genetic resources for cattle. For dairying to continue to be successful and to ensure food security for the growing population, this must be accelerated. Propagating elite germplasm and relying on the idea of producing "a calf per cow per year" through timely insemination and successful conception are the best approaches. An important step in this direction has been the cryopreservation of spermatozoa, which has made cryopreserved spermatozoa widely available for use in artificial insemination (AI) procedures (Ugur *et al*., 2019) [25]. However, insemination with low fertilising capacity cryo injured spermatozoa is responsible for a large loss to the dairy industry (Chung *et al*., 2019; Dewry *et al*., 2020; Singh *et al*., 2020) [9, 10, 23].
The main factors that lead to cryo-injury include temperature changes, the creation of osmotic stress, and the formation of ice crystals, which modify the sperm cell's physiology and structure and cause irreversible damage (Ahmed *et al*., 2019; Upadhyay *et al*., 2021) [2, 26]. While cryopreservation extends the shelf life of spermatozoa, the process can also cause cryo-injury that can lead to changes in plasma membrane permeability, acrosomal destruction, mitochondrial membrane depolarization, and loss of sperm motility (Upadhyay *et al*., 2021) [26]. These elements play a role in the inability of spermatozoa to engage with the female reproductive tract, which leads to the failure of fertilization (Ezzati *et al*., 2020) [11]. The freezing-thawing process alters the physiology and deforms the structure of spermatozoa by causing alterations in the osmotic balance, producing an excessive amount of reactive oxygen species, and forming intracellular ice crystals (Ugur *et al*., 2019) [25].
Numerous factors, including elevated oxidative metabolism and osmotic stress, can cause oxidative stress. A state of oxidative stress develops Because of an imbalance between the sperm cells' capacity to produce reactive oxygen species (ROS) and their antioxidant capacity.

It is distinguished by the presence of DNA breaks in the cryopreserved male gametes and the induction of changes to the sperm membrane. Seminal plasma's antioxidant qualities act as a natural defence against reactive oxygen species (ROS) and control the intracellular redox potential, particularly in stressful situations like low temperatures during cryopreservation. The production of NADPH oxidase in the plasma membrane and modifications to the electron transport chain located in the mitochondria are brought about by the enhanced formation of ROS (Len *et al*., 2019; Peris-Frau *et al*., 2020) [17, 21]. Because of this, oxidative stress is regarded as one of the primary factors that lead to structural and molecular cell damage. Before the freezing procedure, the seminal plasma is completely removed or diluted, which results in a loss in the antioxidant potential. Lipid peroxidation is another oxidative stress-related consequence. Polyunsaturated fatty acids, which are susceptible to ROS-induced peroxidation, are present in relatively high concentrations in sperm membranes. The major cell respiratory system is located in the middle (mitochondrial) region of the sperm flagellum, which is where lipid peroxidation mostly occurs. DNA damage, ATP production reduction, motility loss, and lipid layer damage to cell membranes are possible outcomes of this (Benko *et al*., 2021) [6]. Objective of this study was to study the detrimental effect of Cryopreservation on Frieswal bull spermatozoa.

Materials and Methods

The present study was undertaken at the Semen Freezing Laboratory, Division of Cattle Physiology and Reproduction, ICAR-Central Institute for Research on Cattle, Meerut Cantt., India (29º N, 78° E and altitude 270 m). It has a monsoon-influenced humid subtropical climate characterized by hot summers and cold winters.

For this study, three healthy Frieswal (Holstein-Friesian × Sahiwal) breeding bulls with an average weight of 500–600 kg were kept in uniform housing and fed. Regular normal procedure was followed, and the ejaculates were collected twice a week in the early morning between 8 and 9 AM in graded glass tubes attached to the artificial vagina. Prior to actual collection, each bull was given one false mount. Study samples were chosen based on three criteria: ≥ 2 ml volume, ≥ 600 million spermatozoa per ml concentration, and ≥ 70% individual progressive motility (IPM). Volume was measured by graduated Collecting tube. The Concentration of the spermatozoa (millions/ml) was determined by Accucell Photometer (IMV Technologies, France).

Following the initial evaluation of the semen, the selected samples were extended using a pre-heated (37 °C) glycerol egg yolk tris (GEYT) extender containing the following: tris (3.02 g), citric acid (1.67 g), fructose (1.25 g), penicillin (100,000 IU), streptomycin (100 mg), glycerol (7 ml), and egg yolk (20 ml) in Millipore water. Approximately 80 million progressive motile sperm cells/ml were maintained as the sperm cell concentration. Four characteristics were evaluated at the fresh stage: a) initial progressive motility; b) the percentage of viability of spermatozoa; and c) the integrity of the acrosomes and d) the integrity of the plasma membrane. Progressive motility, live spermatozoa and Acrosome integrity were assessed as per Salisbury *et al*., (1978) [22]; HOST test was performed as described by Jeyendran *et al*., (1984) [13]. Following the filling and sealing of 0.25 Ml French micro straws, the prolonged semen was allowed to acclimate for four hours in a horizontal cold handling cabinet (Minitube).

