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Effect of Trehalose Supplementation to Semen Extender on Quality of Cryopreserved Stallion Semen

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A B S T R A C T

The aim of the present study was to evaluate the cryoprotective effect of trehalose on stallion semen quality like progressive sperm motility, viability, acrosome integrity, membrane integrity. A total of 42 ejaculates from six Marwari stallion (seven ejaculate from each) were used. Immediately after gross evaluation, the semen was filtered into a pre warm, graduated measuring bottle to get gel free semen and it was diluted with primary extender to get sperm pellets. Secondary extender was added and divided into three equal aliquots served as control, treatment 1(50 mM treahalose) and treatment 2 (150mM trehalose). At post-thaw stage there was reduction in the progressive sperm motility, per cent viability, membrane integrity and acrosome integrity in all three groups. In T_1 group per cent progressive sperm motility and viability was higher ($P<0.05$) than control. However, in control group percentage hypo-osmotic swollen spermatozoa were lower ($P<0.05$) than T_1 and T_2. In T_1 group per cent spermatozoa with intact acrosome was higher ($P<0.05$) than control. The deleterious effect of group T_2 on acrosome integrity and membrane integrity was significant. In conclusion, addition of 50 mM trehalose improved post-thawing semen quality.

Introduction

Artificial Insemination (AI) in equine breeding has now been accepted in horse industry. The use of frozen semen AI has increased greatly over last few decades (Kumar, 2008). The use of artificial insemination with frozen semen in equine reproduction has become increasingly popular and several breed registries are approving the foals born from frozen semen (Loomis, 2001).

During cooling and freezing spermatozoa undergo a series of chemical and physical

changes that include partial dehydration, cryoprotectant penetration of cells, reorganization of membrane lipids and proteins, exposure to high salt concentrations and exposure to inter and intracellular ice crystals. Cryopreservation protocols are designed to minimize the negative effects of these stresses (Loomis and Squires, 2005).

Various degrees of damage to sperm occur during freezing and thawing including perturbations to the sperm organelles and change in membrane fluidity and enzymatic activity that result in reduction in sperm motility, viability and freezing ability (Alvarez and Storey, 1983). Oxidative damage is a major factor in sperm cryo-damage (Ghallab *et al.*, 2017). There is increase in ROS production and decrease in antioxidants levels during sperm cryopreservation, which causes increase in susceptibility of frozen thawed to lipid peroxidation (Aitken and Krausz, 2001). Lipid peroxidation is associated with premature aging and DNA fragmentation in cryopreserved equine spermatozoa (Ortega-Ferrusola *et al*., 2008). The semen quality can be improved by preventing sperm cryoinjury during cooling and freezing process by addition of antioxidants in the semen extender.

Trehalose is a non permeating disaccharide which acts as non enzymatic scavenger. Through its osmotic effects trehalose induces its protective effects against oxidative damage rendering a role in protection of spermatozoa against ROS (Reddy *et al*., 2010). Trehalose also act as hypertonic media causing cellular osmotic dehydration before freezing and decreasing the amount of cell injury by crystallization (Bucak *et al*., 2007).

Trehalose supplementation in semen extender has potential to improve the overall quality and freezing ability of semen in different farm animals including stallion. The present study was designed to investigate the effect of trehalose supplementation on post thaw quality of Marwari horse stallion.

Materials and Methods

The study was carried out in the animal reproduction laboratory of equine production campus (EPC), ICAR- National Research Centre on Equine (NRCE) Bikaner. Six adult Marwari stallions managed under uniform conditions of feeding and management were used for the present study.

Preparation of primary extender

Primary extender was prepared by adding 0.15g glucose, 2.6g sodium citrate dehydrate, 0.37g di-sodium EDTA, 0.12g sod-bicarbonate, 0.10g Streptomycin and 50000-100000 unit Benzyl penicillin in distilled water to make 100ml as described previously (Ravi, 2014).

