RESEARCH ARTICLE

Effect of Trehalose on Cryopreservation of Hariana Bull Spermatozoa

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Abstract

The present experiment was designed to study the effect of supplementation of Trehalose on freezability and antioxidant activity of Hariana bull semen. Semen samples were diluted in TFYG extender containing different concentrations of Trehalose (10 mM, 30 mM & 50 mM). The control samples were extended with TFYG alone. Results clearly indicated that, 30 mM Trehalose group had significantly (P<0.05) higher percentage of individual sperm motility, sperm viability, and intact acrosome in comparison to the control and other Trehalose supplemented groups. In biochemical assays, no clear-cut demarcation for antioxidant enzyme was found for Trehalose supplementation. However, Trehalose supplementation has showed increase activity for Glutathione reductase (GR). The results obtained clearly indicated that supplementation of Trehalose (30 mM) to extender prior to cryopreservation improves Hariana bull sperm quality.

Key words: Antioxidant, Cryopreservation, Freezability, Hariana bull, Trehalose.

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Introduction

The quality of frozen semen, composition of the extender, adequate cryoprotectants, and ideal freezing and thawing rates are the important factors affecting semen freezability and fertility (Andrabi, 2009; Malo *et al.*, 2010). Tris-based extenders are commonly used for semen cryopreservation in most farm animals along with cryoprotectants like glycerol (penetrating) or sugars (non-penetrating) (Purdy, 2006). Despite of the higher toxicity of glycerol, it is still the best penetrating cryoprotectant used for semen freezing (Watson, 2000). Cryodamage, which is a byproduct of cryopreservation, affects the spermatozoa's plasma membrane, cytoskeleton, motility system (Bailey *et al.*, 2003), and nucleus (Ragoonanan *et al.*, 2010). Additionally, the high level of unsaturated fatty acids in bovine spermatozoa's membrane and the absence of a large antioxidant system in the cytoplasmic component make them vulnerable to membrane damage (Bailey *et al.*, 2003) by excessive ROS release (Uysal and Bucak, 2007) and needs an effective antioxidant system to counter the peroxidative activity.

Numerous approaches have been attempted to reduce sperm damage by modifying extenders and freezing protocols. Glycerol is an effective permeating cryoprotectant used for decades in semen cryopreservation (Kumar *et al.*, 2003). However, it is toxic to the spermatozoa of some species such as sheep, pigs and horses. Aside from using permeating cryoprotectants, most studies are focusing on non-permeating cryoprotectants particularly disaccharides, which are good at stabilising bio-membrane bilayers by increasing the osmotic dehydration (Platov, 1988). In that respect, trehalose, which contains two glucose molecules linked together by glycosidic linkage, appears to protect cells by strengthening the extender's tonicity and stabilising the plasma membrane, potentially as a result of direct interaction with the polar head groups of the membrane's phospholipids to increase the membrane fluidity (Reddy *et al.*, 2010) and trapping water molecules necessary for hydration (Patist and Zoerb, 2005). Trehalose also has an indirect antioxidant effect by raising antioxidant enzyme levels and lowering lipid peroxide levels (Aisen *et al.,* 2005). So, the current study was carried out to ascertain the effect of Trehalose supplementation on freezability and antioxidant activity of Hariana bull semen.

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Materials and Methods

Present study was conducted on four Hariana bulls of the age group between 8.5 and 9.5 years, weighing 450-500 kg, reared at Semen Biology Lab, Instructional Livestock Farm Complex (ILFC), College of Veterinary Sciences, U.P. Pandit Deendayal Upadhayaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan, Mathura, India. From each bull two ejaculates were taken at 20-30 min gap between two successive ejaculates using artificial vagina. Total 28 ejaculates, 7 ejaculates from each bull were included in this study.

Immediately after collection and routine evaluation, semen samples qualified (>70% progressive motility) the freezing criterion were divided into four equal aliquots. One part of the semen was extended with basic extender (TFYG) and was considered as control group. Other three parts were supplemented with 10 mM, 30 mM and 50 mM of Trehalose and were considered as Treatment groups. The dilution rate was calculated to keep the final concentration of sperm 80 x 10^6 cells/mL. After the final dilution, each semen aliquot was evaluated for post-dilution individual motility under phase contrast microscope (at 40X), sperm viability (eosin and nigrosin stain), acrosomal integrity (Giemsa stain), and enzyme estimation in seminal plasma for oxidative stress using standard procedures. All these parameters were also evaluated at pre-freeze (after equilibration) and post-thaw stage. Thawing of frozen semen straws was done in water-bath at 37°C for 30 seconds.

