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Estimation of Oxidative Stress in Indigenous Bull's Semen during Cryopreservation Following Incorporation of Trehalose

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ABSTRACT

Cryopreservation of sperm is associated with an oxidative stress induced by free radicals formation during freezing process, their by inducing physical and chemical stress on the sperm membrane which in turn reduces sperm viability and fertilizing ability. The objective of this study was to estimate oxidative stress in cryopreserved Kankrej bull's semen samples by using Trehalose as antioxidant semen diluent additive. The pooled semen samples were diluted with Tris-based extender containing different Trehalose concentrations (viz. 50 mM, 100 mM, 150mM) and control(no additive), evaluated for oxidative stress parameters at freezing and frozen thawed stages. Results, clearly indicated the Malondialdehyde (MDA) level were 43.9 ± 0.05 µmol/ml, 34.2 ± 0.09 µmol/ml and 20.06 ± 0.1 µmol/ml at post-dilution, post-equilibration and post-thaw stages of cryopreservation; which were significantly ($P<0.05$) lower in 100mM Trehalose group when compared with the control group. And the glutathione (GSH) level were 55.8 ± 0.1 U/L, 64.0 ± 0.1 U/L and 84.0 ± 0.1 U/L at post-dilution, post-equilibration and post-thaw stages of cryopreservation; which were significantly ($P<0.05$) higher in 100mM Trehalose group when compared with the control group. Conclusively, supplementation of 100mM Trehalose in the Tris-based extender can be best concentration for Kankrej bull's semen cryopreservation.

Key words- Trehalose, Tris-based extender, Cryopreservation, Oxidative stress, Malondialdehyde, Glutathione reductase.

INTRODUCTION

Kankrej Cattle, the heaviest and dual-purpose breed found in the Kankrej area of Banas District in Gujarat State, had shown a sizeable potential with respect to milk production, disease resistance and draughtability at Livestock Research Station, SDAU (Annual Progress Report, 2009). The National Breeding Policy of India is now focusing on the indigenous breed conservation of cattle with the help of artificial insemination in rural areas for the improving the livelihood of people (Shaikh *et al.,* 2016a). Artificial insemination is assisted reproductive technology that has made possible the effective use of best breeding bulls, thus greatly improving the genetic potential of breeding herds (Januskauskas and Zilinskas, 2002). Cryopreservation technique has allowed specific opportunities for the

cryopreservation of semen and widespread dissemination of precious genetic resources through sperm banks that collaboration in breed improvement programs by means of artificial insemination (Holt, 1997).

Semen cryopreservation associates with an oxidative stress induced by free radicals formation during different processing stages (Salvador *et al*., 2006; Shaikh *et al.,* 2016b). The plasma membranes of sperm cells had supplementary content of unsaturated fatty acids and their cytoplasmic components are deficient in antioxidants. Therefore, sperm cells are highly susceptible to lipid peroxidation (LPO) in presence of ROS, leading to impaired function activity (Hu *et al*., 2009; Hu *et al*., 2010; Shaikh *et al.,* 2016b). In past few decades, antioxidants are used to protect sperm cells from the deleterious effects of cryopreservation and free radicals formation with the incorporation of antioxidants (Stradaloli *et al*., 2007; Umut *et al*., 2013; Shaikh *et al.,* 2016b). So, cryoprotectants are incorporated in tris-based extender to reduce the damage to sperm during the process of freezing and cryopreservation (Badr *et al*., 2010; Purdy, 2006; Bucak *et al*., 2007).

Trehalose, as non-permeating and non-reducing disaccharide which contains two glucose molecules linked together as 1, 1-glycosidic linkage (α-d-glucopyranosyl-l, 1-α-d-glucopyranoside), mostly found in yeast and fungi at higher concentrations (Woelders *et al*., 1997; Aisen *et al*., 2000; Aisen *et al*., 2002; Shaikh *et al.,* 2016a). Trehalose probably plays a crucial role in preventing deleterious effects to the sperm membrane by maintaining the osmotic pressure of the diluent, acting as a non-reducing cryoprotectant and providing energy substrate for the sperm cell during dilution, equilibration, cryopreservation and post-thawing (Liu *et al*., 1998; Uysal and Bucak, 2009; Shaikh *et al.,* 2016a). Incorporation of trehalose to semen extenders is known to improve the individual motility and sperm viability during cryopreservation (Matsuoka *et al*., 2006; Sztein *et al*., 2001). Trehalose showed a synergic effect with glycerol and prevented intracellular ice crystal formation, when added in hypertonic condition (Gutierrez *et al*., 2009; Shaikh *et al.,* 2016a).