Preparation of secondary extender

Solution A + Solution B + egg yolk + cryoprotectant

Solution A (EDTA-glucose) was prepared by adding Glucose 6.00g, Sodium citrate dehydrates 0.37g, Di-sodium EDTA 0.37g, Sod-bicarbonate 0.12g, Streptomycin 0.10g, Benzyl penicillin 0.10g. Above all ingredients were dissolved in conical flask with addition of double distilled water and the final volume was made up to 100 ml.

Solution B (Lactose) was prepared by adding Lactose 11.00g, Streptomycin 0.08g, Benzyl penicillin 0. 08g. in a conical flask with addition of double distilled water and the final volume was made up to 100 ml as per Ravi, 2014.

Solution A 50 ml, Solution B 100ml and egg yolk 40 ml was mixed and centrifuged at 3000 RPM (10^0C) for 30 min. The supernatant was filtered through a sterile gauge to prepare the final extender, DMF was added as cryoprotectant at rate of 5% to the total volume.

Semen collection, evaluation and freezing and post thaw evaluation

Semen from Six Marwari stallions were collected twice a week during morning hours using Colorado type artificial vagina and a mare in estrus as dummy. Seven ejaculates were collected each from all six stallions. Total Forty two ejaculates were collected. Each ejaculate was filtered through a sterile piece of gauge to measure the gel free semen volume.

Total ejaculate volume and gel free volume were recorded immediately after collection as per standard methods described previously (Kumar, 2017).

The gel free semen was evaluated for progressive sperm motility by CASA (Kumar, 2019), sperm concentration (Kant, 2016), live sperm count (Pintado *et al*., 2000), membrane integrity (Nie and Wenzel, 2001) and acrosomal integrity (Watson, 1975).

Primary extender in the proportion equal to volume of gel free semen taken into centrifuge tube, subjected to centrifugation (1700-1800 rpm for 3 min) to get a sperm pellet at the base after discarding the supernatant.

The sperm pellet which was added with secondary extender containing additives as described below to get a final concentration 150×10^6 spermtaozoa/ml.

The supplementation of additives to the secondary extender was as follows.

Control: Secondary extender (SE) without any additives
Treatment 1: SE with trehalose at 50mM concentration
Treatment 2: SE with trehalose at 150mM concentration

Extended semen samples of all the above three aliquots were filled and sealed in 0.5 ml French medium straws of different colors and was sealed with automatic filling and sealing machine, then kept for 2 hr equilibration at 4°C. Following equilibration, the semen was frozen by vapour freeze technique (Pal *et al*., 2011) .and the straws containing frozen semen were stored in goblets and kept immersed in liquid nitrogen cryocans.

After more than 24 hours of cryopreservation or storage semen straws were taken out and thawed at 37°C for 30 seconds. Control, T_1 andT_2 groups were evaluated for post thaw motility, viability, membrane integrity and acrosome integrity as done for fresh semen.

Evaluation of progressive sperm motility

Evaluation of progressive sperm motility was done as per Kumar, 2019 with minor modifications. CASA system (HTB CEROS II, Version 1.3, Hamilton Thorne Research, Beverly, MA, USA, Plate 20) equipped with a thermo stage (MiniTherm®, Hamilton Thorne Inc. Beverly, MA, USA, Plate 21) was used to analyse the progressive sperm motility. Semen sample (4 µl diluted aliquots; 50 µl of semen sample dissolved in 1 ml of sodium citrate diluting fluid to make a 1:20 dilution) was loaded in disposable chambers with a 20 µm chamber depth (Leja® Standard Count 8 Chamber Slide, 20 µm, Leja® Products B.V., Netherlands). For each evaluation, seven microscopic fields, each including at least 250 cells, were analysed. CASA settings used were progressive STR (%) 50, Progressive VAP (µm/s) 37. System was set to measure

30 frames per field at frame rate of 60Hz. Sperm track with a straightness value more than 50% and velocity average path more than 37µm/s were progressive motile. Stage temperature was 38ºC.

