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# Trehalose with a modified freezing protocol can be an alternative to improve the sperm motility of poor freezable dairy bulls

**Manisha Sethi, Tushar K Mohanty, Mukesh Bhakat, Nadeem Shah, Dileep K Yadav, Rubina K Baithalu and Dilip K Swain**

## Abstract
The present study aimed to determine the effect of trehalose and glycerol as a modified freezing diluent on the post-thaw motility of poor freezable bull semen samples at different time intervals (0 min, 30 min, 60 min, 90 min and 120 min). In the proposed study semen was collected from four Sahiwal bulls (n=6) using an artificial vagina. Split samples were used and two groups were made. Group 1 was considered as a control sample (C) in which the semen was extended with a TEYC extender containing (6.4%) and no trehalose. Group 2 was considered as a treatment sample (T) in which semen was extended in a TEYC extender containing trehalose (25%) and glycerol (5%). The treatment group underwent modified freezing whereas the control group was subjected to conventional freezing. Post-thaw incubation test was done in both groups and it was seen that the treatment sample preserved the post-thaw sperm motility of poor freezable ejaculates significantly better than the control sample at different time intervals up to 2 hrs. In conclusion, a modified freezing approach combined with trehalose and glycerol proved useful in maintaining sperm motility.

**Keywords:** Cryopreservation, modified freezing, poor freezable bull, sperm, trehalose

## Introduction
AI is largely responsible for enhancing milk production and also enhances genetic improvement by using superior male germplasm. Despite a good amount of doses prepared still, some animals have not calved yet, which means there is a gap between demand and availability of semen. Not only this but also there is a gap between the freezable semen dose produced (120 million FSD) to the actual number of freezable doses required (~155 million doses) (DAHD, 2019) [1]. One way to rectify this problem is by using semen from the poor freezable bulls. Terminology such as a good freezer or a bad freezer is generally used for a bull. Sometimes a bad freezer bull has good pre-freezing sperm parameters when initially evaluated. It is after freezing that when sperm parameters are seen they get deteriorated. So to call a bull a bad freezer or a good freezer does not purely means that a bull inherently does not produce good sperms. It can be said that the freezing protocols used may make the bull's sperm lose their capacity to fertilize. Thus by modifying the freezing protocol we can help a bad freezer bull to produce sperms with good freezing and fertilizing potential (Rajak *et al*., 2016) [2].

Trehalose is a disaccharide of glucose and is found in high concentrations in many organisms which are capable of surviving complete dehydration (Iturriaga *et al*., 2009) [3]. It is well known that water plays an important role in the maintenance of structural and functional integrity of biological membranes and the removal of this water by freezing results in functional alterations of biological membranes. Trehalose protects the membrane functionality better than glycerol and sucrose (Starciuc *et al*., 2020) [4]. Trehalose activity is thought to be linked to its ability to replace water at the membrane/solution interface (Jain and Roy, 2009) [5]. Hence modifying the conventional freezing protocol and including cryoprotectants such as trehalose and glycerol may prove to be beneficial to retrieving more fertile sperms after cryopreservation and also pave a useful way for enhancing the survivability of sperm from poor freezable bulls. Hence in our study, we used a combination of cryoprotectants namely trehalose and glycerol and modified the freezing protocol to see the effect of the same on sperm from poor freezable ejaculates.

## Material and Methods

### Chemicals and reagents

Tris, glycerol, Benzylpenicillin, citric acid, fructose and Streptomycin were procured from Sisco Research Laboratories (SRL, Mumbai), and ethanol (Merck). Trehalose was purchased from SRL, Mumbai (Product Code: 91094, CAS No. 6138-23-4);

### Selection of poor freezable bulls

The study was carried out at Artificial Breeding Research Centre (ABRC), ICAR-National Dairy Research Institute, Karnal (Haryana). Poor freezable bulls were selected based on the history of low post-thaw parameters, especially those with post-thaw progressive motility of < 50%. Three poor freezable bulls were selected based on the above-mentioned criteria, and six ejaculates were taken from each bull.

### Semen dilution and cryopreservation

After the initial assessment of semen quality, the sperm concentration of all the samples was evaluated using a photometer (IVM, L'Aigle, France). From each ejaculate, two splits were made. The first split was taken as the control in which semen was extended with Egg Yolk Tris Glycerol extender. The extended semen was subjected to sealing and filling at room temperature, after which it was equilibrated at 4 °C for 4 hours. The equilibrated sample was then subjected to conventional freezing. The second split was extended with a TEYC extender containing 25% trehalose concentration (5% glycerol). Extended semen was filled and sealed in straws and straws were put inside the depressions made in aluminum blocks and a modified freezing protocol was performed. The equilibration and freezing protocol differed for control and treatment samples.

### Assessment of sperm progressive motility at a different time interval

Individual sperm progressive sperm motility was evaluated after post-thaw stages. A drop of extended semen was placed on a pre-warmed glass slide, covered with a cover slip, and examined using a phase-contrast microscope with high power (40X magnification) objective. On a thermostatically controlled stage maintained at 37 °C, sperm were observed in at least five separate fields at 30-minute time intervals up to 2 hours and expressed as a percentage progressive motility with a 5% accuracy.