In view to the facts above, the present study was carried out to determine suitable concentration following incorporation of Trehalose to improve the Kankrej bull semen quality during cryopreservation and frozen thawed stages. This might not only help in improving preservability of semen but also provide a way for fastest utilization of Kankrej bull semen towards indigenous breed improvement.

MATERIALS AND METHODS

A total of 36 ejaculates, 12 ejaculates per bull were obtained once in a week using artificial vagina from three healthy Kankrej bulls aged between 4 to 5 years, of Dama Semen Production Unit, Banas dairy, Palanpur during research work. Immediately after collection, semen collection tubes were placed in water bath at 37^{o}C until their assessment in the laboratory.

Ejaculates of semen with more than 70 per cent initial motility were used for the research work. The collected semen samples were pooled to split further into 4 equal aliquots and each one was diluted with Tris-Fructose Egg Yolk Citrate Glycerol (TFYG) freezing extender containing different Trehalose concentrations viz. 50mM, 100mM, 150mM and no additive (control) so as to obtain a final sperm concentration of 80 million sperms per ml. Extended semen aliquots were filled, sealed and printed in French Mini Straw of 0.25 ml capacity using automatic machine (IS-4, IMV-France) and were stored in Liquid Nitrogen at -196^{0}C. After cryopreservation period of 24 hrs, straws were thawed at 37^{0}C for 30 seconds in a water bath for post thaw evaluation.

The seminal plasma was separated from processed semen straws at different stages of cryopreservation by centrifugation at 5000 rpm for 10 min. and stored at -20° C before being assayed. The seminal plasma samples were thawed before analyzing the lipid peroxidation and glutathione reductase values. Membrane peroxidative damage in seminal plasma was determined in terms of malondialdehyde (MDA) by using the method of (Placer *et al*., 1966). The values of MDA were expressed as µmol/ml. The GSH content of sperm was measured using the method of (Sedlak and Lindsay, 1968). The values of GSH were expressed as U/L. The data were statistically analyzed using Completely Randomized Design (CRD) and Duncan New Multiple Range Test to determine levels of significance. The interrelationship was worked out as per the procedure described by (Snedecor and Cochran, 1994).

RESULT AND DISCUSSION

The overall mean Malondialdehyde (MDA) values using different concentrations of Trehalose were 57.7 ± 0.1,

40.1 ± 0.06 and 32.1 ± 0.09 µmol/ml in 50mM group; 43.9 ± 0.05, 34.2 ± 0.09 and 20.06 ± 0.1 µmol/ml in 100mM group; 50.06 ± 0.08, 39.01 ± 0.1 and 28.04 ± 0.08 µmol/ml in 150mM group and 58.01 ± 0.08, 40.1 ± 0.07 and 31.9 ± 0.07 µmol/ml in control group at post-dilution, post-equilibration and post-thaw stages of cryopreservation. The overall mean Malondialdehyde (MDA) value in 100mM Trehalose group was significantly (P<0.05) lower in post-dilution, post-equilibration and post-thaw stages of cryopreservation as compared to that of the 50mM Trehalose, 150mM Trehalose and control groups. Whereas, Malondialdehyde (MDA) values was found to be significantly (P<0.05) higher in 50mM Trehalose and control groups as compared to that of the 150mM Trehalose group (Table-1). These findings are in accordance with (Badr *et al*., 2010) who have reported that the addition of 100mM Trehalose to the freezing extender resulted in decreased Malondialdehyde (MDA) values in buffalo bulls and also with that of the similar findings in Karan-Fries bulls (Chhillar *et al*., 2012; Kumar *et al*., 2012; Shaikh *et al.,* 2016a; Shaikh *et al.,* 2016b).

Due to higher production of reactive oxygen species, during cryopreservation the semen is exposed to cold shock at atmospheric oxygen which in turn increases the susceptibility to lipid peroxidation (Perumal *et al*., 2009). The free radicals are known to be involved in lipid peroxidation as well as DNA and sperm membrane damages which may lead to decreased sperm motility or cell death (Shaikh *et al.,* 2016a). Therefore, in the present study addition of Trehalose in semen might be a beneficial factor in preventing the damage to sperm cells and reduced generation of ROS, which otherwise had negatively affected the spermatozoa (Uysal *et al*., 2007).

The proper mechanism of Trehalose reacting with the sperm membrane is not known, but theoretically it form hydrogen bonds with the polar head of the phospholipids and its introduction into the sperm membrane limits the amount of dehydration that can occurs due to cryopreservation and thawing (Liu *et al*., 1998). Trehalose has a protective action in relation to the osmotic effect and specific interactions with the membrane phospholipids, which renders the media hypertonic, thereby reduces the degree of sperm cell injury during the freeze-thaw process (Molinia *et al*., 1994; Storey *et al*., 1998; Shaikh *et al.,* 2016a).