Evaluation of Sperm concentration

The sperm concentration, in semen sample mixed with diluting sample (1:20) was estimated by hemocytometer according to Kant, 2016.

Evaluation of live sperm count

For live sperm count propidium iodide staining procedure given by (Pintado *et al*., 2000) was used. A stock solution of 0.5 mg propidium iodide per ml water was prepared, aliquoted and stored frozen at –20°C in the dark. Spermatozoa were diluted in the saline medium and the propidium iodide stock solution was added, so that the final concentration was 5 µg/ml. The suspension was incubated for 5 min at room temperature and spermatozoa were immobilized with formaldehyde 0.003% (w/v), BSA (5 µl of 100 mg/ml) was added and stained samples were placed between a slide and coverslip. Spermatozoa were examined under phase-contrast optics and using the filter for propidium iodide (Fig.2).

Evaluation of membrane integrity

The procedure described by Nie and Wenzel, 2001 was used to determine the percentage of hypo-osmotic stress test-positive cells in each sample. A 1µL aliquot of each semen sample was mixed in 1.0 mL of a prewarmed 100 mOsm sucrose solution (1.712 g sucrose dissolved in 50 mL of sterile, deionized water). The mixture was incubated at 37^0C for 60 minutes in a 1.5-mL microcentrifuge tube. Following incubation, a small drop of sample was placed on a microscope slide and cover slipped for examination by using phase-contrast microscopy (400) to evaluate 100 spermatozoa for evidence of swelling and curling changes.

Evaluation of Acrosomal integrity test

The acrosome integrity of the sperms was determined by Giemsa stain as per method described by Watson (1975) was followed.

Results and Discussion

Overall mean total ejaculate volume, overall mean gel volume and overall mean gel free semen volume recorded for fresh semen were 49.47±5.31 ml, 18±3.9 ml and 31±2.04 ml respectively. Overall mean progressive sperm motility recorded was 76.34±0.95%. Overall mean sperm concentration was 143.49±8.29 millions per ml. Overall mean sperm viability, membrane integrity and acrosome integrity recorded were 85.00±0.87%. 58.1±0.86%. 86.97±0.76%, respectively.

At post-thaw stage there was reduction in the progressive sperm motility in all three groups. In T_1 group per cent progressive sperm motility was significantly ($P<0.05$) higher than control (Table.1). The percentage reduction in progressive sperm motility from fresh to post-thaw stage was 44.31, 28.26 and 46.80 in C, T_1 and T_2 group respectively, indicating beneficial effect of 50mM trehalose.

El Badry *et al*., 2017 reported improved motility rate of post-thaw stallion spermatozoa frozen by 50 mM trehalose, while higher concentrations (100 mM and 200 mM) reduced post-thaw motility. Ghallab *et al*., (2017) reported beneficial effect of 150 mM trehalose on motility post thaw stallion spermatozoa. Vafaei *et al*., (2019) reported toxic effect of 150 mM trehalose on motility of post thaw stallion spermatozoa.

Similarly beneficial effect of different concentrations of trehalose supplementation for different species has been reported. Aisen *et al.*, 2002 (Ram, 100mOsm), Bucak *et al.*, 2007 (Ram, 50mM), Matsuoka *et al*., 2006 (Ram, 435mM), Hu *et al.*, 2009 (Boar, 25,50,100 and 200mM), Reddy *et al.*, 2010 (Buffalo, 100mM), Ozturk *et al*., 2017 (Bull, 25mM) and Bittencourt *et al.*, 2018 (Ram,100mOsm).While deleterious effect of trehalose has also been reported by Aisen *et al*., 2002 (Ram, 200mOsm).

At post-thaw stage there was reduction in the per cent viable sperm in all three groups(Table.1). In T_1 group per cent viable sperm was significantly ($P<0.05$) higher than control. The percentage decease in per cent viable sperm from fresh to post-thaw stage was 43.4, 29.74 and 43.81 in C, T_1 and T_2 group respectively, pointing to advantageous effect of 50mM trehalose.