### Statistics

Sperm motility parameters at different time intervals were analyzed by 't-test' using SPSS v.22 software

## Result and Discussion

It has been known that more than half (54.96%) of crossbred bulls are not able to produce ejaculates that meet the minimum standards required for semen freezing and their ejaculates are regarded as poor freezable ejaculates (Chacon *et al*., 1999; Mathur *et al*., 2002; Sarder, 2003; Mukhopadhyay *et al*., 2010; Singh *et al*., 2013) [6-10]. Studies have been done in which trehalose preserved the post-thaw motility of poor freezable ejaculates from cross bred bulls (Chillar *et al*., 2012) [11] which is in agreement with our study in which trehalose provided cryoprotection to sperm during freezing and helped to increase the post-thaw motility of sperm in poor freezable ejaculates from Sahiwal bulls.

In the present study post-thaw motility of poor freezable sperm at different time intervals (Tab.1; Fig:1) was significantly higher ($p<0.05$) in treatment group ($51.11^{b} \pm 0.64$, $45.83^{b} \pm 0.72$, $40.83^{b} \pm 0.72$, $32.77^{b} \pm 0.83$ and $23.88^{b} \pm 0.86$) than control ($47.22^{a} \pm 0.83$, $41.66^{a} \pm 0.90$, $36.38^{a} \pm 0.88$, $27.77^{a} \pm 0.83$ and$19.72^{a} \pm 0.94$) at 0min, 30 min, 60min, 90min and 120 min. Aisen *et al*. (2005) [12] reported that trehalose as an additive in ram semen preserved sperm motility after 4-h post-thaw resistance test better than control which is in agreement with our study.

In our study sperm motility reduction after 2 hours (%) in the treatment group was 53.25 whereas it was 58.23 in the control. Our results may be because trehalose, a naturally occurring osmolyte, exceptionally stabilizes the proteins and helps to retain the activity of enzymes in the solution as well as in the freeze-dried state. Trehalose has also been found to be very effective in the stabilization of labile proteins during lyophilization (Back *et al*., 1979; Sola-Penna *et al*., 1997) [13, 14] and exposure to high temperatures in solution (Kaushik and Bhat, 1998) [15]. Kaushik and Bhat (2003) [16] did a thermal denaturation experiment of proteins using trehalose in which trehalose solution provided thermostabilization and thermoprotection of proteins better than any other sugar. Hence it may be due to this exceptional stabilization action of trehalose that it preserved the sperm motility for up to 2 hours in our experiment. But this action of trehalose is concentration dependent such that at higher concentrations (Chillar *et al*., 2012; Aisen *et al*., 2002; Al-Badrany *et al*., 2017) [11, 17, 18]. Trehalose also boosts the antioxidant enzyme activity of bull semen (Iqbal *et al*., 2016) [19] and it is well known that antioxidants such as glutathione improve the fertilization potential of sperm (Shah *et al*., 2017; Satorre *et al*., 2007) [20, 21]. The effect of modified freezing with trehalose on post-thaw motility of bull sperm at different time intervals has not been studied to date. It is the first study to depict the effect of a combination of cryoprotectants (trehalose and glycerol) along with modified freezing on post-thaw motility of sperm at different time intervals.

**Table 1:** Effect of Extender with 25% Trehalose and 5% glycerol along with modified freezing protocol on post-thaw motility of) at different time intervals (Mean ± SE, n=18)

| Time | Control (C) | Treatment (T) |
|---|---|---|
| 0 min | $47.22^{a} \pm 0.83$ | $51.11^{b} \pm 0.64$ |
| 30 min | $41.66^{a} \pm 0.90$ | $45.83^{b} \pm 0.72$ |
| 60 min | $36.38^{a} \pm 0.88$ | $40.83^{b} \pm 0.72$ |
| 90 min | $27.77^{a} \pm 0.83$ | $32.77^{b} \pm 0.83$ |
| 120 min | $19.72^{a} \pm 0.94$ | $23.88^{b} \pm 0.86$ |

Means bearing different superscripts (a, b) in row differ significantly ($p<0.05$)

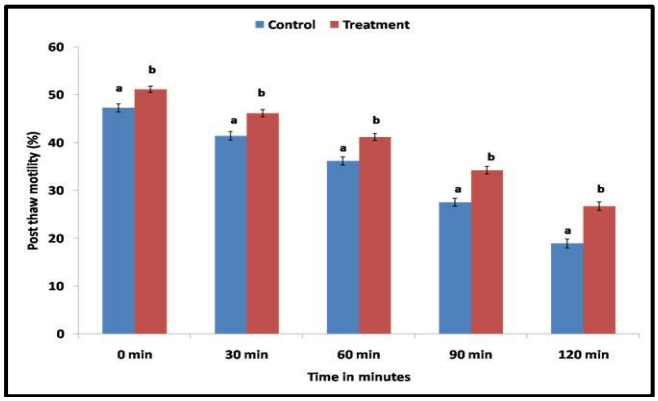


**Fig 1:** Effect of Extender with 25% Trehalose and 5% glycerol along with modified freezing protocol on post-thaw motility of Sahiwal bulls (with poor freezability) at different time intervals

## Conclusion
In our investigation, ejaculates from poor freezable Sahiwal bulls showed excellent post-thaw motility improvement after a novel freezing approach with trehalose and glycerol as cryoprotectants in the extender. Hence as it is known that the number of high fertile bulls is less in our country and using sperms from poor freezable bulls might help to increase the number of AI doses which may cover a huge population of bovines that are not covered by AI.

## Conflict of Interest
None of the authors have any conflict of interest to declare.

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## Ethical permission
Ethical permission was taken from the Institutional Animal Ethical Committee for all experimental protocol and semen collection using an artificial vagina.

## Data Availability Statement
The data that support the findings of this study are available from the corresponding author upon reasonable request. We are not disclosing the freezing equipment and protocol used in this study, which is under patent filing; however, if required it can be disclosed on a confidential basis to the editor or reviewer.

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