The malondialdehyde (MDA) assay was performed by the TBARS (thio-barbituric acid reactive substance) method to assess LPO activity (Ohkawa *et al.,* 1979), and Glutathione Reductase (GR) activity was measured using the method described by Sedlak and Lindsay (1968). The data were analyzed statistically using one-way ANOVA by SPSS version 16.00.

Results and Discussion

Impact on Sperm Quality Parameters

The mean values of percent individual sperm motility, viability and acrosome integrity were significantly ($P<0.05$) higher in 30 mM Trehalose group followed by 10 mM Trehalose and control groups at all three stages, *i.e.,* on initial dilution, post-equilibration and post-thaw stages of cryopreservation, while 50 mM Trehalose significantly suppressed all these sperm parameters suggesting its toxicity at higher level in the extender. Further at post-thaw stage, the progressive sperm motility, viability and acrosome integrity were statistically similar with 10 mM and 30 mM Trehalose supplemented groups, and both were significantly ($p<0.05$) higher as compared to 50 mM Trehalose and control groups (Table 1).

The effect of Trehalose on post-thaw sperm quality parameters have been reported in cryopreserved semen of ram (Aisen *et al.*, 2002), boar (Hu *et al.*, 2009), and bull (Hu *et al.*, 2010; Kumar *et al.*, 2022). Trehalose probably plays a vital role in preventing deleterious alteration to the sperm membrane by increasing the fluidity of membrane, maintaining the osmotic pressure of the diluent, acting as a non-reducing cryoprotectant and providing energy substrate for the sperm cell during cryopreservation (Uysal and Bucak, 2009). In Nili Ravi buffaloes, 30-mM Trehalose has been reported with higher progressive sperm motility and acrosome integrity of post-thawed semen compared to 15 mM, 45 mM and 60 mM Trehalose supplemented groups and control, probably due to increased osmolarity of extender in a dose dependent manner (Iqbal *et al.,* 2016). This might be due to the cryoprotective effects of Trehalose on the sperm organelles like mitochondria and acrosome, which are in charge of producing energy and improving post-thaw sperm motility (Reddy *et al.,* 2010), which was evidenced in the supplemented groups. However, the findings of Hu *et al.* (2010) and Shaikh *et al.* (2016) conflicted with the present results, that the extender supplemented with 100

Table 1: Effect of trehalose concentration on progressive sperm motility, sperm viability and acrosomal integrity on dilution, pre-freeze and post thaw stages of cryopreservation (Mean ± SEM, n=28)

Sperm quality parameters	Freezing stages	TFYG	TFYG + Trehalose (mM)		
			10 mM	30 mM	50 mM
Progressive sperm motility (%)	On dilution	75.54 ± 0.79^b	77.68 ± 0.65^b	82.5 ± 0.91^a	72.93 ± 1.09^c
	Pre-freeze	62.50 ± 0.66^c	64.89 ± 0.69^b	67.86 ± 0.83^a	55.89 ± 1.00^d
	Post thaw	41.14 ± 0.77^b	43.86 ± 0.73^a	46.00 ± 0.87^a	30.71 ± 0.95^c
Sperm viability (%)	On dilution	83.50 ± 0.65^b	85.21 ± 0.71^b	86.64 ± 0.67^a	81.71 ± 0.65^c
	Pre-freeze	72.79 ± 0.86^b	75.07 ± 0.80^{ab}	78.21 ± 0.71^a	71.11 ± 1.06^c
	Post thaw	58.29 ± 1.05^b	61.32 ± 1.19^a	65.11 ± 0.98^a	56.11 ± 1.25^c
Acrosome integrity (%)	On dilution	80.04 ± 0.68^b	82.57 ± 0.70^a	84.36 ± 0.66^a	78.25 ± 0.89^b
	Pre-freeze	70.54 ± 0.84^b	72.21 ± 0.88^b	75.36 ± 0.72^a	64.50 ± 1.07^c
	Post thaw	54.71 ± 1.25^b	59.29 ± 1.03^a	62.64 ± 1.13^a	53.04 ± 1.62^b

Mean ± SE values bearing common superscript within the row do not differ significantly ($P>0.05$).