A programmable automated freezing system (IMV, France) was used to freeze the samples. It had a rate freezing range of 4 °C to -10 °C @ 5 °C, -10 °C to -100 °C @ 40 °C, and -100 °C to -140 °C @ 20 °C once the temperature reached -140 °C. The straws were frozen and then kept until they were tested in cryogenic containers submerged in liquid nitrogen (-196 °C). In a thawing unit (CITO thawer 12/220V, IMV), frozen samples were thawed for at least 30 seconds at 37 °C. At the post-dilution and post-thaw stages of the semen processing, total ejaculates (n = 18) were assessed for percentage Live spermatozoa, acrosomal integrity, progressive motility, and hypo-osmotic swelling test. However, only when thawing is complete test for oxidative stress (ROS) was performed. Oxidative Stress test *viz*. ROS assessment in seminal plasma was done via a procedure described by Hayashi *et al*. (2007) [12]. The Results were analysed statistically using Analysis of Variance (ANOVA) (Snedecor And Cochran, 1989) [24].

Results

The range of initial progressive motility of spermatozoa, percentage of live spermatozoa, acrosome integrity, plasma membrane integrity were with a mean value of 71.11±0.76%, 85.170.65%, 77.50±1.20% and 81.44±0.56%, respectively. As compared to the fresh, the progressive motility was found to be significantly ($p<0.001$) reducedat post-thaw stage (33.06±1.81), the viability was found to be significantly ($p<0.05$) reduced at the post-thaw stage (38.94±2.09) & the mean percentage of spermatozoa with intact acrosome was significantly reduced (62.17±1.98) after freeze-thaw process & mean percentage of spermatozoa with intact plasma membrane was significantly reduced (35.50±1.76) after freeze-thaw process. Percent decrease in progressive motility, viability, acrosome integrity and plasma membrane integrity after thawed semen were in accordance with Pande *et al*., (2019) [19]. Reactive oxygen species value was 212.91±3.17 (unit of H_2O_2) in post thaw semen.

Table 1: Effect of cryopreservation on progressive motility, viability, acrosome integrity, plasma membrane integrity and ROS level in bull semen (18 ejaculates from three bulls)

Parameter	Fresh	Post thaw	Decrease by
Progressive motility % (Mean± SEM)	71.11±0.76^a	33.06±1.81^b	38.05
Viability % (Mean± SEM)	85.17±0.65^a	38.94±2.09^b	46.23
Intact acrosome % (Mean± SEM)	77.50±1.20^a	62.17±1.98^b	15.33
Plasma membrane Integrity % (Mean± SEM)	81.44±0.56^a	35.50±1.76^b	45.94
ROS (Units of H_2O_2)	-	212.91±3.17	-

Discussion

Numerous factors contribute to the cryopreservation of semen, which is known to harm sperm in various ways. Reduced survival rates, motility, changes to the plasma membrane, mitochondria, acrosomes, DNA integrity, and oxidative stress are the results of such damages (Lemma, 2011; Upadhyay *et al*., 2021) [16, 26]. Thus, it is not unexpected that in our investigation, frozen semen samples had lower values of the sperm parameters progressive motilityl, viability, acrosome integrity, and plasma membrane integrity than fresh semen samples. One of the main underlying causes of sperm damage in cryopreservation is the production of ROS, which subsequently affects sperm characteristics such

as viability and post-thaw motility. The precise role that superoxide anion (SO) and H_2O_2 play in male fertility is yet unknown (Cheng *et al*., 2022) [8]. H_2O_2damages DNA in bovine sperm cells without altering other ROS levels. Several other studies have already shown that the cryopreservation process causes DNA damage to mammalian sperm in human, boar, bull and ram (Ezzati *et al*., 2020; Keskin *et al*., 2020; Aitken *et al*., 2021; Yánez-Ortiz *et al*., 2022) [11, 15, 3, 27]. There is strong evidence indicating that the freezing–thawing process induces oxidative stress associated with free radical-mediated damage to sperm nuclear DNA (Ozimic *et al*., 2023; Abdulkareem *et al*., 2024) [18, 1].

Our present study was in accordance with the he previous studies from our laboratory also reported a reduction in post-thaw sperm motility, viability, acrosome integrity and plasma membrane integrity with elevation of oxidative stress markers likereactive oxygen species (Pande *et al*., 2018; Pande *et al*., 2019) [20, 19].

Conclusion

We observed that dilution, equilibration and freezing with cryoprotectants do not produce ultrastructural damage despite lowering sperm motility, viability, acrosome integrity and plasma membrane integrity using conventional cryopreservation technique. The structure of the plasma membrane showed the most obvious damage, with more enlarged and missing membranes during the freeze-thawing phase. Following freezing, there was also noticeable chromatin, mitochondrial, and acrosomal damage. In order to improve the cryopreservation procedures for bull semen used in artificial intelligence, we have emphasized that mitigating damage to the sperm plasma membrane, and DNA should be evaluated and targeted during equilibration and upon freezing, before the majority of the damage is began.

It is concluded that the process of freezing and thawing significantly reduced semen quality of Frieswal bull in terms of various functional parameters including motilities, plasma membrane integrity, acrosome integrity of Frieswal bull spermatozoa.

Conflict of interest

The authors have no conflict of interest in this study.

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