RESEARCH ARTICLE

Effect of Modified Freezing Diluent on Post-Thaw Sperm Characteristics of Poor Freezable Sahiwal Bull

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Abstract

The study aimed to enhance the cryo-survivability and fertilization potential of poor freezable Sahiwal bull semen by devising a modified extender and freezing protocol. This protocol incorporated a combination of trehalose and glycerol as cryoprotectants and freezing with an aluminium block. The semen samples were split into three; the split 1 was extended in routine tris fructose egg yolk glycerol (TFEG) extender and underwent the conventional vapour freezing (Control group). The other split samples considered as treatment samples were extended with diluent having 75 % of TFEG and 25 % of trehalose (280 mOsm/l both) with glycerol percentage being 5, and frozen using a conventional vapour freezing (T1) and modified freezing protocol with an aluminium block (T2). After freezing-thawing, the progressive sperm motility, viability, HOS response and acrosome integrity were significantly ($p \leq 0.05$) improved in treatment 2 compared to the control and treatment 1 groups. Along with this, the degree of oxidative stress (MDA production) was substantially lower in treatment groups (T1 and T2) compared to control group. The results highlight the promising effectiveness of the modified freezing method incorporating trehalose and glycerol as cryoprotectants and using an aluminium block. The significance of these findings lies in the potential application of this technique for freezing-thawing semen, particularly from bulls with poor freezability.

Key words: Bull, Glycerol, Modified freezing, Sperm, Trehalose.

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INTRODUCTION

The implementation of artificial insemination (AI) has led to a significant increase in milk production, along with advancements in genetic improvement through the use of superior male germplasm. However, despite the preparation of a substantial number of frozen semen doses (122 million doses), there remains a deficit over the required number of doses (~156 million doses) (DAHD, 2022-23). To address this issue, it is possible to rectify the problem by utilizing poor freezable ejaculates by incorporating certain cryoprotectants in the freezing medium and modifying the freezing process. Various strategies and techniques have been developed to address the issue of cryoinjuries to sperm in different animal species such as vitrification with a combination of cryoprotectants in stallions (Consuegra *et al.,* 2019), as well as vitrification without cryoprotectants in humans (Isachenko *et al.,* 2003). In addition to altering the freezing process, different cryoprotectants such as Trehalose are used to enhance the quality of post-thaw semen.

Trehalose has several beneficial effects during cryopreservation, dehydration, and rehydration of sperm. It increases fluidity, rearranges the sperm membrane, and reduces the membrane's phase transition temperature of dry lipids by interacting with plasma membrane phospholipids (Fernández-Santos *et al.,* 2007). The unique interactions between trehalose and membrane phospholipids contribute to its protective effects, as it acts as a water substitute at the membrane solution interface due to its osmotic properties (Bakas and Disalvo, 1991). Comparatively, trehalose provides better protection for membrane functionality than glycerol and sucrose (El-Sheshtawy *et al.,* 2015; Starciuc *et al.,* 2020). Glycerol, when used above a specific quantity, has been observed to cause depolymerization of actin in the sperm cytoskeleton and exhibit cytotoxic effects (García *et al.,* 2012). Ahmed *et al*. (2020) demonstrated that 5% glycerol (with 1% fructose) had a positive impact on improving the quality of frozen-thawed sperm and the fertility of buffalo bull sperm *in vivo*. Baruah *et al.* (2013) in Mithun also noted favorable effects of sperm cryopreservation using 5% glycerol.

The significance of cryoprotectants in the freezing of semen cannot be overstated. Both permeable and non-permeable cryoprotectants are gaining importance due to their reduced toxicity to sperm (Isachenko *et al.,* 2003).

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No prior information exists regarding the combination of permeable and non-permeable cryoprotectants, along with a modified freezing approach, for the preservation of poorly freezable bull semen. Therefore, this experiment was aimed to assess the effectiveness of modified freezing in comparison to conventional vapour freezing in preserving the semen parameters of Sahiwal bulls with poor freezability, with the goal of identifying the more efficient method.

Materials and Methods

Ethical Permission and Procurement of Chemicals/ Reagents

Institutional Animal Ethical Committee approved all experimental protocols and semen collection using artificial vagina (45-IAEC-19-4). All the required chemicals including benzylpenicillin, streptomycin and trehalose were procured from Sisco Research Laboratories (SRL, Mumbai, India), and fluorescent stains were procured from Sigma-Aldrich® (Milano, Italy).