Table 2: Effect of trehalose concentration on Lipid peroxidation/MDA activity and Glutathione reductase activity in the seminal plasma at initial dilution, pre-freeze and post-thaw stages of Harian bull semen (Mean ± SEM, n=28)

Enzyme	Freezing stages	TFYG	TFYG + Trehalose (mM)		
			10mM	30mM	50mM
Lipid peroxidation / MDA activity (nM/µL)	On dilution	0.042±0.006[a]	0.034±0.004[a]	0.037±0.005[a]	0.039±0.003[a]
	Pre-freeze	0.039±0.007[a]	0.029±0.004[a]	0.035±0.006[a]	0.032±0.004[a]
	Post thaw	0.032±0.002[ab]	0.026±0.003[b]	0.032±0.006[ab]	0.039±0.004[a]
Glutathione reductase activity (U/L)	On dilution	6.587±1.42[ab]	3.053±0.66[b]	13.314±2.37[a]	8.933±3.79[ab]
	Pre-freeze	5.314±2.49[a]	6.841±2.03[a]	14.078±2.41[a]	13.032±4.82[a]
	Post thaw	3.336±1.07[b]	15.378±4.94[a]	20.099±0.96[a]	20.155±2.96[a]

Mean ± SE values bearing common superscript within the row do not differ significantly (P>0.05).

mM Trehalose resulted in greater (p<0.05) motility and acrosomal membrane integrity as compared to control group. Likewise, Reda *et al.* (2015) reported that addition of 50 mM of Trehalose/L maintained the membrane integrity which was a level higher than that of the control tris-based diluent. The reduced sperm motility at higher Trehalose concentration may be due to increase in osmolarity of extender.

Aisen *et al.* (2002) opined that high concentrations of Trehalose (200-400 mOsm) might have a deleterious effect on ram spermatozoa during the cooling process. This damage was also evident in the freeze-thaw step in our study, which may be due to increased osmolarity of extender, release of toxic substances, ultralow temperature exposure, enzymatic leakage, aging effect of sperm and individual variation. Trehalose in low concentration, acts as cryoprotectant, causes mild cellular dehydration of spermatozoa due to the osmotically driven flow of water thus reducing intracellular ice crystal formation (Chhillar *et al.,* 2012). Additionally, Trehalose shows a synergistic effect with glycerol and prevents intracellular ice crystal formation (Gutierrez *et al.,* 2009). Overall, the addition of Trehalose at 30 mM improved the motility, viability and acrosome integrity of sperm cells during cryopreservation, as been reported by Matsuoka *et al.* (2006).

Impact on Antioxidant Activity

Sperm membrane has a large content of unsaturated fatty acids and lack a significant cytoplasmic component containing antioxidants and is particularly susceptible to lipid peroxidation (LPO) in the presence of ROS, leading to impaired cell function and decreased sperm motility (Lenzi *et al.,* 2002; Bucak *et al.,* 2007). In present study, the production of malondialdehyde (MDA) was found to be the lowest in 10 mM trehalose supplemented group followed by 30 mM, 50 mM and control groups at all three stages of cryopreservation process, with significant difference between 10 mM and 50 mM Trehalose at post-thaw stage only. On the other hand, glutathione reductase (GSH) levels in Trehalose supplemented groups (10 mM, 30 mM and 50 mM) were significantly (p<0.05) higher than that of control group at post-thaw stage of cryopreservation, but there was no significant difference between treatment and control groups at pre-freeze stage (Table 2). The reduced MDA and increased glutathione reductase activity in post-thawed semen is an indication of enhanced antioxidant ability of trehalose extender during ultra-low temperature freezing process (Serpil *et al.,* 2009).

The present findings of significantly reduced lipid peroxidation (MDA) in post-thawed semen upon supplementation of extender with Trehalose at 10 mM concentration closely agreed with the reports of Badr *et al.* (2010) and Chhillar *et al.* (2012) on bull semen. Shaikh *et al.* (2016) also reported overall significantly (p<0.05) lower mean MDA value in 100 mM Trehalose group at post-dilution, post-equilibration and post-thaw stages of cryopreservation as compared to that of control group of Kankrej bull semen. Our findings on increased GSH activity in post-thawed semen with Trehalose supplementation concurred with the reports of Hu *et al.* (2010) and Shaikh *et al.* (2016). Aisen *et al.* (2005) in ram semen reported that the extender containing Trehalose enhanced the level of GSH and decreased the oxidative stress provoked by the freeze-thaw process. Thus, the addition of Trehalose at 10 mM concentration in present study reduced the MDA production and increased the antioxidant enzyme (GSH) level to protect the spermatozoa from oxidative stress during cryostress.

Conclusion

The findings of present study revealed that the Tris extender supplemented with Trehalose @ 30 mM improved the post-thaw semen quality and was found to be having more beneficial effect on freezability of Hariana bull spermatozoa as compared to control extender and low (10 mM) or high (50 mM) concentration of Trehalose.

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