The overall mean glutathione reductase (GSH) values using different concentrations of Trehalose were 38.1 ± 0.1, 50.2 ± 0.07 and 62.1 ± 0.08 U/L in 50mM group; 55.8 ± 0.1, 64.0 ± 0.1 and 84.0 ± 0.1 U/L in 100mM group; 42.1 ± 0.07, 50.1 ± 0.07 and 57.07 ± 0.06 U/L in 150mM group and 38.3 ± 0.1, 49.1 ± 0.07 and 62.04 ± 0.07 U/L in control group at post-dilution, post-equilibration and post-thaw stages of cryopreservation.

Table 1: Lipid Peroxidation(µmol/ml) values in Different Groups of Additive at Various Stages of Cryopreservation (Mean ± S.E.)

Semen Additive Concentration	Post-Dilution Stage (PDS)	Post-Equilibration Stage (PES)	Post-Thaw Stage (PTS)
Trehalose 50mM	57.7 ± 0.1^{c}	40.1 ± 0.06^{c}	32.1 ± 0.09^{c}
Trehalose 100mM	43.9 ± 0.05^{a}	34.2 ± 0.09^{a}	20.06 ± 0.1^{a}
Trehalose 150mM	50.06 ± 0.08^{b}	39.01 ± 0.1^{b}	28.04 ± 0.08^{b}
Control	58.01 ± 0.08^{c}	40.1 ± 0.07^{c}	31.9 ± 0.07^{c}

Means with different superscripts within column differ significantly at (P<0.05) level.

Table 2: Glutathione Reductase(U/L) values in Different Groups of Additive at Various Stages of Cryopreservation (Mean ± S.E.)

Semen Additive Concentration	Post-Dilution Stage (PDS)	Post-Equilibration Stage (PES)	Post-Thaw Stage (PTS)
Trehalose 50mM	38.1 ± 0.1^{a}	50.2 ± 0.07^{b}	62.1 ± 0.08^{b}
Trehalose 100mM	55.8 ± 0.1^{c}	64.0 ± 0.1^{c}	84.0 ± 0.1^{c}
Trehalose 150mM	42.1 ± 0.07^{b}	50.1 ± 0.07^{b}	57.07 ± 0.06^{a}
Control	38.3 ± 0.1^{a}	49.1 ± 0.07^{a}	62.04 ± 0.07^{b}

Means with different superscripts within column differ significantly at (P<0.05) level.

The overall mean glutathione reductase (GSH) value in 100mM Trehalose group was significantly (P<0.05) higher in post-dilution, post-equilibration and post-thaw stages of cryopreservation as compared to that of the 50mM Trehalose, 150mM Trehalose and control groups. Whereas, glutathione reductase (GSH) values was found to be significantly (P<0.05) lower in 50mM Trehalose and control groups as compared to that of the 150mM Trehalose group (Table-2). Present findings are in harmony with (Badr *et al*., 2010; Hu *et al*., 2010; Shaikh *et al.,* 2016a; Shaikh *et al.,* 2016b) who have reported that the addition of 100mM Trehalose to the freezing extender resulted in increase glutathione reductase levels in buffalo and bovine bulls, respectively.

Glutathione, a naturally occurring tri-peptide plays an integral role on scavenger ROS and free radicals with the help of the glutathione reductase in semen cycle (Meister and Anderson, 1983; Shaikh *et al.,* 2016b). A well known fact of glutathione reductase, it plays an integral role in protecting mammalian cells from oxidative damages. The enhanced antioxidant ability indicated the increase in glutathione reductase activity (Perumal *et al*., 2009; Serpil *et al*., 2009). Therefore, higher GSH values in the semen are factor in making the sperm membrane more resistant to the spontaneous lipid peroxidation that destroys the structure of the lipid matrix (Mohanty and Ansari, 2004). Trapping of the free radicals by Trehalose, thereby alleviating GSH consumption by the enzymatic antioxidant defenses might be implicated in higher GSH values observed in the present study.

Conclusion

From the above observations, it was concluded that semen extender supplemented with 100mM trehalose resulted in higher GSH values and lower MDA values, during the cryopreservation process and are beneficial in minimizing the oxidative stress provoked by freeze thaw process. The optimum trehalose concentration determined to be 100mM in tris-extender for cryopreservation of Kankrej bull's semen.

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