Experimental Bulls and Semen Collection

The study was carried out at Artificial Breeding Research Centre (ABRC), ICAR-National Dairy Research Institute, Karnal (Haryana, India). A total of eighteen ejaculates were collected from three poor freezable Sahiwal bulls twice a week using artificial vagina under standard semen collection procedures. Poor freezability of bulls was defined based on data of last 2-years' maintained at ABRC (post-thaw motility ranging from 25 % to 45 %). Immediately after collection, the ejaculates were transferred to the water bath at 32-34°C for further processing. The collected ejaculates were screened and after qualifying the optimal standards (progressive motile spermatozoa >70%, viability >85%, and sperm concentration >800 million/mL using a photometer, IVM, L'Aigle, France) were further processed for freezing-thawing.

Semen Dilution and Addition of Trehalose

The trehalose concentration and protocol for modified freezing were finalized based on our pilot study. In the pilot study, two extenders were prepared separately: extender E1 with trehalose (280 mOsm/l) and extender E2 with tris buffer (280 mOsm/l), both extender contained 20% egg yolk and 5% glycerol and the different combinations of these solutions were prepared by substituting extender E2 with extender E1 (that gave a final solution of molarity 280 mOsm/l). Combinations with 25% (1:3), 50% (1:1), 75% (3:1), and 100% trehalose extender E1 with E2 were utilized. In all combinations, glycerol was maintained at 5%, and modified freezing was performed using an aluminium block. After dilution, semen samples of all the groups were packaged in 0.25 mL TBS straw by MRS I filling sealing machine (IVM, L'Aigle, France). The straws of all groups were put inside holes made in the aluminium block and were kept at 4°C at different equilibration time intervals (1-4 h). Modified freezing of straws was performed by reducing the distance between LN_2 and straws (from 4 cm in conventional freezing, Baracaldo *et al.,* 2006) to 1 cm, 2 cm, and 3 cm in order to find the best distance, and by reducing the exposure time of straws to LN_2 (from 10 min in conventional LN_2 vapour freezing, Baracaldo *et al.,* 2006) to 6 min and 8 min in order to find the best vapour freezing time (which preserved the sperm motility better). Straws were exposed to LN_2 vapour within the aluminium block, after exposure straws were immediately dipped in LN_2. Post-thaw sperm evaluation revealed that trehalose at 25% with a modified freezing protocol with aluminium block preserved sperm motility better than any other trehalose concentration. Hence, 25% concentration of trehalose, along with a modified freezing protocol, was used for this experiment.

From each ejaculate, three split samples were obtained. The first split semen sample was extended in routine Tris fructose egg yolk glycerol extender (TFEG: 0.20 M tris, 20% egg yolk, 1% fructose and 6.4% glycerol) (Foote, 1970) with the conventional freezing protocol to 80 million sperm/mL to be frozen in 0.25 mL IMV TBI mini straws (Control C group). An extender containing 25% trehalose was prepared by mixing standard tris fructose egg yolk glycerol and a trehalose (25% of trehalose and 75% of TFEG, both with 280 mOsm/l) such that the glycerol percentage was 5% in it, and was used for cryo-preservation of 2nd and 3rd split fraction by the conventional protocol (T1 group) and a modified freezing protocol with an aluminium block (T2 group).

Semen Equilibration and Freezing

The equilibration and freezing protocol differed for control and treatment groups. The control and treatment 1 groups (C and T1) were subjected to filling and sealing in French mini TBS straws (0.25 mL, IVM, France) at room temperature. The samples were equilibrated at 4˚C for 4 h; and then vapour freezing for 10 min was done in a straw distribution rack. The distance between the level of LN_2 and the rack containing straws was 4 cm. The straws were immersed in liquid nitrogen (-196º C) and kept until thawing.

The straws of treatment 2 group (T2) were equilibrated at 4ºC for 2 h and finally underwent modified freezing. For vapour freezing, straws were put inside the holes made of aluminium blocks (Fig. 1). The block was kept for vapour freezing in a wide-mouth LN_2 container 2 cm above the LN_2 for 6 min, after which the straws were immediately dipped into LN_2.