El Badry *et al.*, 2017 reported that 25mM and 50mM trehalose improved viability of post-thaw stallion spermatozoa but 200 mM proved to be deleterious. Ghallab *et al.*, 2017 also reported beneficial effect of 150 mM trehalose on viability of post thaw stallion spermatozoa. Vafaei *et al.*, 2019 reported toxic effect of 150 mM trehalose on motility of post thaw stallion spermatozoa. Similarly, beneficial effect of different concentrations of trehalose supplementation on post-thaw viability for different species has been reported. Matsuoka *et al.*, 2006 (Ram, 435mM), Reddy *et al.*, 2010 (Buffalo, 100mM), El Badry *et al*., 2017 (Equine 50mM and 25mM), Ozturk *et al*., 2017 (Bull, 25mM). Bittencourt *et al.*, 2018 (Ram, 100mOsm).

At post-thaw stage there was reduction in the per cent hypo-osmotic swollen spermatozoa in all three groups. However, in control group per cent hypo-osmotic swollen spermatozoa were significantly ($P<0.05$) lower than T_1 and T_2 (Table.1). The deleterious effect of group T_2 on percentage hypo-osmotic swollen spermatozoa was significant. The percentage decease in per cent hypo-osmotic swollen spermatozoa from fresh to post-thaw stage was 43.81, 31.81 and 47.25 in C, T_1 and T_2 group, respectively supporting supplementation of low dose of trehalose.

In concord with our results beneficial effect of 50 mM and 100 mM trehalose on membrane integrity has been reported by El Badry *et al*., 2017 for post-thaw equine semen and deleterious effect observed on 200 mM trehalose. Ghallab et *al*., 2017 reported beneficial effect of 100 mM and 150mM trehalose on membrane integrity on post thaw stallion spermatozoa. In agreement with our finding, Aisen *et al.*, 2002 (Ram, 100mOsm), Matsuoka *et al.*, 2006 (Ram, 435mM), Hu *et al.*, 2009 (Boar, 25,50,100 and 200 mmol/liter), Reddy *et al.*, 2010 (Buffalo, 100mM) beneficial effect of trehalose has been reported. Contrary to present study no significant beneficial effect was reported by Bucak *et al.*, 2007 (Ram, 100mM).

At post-thaw stage there was reduction in the per cent spermatozoa with intact acrosome in all three groups. In T_1 group per cent spermatozoa with intact acrosome was significantly ($P<0.05$) higher than control (Table.1).

The deleterious effect of group T_2 on acrosome integrity was significant. The percentage decease in per cent intact acrosome spermatozoa from fresh to post-thaw stage was 16.21, 11.6 and 19.32 in C, T_1 and T_2 group respectively, demonstrating beneficial effect of 50mM trehalose.

In concord with our results beneficial effect of 50 mM and 100 mM trehalose on acrosome integrity has been reported by El Badry *et al.*,

2017 for post-thaw equine semen while deleterious effect on acrosome integrity by 200mM trehalose. However, Ghallab *et al.*, 2017 reported improved acrosome integrity using 75 mM, 100 mM and 150 mM trehalose. Similarly, beneficial effect of trehalose has been reported by Aisen *et al.*, 2002 (Ram, 100mOsm improved), Hu *et al.*, 2009 (Boar, 25,50,100 and 200 mmol/liter) and Reddy *et al.*, 2010 (Buffalo, 100mM)

Contrary to our finding, Bucak *et al.*, 2007 reported no improvement in post-thaw acrosome integrity by 50mM and 100 mM trehalose on ram semen, like wise Bittencourt *et al.*, 2018 (Ram, 100mOsm) and Ozturk *et al.*, 2017 (Bull, 25mM) reported no significant benefit on acrosome integrity post-thaw while harmful effects of trehalose has been reported by Aisen *et al.*, 2002 (Ram, 200mOsm).

