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Research Article

EFFECT OF TREHALOSE ADDITION IN SEMEN EXTENDER ON SPERM ABNORMALITIES OF CRYOPRESERVED STALLION SEMEN

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ABSTRACT

The aim of the present study was to evaluate the cryoprotective effect of trehalose on post-thaw sperm abnormality in stallion. A total of 42 ejaculates from six Marwari stallions (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 and centrifuged to get sperm pellets. Secondary extender was added and divided into three equal aliquots that served as control, treatment 1 (50 mM treahalose) and treatment 2 (150mM trehalose). At post-thaw stage, there was increase in the per cent sperm with abnormal head, midpiece, tail and total abnormality in all three groups. On an average, 2% decrease in per cent sperm with total abnormality was observed in T_1 group as compared to control. In control group, per cent sperm with abnormal head, midpiece, tail and total abnormality were significantly ($P<0.05$) higher than T_1 group. T_2 group had significant ($P<0.05$) deleterious effect on abnormality as compared to T_1. In conclusion, addition of 50 mM trehalose improved post-thawing semen quality.

Keywords: Trehalose, stallion, cryopreservation, sperm abnormality

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The use of Artificial Insemination (AI) with frozen semen in equine reproduction has become increasingly popular and several breed registries are approving the foals born from frozen semen (Loomis, 2001). Spermatozoa undergo a series of chemical and physical changes like 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 during cryopreservation procedure. Cryopreservation protocols are designed to minimize the negative effects of these stresses (Loomis and Squires, 2005). Cryopreservation has been reported to cause changes in sperm morphology, including damage to mitochondria, the acrosome and the sperm tail (Wooley and Richardson, 1978). Therefore, the proportion of fully functional sperms that retain intact membranes, tail and mitochondrial activity after freeze–thawing is low (Holt, 1997).

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). Trehalose has a 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 and therefore rendering the membrane more stable during freezing (Aboagla and Terada, 2003). Present study is designed to investigate the effect of trehalose supplementation on post thaw quality of semen of Marwari horse stallion. Trehalose acts as non enzymatic scavenger. Through its osmotic effect, 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). The significant reduction in sperm abnormalities at high concentration compared to low concentration at pre-freeze and post-thaw stage can be explained by concept that 150 mM trehalose may change the media hypertonic and therefore increase external pressure on sperm and altering cell architecture by membrane protein denaturation, lipid phase transition and reduce membrane fluidity (Ahmad *et al.*, 2013).

MATERIAL 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.

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Semen collection, evaluation and freezing and post thaw evaluation: Semen from six Marwari stallions was collected twice a week during morning hours using Colorado type artificial vagina and a mare in estrus as dummy. Total forty-two ejaculates were collected each from all six stallions. Each ejaculate was filtered through a sterile piece of gauge to measure gel free semen volume. The gel free semen was evaluated for sperm abnormalities (Soni, 2016).

Primary extender in the proportion equal to volume of gel free semen was taken in centrifuge tube, to get a sperm pellet.The sperm pellet which was added with secondary extender containing additives as described below to get a final concentration of 150×10^6 spermtaozoa /ml as per Ravi (2014).

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 cryopreserved by standard procedure (Pal *et al.*, 2011). Fresh, pre-freeze and post-thaw evaluation of sperm abnormalities was done according to Soni (2016) using Eosin Nigrosin stain.

Statistical Analysis

Data were collected and statistically analyzed. The data were analyzed using conventional statistical procedures as described by Snedecor and Cochran (1994).

RESULTSAND DISCUSSION

The mean value of per cent sperm with head, midpiece, tail and total abnormalities in each of the stallions are presented in Table 1. The mean values of per cent sperm having head, midpiece, tail and total abnormalities recorded in each of the stallion at pre-freeze and post-thaw stage pretreated with different concentrations of trehalose are presented in Table 2 and 3, respectively. Analysis of variance of this parameter revealed that overall mean sperm having head, mid piece, tail and total abnormalities were significantly lower ($P<0.05$) in group T_1 as compared to control at both pre-freeze and post-thaw stage.

The percentage increment in sperm with total abnormalities from fresh to post-thaw stage was 87.72, 67.27 and 86.25% in C, T_1 and T_2 group, respectively, pointing to favourable effect of 50mM trehalose on maintaining normal morphology on spermatozoa.

Similar beneficial effects of addition of trehalose at concentration of 100 mM and 150 mM on total abnormalities reduction during pre-freeze and post-freeze thaw stage in stallion spermatozoa had been recorded by Ghallab *et al.* (2017). In concord with our results, protective effect of trehalose has been reported in ram at 50 mM (Dolti *et al.*, 2016), 100 mM and best at 50 mM and 150 mM concentration (Uysal and Bucak, 2009), in buck at 50 mM (Tuncer *et al.*, 2013) and in equine at 100 mM and 150 mM concentration (Ghallab *et al.*, 2017). However, no significant benefit has been reported by addition of trehalose at different concentration of 435 mM (Matsuoka *et al.*, 2006), 50 mM and 100 mM (Bucak *et al.*, 2007) in ram and 25 mM and 75 mM in buck (Tuncer *et al.*, 2013).In contrary to our findings, deleterious effect have been reported on sperm abnormalities by Vafaei *et al.* (2019) in post-thaw equine semen frozen by 50 mM trehalose supplementation. Similarly, Tuncer *et al.*, 2013 also reported toxic effects in post-thaw buck semen by 100 mM and 150 mM trehalose. Conflicting results reported regarding effect of trehalose by other researchers may be due to species tolerance of trehalose concentration and type of extender used.