Fig. 1: Aluminium block with depressions made according to length and width of straws

Evaluation of Semen

All semen samples were assessed following equilibration, *i.e.*,at pre-freezing and after thawing.Frozen semen

straws were thawed at 37°C for 30 sec following 24 h of cryopreservation. The pre-freeze and post-thawed semen samples were analysed for progressive motility, viability, membrane integrity, acrosome reaction, and lipid peroxidation adopting standard procedures. The sperm progressive motility was assessed under a phase-contrast microscope with a high power objective (40X) and was expressed with 5% accuracy. Sperm viability was assessed by Carboxyfluorescein Diacetate-Propidium Iodide (CFDA-PI) fluorescence staining (Harrison and Vickers, 1990). The viable sperm cells with intact membrane showed green fluorescence and dead sperm cells with lost membrane intactness showed red fluorescence. HOS (Hypo-osmotic swelling) response was evaluated at 150 mOsm/L HOS solution after 1 h of incubation (Jeyendran *et al.,* 1984). The acrosome integrity of spermatozoa was assessed using the fluorescent FITC-PSA staining method (Swain *et al.,* 2017). The spermatic lipid peroxidation was measured by malondialdehyde (MDA) concentration using the modified procedure of Suleiman *et al.* (1996). The MDA concentration was calculated by the specific absorbance coefficient of $1.56\times105/mol/cm^3$ using formula LPO (nM MDA/100 $\times$ 10^6 sperm) = OD x 10 x test volume/1.56 x test volume.

Statistical Analysis

The SPSS 22 statistical package was used to conduct all the statistical analyses. Two-way analysis of variance (ANOVA) was used to assess the sperm function tests, and Tukey's post hoc test was used to compare means and identify significant changes between the treatment groups. The collected data were presented as Mean ± SE. Statistics were used to determine whether differences were statistically significant at $p<0.05$.

Results and Discussion

Initial Seminal Attributes

The mean ± SE values of the parameters determined in fresh neat and initial diluted semen samples of three poor freezability Sahiwal bulls are depicted in Table 1. The initial sperm quality was quite good and within normal range for the breed.

Table 1: Physical seminal attributes of fresh semen (after initial dilution) of Sahiwal bulls with poor freezability (Mean ± SE; n=18)

Seminal Attributes	Mean ± SE	Range
Mass motility (0-5 scale)	3.02 ±0.08	2.5 - 4.0
Sperm concentration (million/mL)	1441.88 ±95.70	962 - 2135
Progressive motility (%)	72.77 ±0.83	70.0 - 80.0
Live spermatozoa (%)	83.27 ±0.83	78.5 - 89.5
HOS response (%)	68.77 ±0.66	65.0 - 73.5
Acrosome integrity (%)	87.30 ±0.49	84.5 - 91.5

Functional Sperm Attributes

The effect of the extender with trehalose and glycerol, along with modified freezing on pre-freeze and post-thaw sperm quality of poor freezable ejaculates is shown in Table 2.

Both the pre-freeze and post-thaw sperm progressive motility, viability, HOS response and acrosome integrity were significantly higher ($p<0.05$) in T2 than in Control (C) and T1 groups. The study aimed to explore the benefits of incorporating trehalose and modifying the freezing protocol in comparison to traditional freezing, focusing on the functional characteristics of poorly freezable bovine sperm after thawing. The results demonstrated that the modified freezing protocol with 25% trehalose and 5% glycerol significantly enhanced post-thaw sperm quality in terms of all parameters studied.

The poor freezability of ejaculates from crossbred bulls has been well-documented in previous studies, with a considerable percentage failing to meet the minimum standards required for semen freezing (Mathur *et al.,* 2002; Mukhopadhyay *et al.,* 2010). However, our findings in Sahiwal bulls align with previous research indicating that trehalose can effectively preserve the post-thaw motility of poor freezable ejaculates from Karan Fries (HF × Tharparkar) bulls (Chhillar *et al.,* 2012).