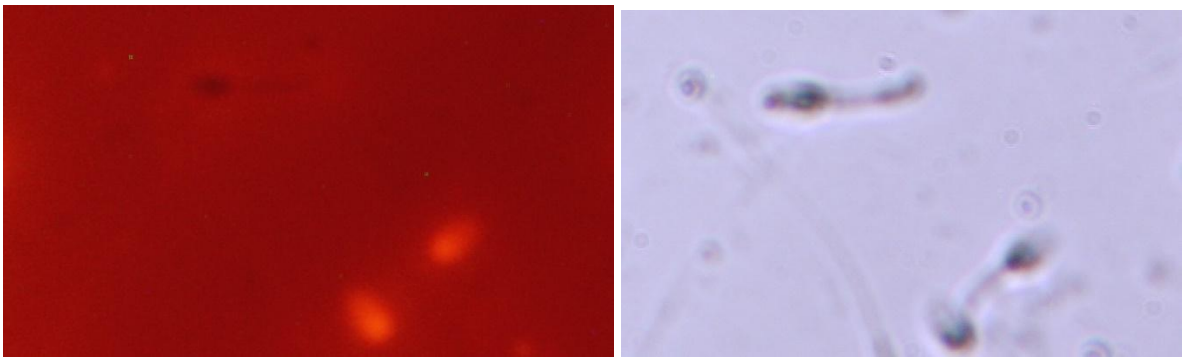
Table.1 Progressive motility, viability, membrane integrity and acrosome integrity in post-thaw semen of stallions treated with different concentrations of trehalose

Group	Post-thaw parameters			
	Progressive sperm motility (%)	Viability (%)	Membrane integrity (%)	Acrosome integrity (%)
C	42.51^{a}±0.98	48.36^{a}±0.89	32.65^{b}±0.43	72.87^{b}±0.50
T_1	54.76^{b}±1.11	60.03^{c}±1.28	39.62^{d}±0.61	76.89^{d}±0.49
T_2	40.61^{a}±0.98	48.01^{a}±1.00	30.65^{a}±0.45	70.16^{a}±0.46

Mean values with different superscripts between treatment groups differ significantly ($P<0.05$). Group C, T_1, T_2, contain 0 mM trehalose, 50 mM trehalose and 150 mM trehalose respectively.

Fig.1 Semen collection by Colorado model Artificial vagina

Fig.2 Sperm viability examination using filter for propidium iodide (left) and under phase contrast optics (right)



Trehalose exert cryoprotective role by decreasing intracellular ice crystal formation, thus maintaining the osmotic balance of the diluent (Garde *et al.*, 2008). Trehalose is a non-penetrating disaccharide, which has a protective action on cells both by increasing the tonicity of the extender and by stabilising the plasma membrane, possibly due to specific interactions with head groups of membrane phospholipids (Crowe *et al.*, 1987). Trehalose inserts itself into the membrane phospholipids bilayer, thus modulating membrane fluidity and therefore rendering the membrane more stable during freezing (Aboagla and Terada, 2003).

Trehalose is a non permeating disaccharide which acts as non enzymatic scavenger. Through its osmotic effects trehalose induces its protective effects against oxidative damage rendering a role in protection of spermatozoa against ROS (Reddy *et al*., 2010). Trehalose also act as hypertonic media causing cellular osmotic dehydration before freezing and decreasing the amount of cell injury by crystallization (Bucak *et al*., 2007). Difference may be ascribed to individual variation in stallion spermatozoa, breeds, type of diluents used in each study (Ghallab *et al.*, 2017). Deleterious effect on sperms by high concentration of trehalose might be due to the incompatibility between water efflux and cellular integrity, further the variable results of reported studies might be due to different extender compositions used or different species exhibiting different tolerance level for trehalose (Ahmad and Aksoy, 2012).

Further study is required regarding fertility of semen frozen by supplementing freezing media by trehalose.

Conflict of interest

The authors have no conflict of interest.

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