Table 2: Seminal attributes of Sahiwal bull spermatozoa with poor freezability at different stages of freezing (Mean ± SE; n=18)

Seminal attributes	Stage of freezing	Control (C)	Treatment 1 (T1)	Treatment 2 (T2)
Progressive motility (%)	Pre-freeze	66.67^{a} ±0.99	67.00^{a} ±1.01	70.56^{b} ±1.06
	Post-thaw	47.22^{a} ±0.83	49.44^{b} ±0.69	51.10^{c} ±0.64
Viability (%)	Pre-freeze	78.53^{a} ±0.93	79.55^{a} ±0.92	83.06^{b} ±0.89
	Post-thaw	56.80^{a} ±0.62	58.25^{b}±0.62	61.44^{c} ±0.69
HOS response (%)	Pre-freeze	52.75^{a} ±0.65	54.81^{b} ±0.54	56.69^{c} ±0.51
	Post-thaw	39.77^{a} ±0.39	40.42^{a} ±0.37	41.42^{b} ±0.35
Acrosome integrity (%)	Pre-freeze	78.36^{a} ±0.48	80.03^{b} ±0.56	82.44^{c} ±0.61
	Post-thaw	65.02^{a} ±0.71	66.19^{ab} ±0.72	67.22^{b} ±0.67

Mean values bearing different superscripts (a,b,c) within the row differ significantly ($p<0.05$).

Similarly, the freezing capacity of sperm from crossbred and buffalo bulls has been a challenge due to their susceptibility to freezing-induced stress. Previous studies have highlighted the beneficial effects of trehalose in preserving acrosome integrity in buffalo and Karan-Fires bull sperm (Badr *et al.,* 2010; Hu *et al.,* 2010; Chhillar *et al.,* 2012; Shaikh *et al.,* 2016; Iqbal *et al.,* 2018). In line with these reports, our study demonstrated improved preservation of acrosome integrity in poor freezable ejaculates from Sahiwal bulls when trehalose was incorporated in the extender. Furthermore, various studies have reported that trehalose at lower concentrations preserves sperm parameters better than higher concentrations (Reddy *et al.,* 2010; Chhillar *et al.,* 2012; Shaikh *et al.,* 2016; El-Badry *et al.,* 2017). This could be attributed to cryoprotective action of trehalose on mitochondrial functional integrity, reducing its susceptibility to damage and preserving ATP production for post-thaw sperm motility.

The evaluation of sperm viability improvement after freezing-thawing using trehalose, as compared to control, is consistent with studies conducted in stallions, buffalo bulls, and bulls (Reddy *et al.,* 2010; Chhillar *et al.,* 2012; Shaikh *et al.,* 2016; Consuegra *et al.,* 2019). Trehalose also exhibits a synergistic effect with glycerol, preventing intracellular ice crystal formation and further improving sperm parameters (Najafi *et al.,* 2013). Its non-permeating nature causes mild dehydration of spermatozoa through osmotically driven water flow, reducing intracellular ice crystal formation, which is detrimental to sperm viability.

Post-Thaw Lipid Peroxidation

Post-thaw semen evaluation showed that mean value of the rate of lipid peroxidation was significantly lower ($p<0.05$) in T2 group (3.33±0.29 nM MDA/100 million sperm) than in control and T1 group (3.79±0.20; 3.63±0.21 nM MDA/100 million sperm, respectively) of Sahiwal bulls with poor semen freezability. The indirect antioxidant effect of trehalose involves increasing glutathione levels and reducing lipid peroxide, further benefiting the combat against oxidative stress in poor freezable ejaculates. This result aligns with previous studies indicating that trehalose supplementation increases antioxidant enzyme activity and reduces lipid peroxidation in bovine, buffalo, and ram sperm (Aisen *et al.,* 2005; Bucak *et al.,* 2007; Hu *et al.,* 2010; Reddy *et al.,* 2010). During cryopreservation, exposure to cold shock and atmospheric oxygen increases sperm susceptibility to lipid peroxidation due to ROS production (Perumal *et al.,* 2011). Trehalose is known for its protective properties against various environmental stresses, including dehydration, heat, cold, and oxidation (Chen and Haddad, 2004). By reducing the generation of ROS, trehalose in our study helped preserve spermatozoa against cryo-damage.

Conclusion

In conclusion, the modified freezing protocol using an aluminium holding block for straws and incorporating 25% trehalose and 5% glycerol in extender as cryoprotectants proved highly beneficial in improving the post-thaw functional parameters of poorly freezable Sahiwal bull ejaculates, surpassing traditional freezing method.

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