Table 1

Sperm head, mid-piece, tail and total sperm morphological abnormalities (Mean±SE) in freshly ejaculated semen of Marwari stallions

Stallion	Head (%)	Mid-piece (%)	Tail (%)	Total (%)
S1	$1.66^{a}\pm0.12$	$3.08^{a}\pm0.27$	3.16 ± 0.36	$7.91^{a}\pm0.43$
S2	$1.91^{ab}\pm0.12$	$3.36^{a}\pm0.10$	3.42 ± 0.11	$8.70^{ab}\pm0.18$
S3	$2.21^{bc}\pm0.09$	$3.04^{a}\pm0.15$	3.50 ± 0.12	$8.75^{ab}\pm0.30$
S4	$2.54^{c}\pm0.14$	$3.02^{a}\pm0.17$	3.36 ± 0.15	$8.92^{b}\pm0.12$
S5	$2.49^{c}\pm013$	$3.96^{b}\pm0.13$	3.51 ± 0.08	$9.97^{c}\pm0.27$
S6	$2.07^{b}\pm0.09$	$3.18^{a}\pm0.16$	3.28 ± 0.07	$8.53^{ab}\pm0.25$
Overall	2.14 ± 0.06	3.27 ± 0.08	3.37 ± 0.07	8.80 ± 0.14

Note: Mean values with different superscripts between different stallions differ statistically in column. ($P<0.05$)

Table 2

Sperm morphological abnormalities in head, mid-piece and tail (%) (Mean±SE) in pre-freeze semen of stallion pretreated with different concentrations of trehalose

Marwari stallion→ groups↓		S1	S2	S3	S4	S5	S6	Over all
C	Head (%)	2.27^{b}±0.09	3.05^{b}±0.22	3.58^{b}±0.10	3.47^{bc}±0.13	3.51^{b}±0.13	3.14^{b}±0.11	3.17^{b}±0.09
	Mid-piece (%)	4.12±0.25	4.55^{c}±0.11	4.13^{bc}±0.17	3.93^{bc}±0.16	3.96^{b}±0.13	4.08^{b}±0.17	4.13^{b}±0.07
	Tail (%)	4.42±0.36	4.35^{b}±0.10	4.36^{cd}±0.11	4.23^{b}±0.16	4.34^{c}±0.06	4.11^{b}±0.12	4.30^{b}±0.07
	Total (%)	10.81±0.44^{b}	11.95^{c} ±0.25	12.07^{c}±0.27	11.63^{c}±0.23	11.82^{b}±0.25	11.33±0.21^{c}	11.60^{b}±0.13
T_1	Head (%)	2.17^{b}±0.13	2.29^{a}±0.14	2.89^{a}±0.16	3.00^{a}±0.15	2.74^{a}±0.12	2.46^{a}±0.07	2.59^{a}±0.07
	Mid-piece (%)	3.75±0.26	3.79^{ab}±0.08	3.60^{a}±0.13	3.31^{a}±0.15	3.47^{a}±0.11	3.44^{a}±0.10	3.56^{a}±0.06
	Tail (%)	4.02±0.40	3.80^{a}±0.12	3.73^{a}±0.12	3.67^{a}±0.08	3.71^{a}±0.08	3.45^{a}±0.07	3.73^{a}±0.08
	Total (%)	9.94^{ab}±0.50	9.89±0.18	10.23^{ab}±0.21	9.98^{ab}±0.14	9.92^{a}±0.26	9.35^{a}±0.17	9.88^{a}±0.11
T_2	Head (%)	1.81^{a}±0.10	2.99^{b}±0.20	3.76^{b} ±0.09	3.67^{c}±0.15	3.91^{b}±0.17	3.11^{b}±0.11	3.21^{b}±0.12
	Mid-piece (%)	3.36±0.27	4.93^{d}±0.14	4.31^{c}±0.15	4.15^{c}±0.14	4.03^{b}±0.21	4.11^{b}±0.16	4.15^{b}±0.10
	Tail (%)	3.54±0.38	4.59^{b}±0.13	4.60^{d}±0.10	4.37^{b}±0.14	4.29^{b}±0.08	4.15^{b}±0.11	4.26^{b}±0.09
	Total (%)	8.71^{a}±0.40	12.51±0.35	12.67^{c}±0.23	12.19^{c}±0.23	12.23^{b}±0.34	11.37^{c}±0.22	11.61^{b}±0.24

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

Table 3

Sperm morphological abnormalities in head, mid-piece and tail (%) (Mean±SE) in post thawed semen of Marwari stallions

Marwari stallion→ groups↓		S1	S2	S3	S4	S5	S6	Over all
C	Head (%)	2.93^{c}±0.11	3.62^{c}±0.19	4.15^{b}±0.10	3.99^{bc}±0.12	4.18^{b}±0.17	3.63^{b}±0.10	3.75^{b}±0.08
	Mid-piece (%)	6.23^{c}±0.23	6.64^{c}±0.07	6.03^{c}±0.14	6.42^{d}±0.16	6.49$^{a±}$0.17	6.60^{c}±0.16	6.40^{c}±0.07
	Tail (%)	6.35±0.32	6.35^{c}±0.11	6.17^{bc}±0.09	6.25^{cd}±0.16	6.51^{b}±0.12	6.59^{b}±0.11	6.37^{b}±0.07
	Total (%)	15.51^{b}±0.39	16.62^{c}±0.21	16.36^{c}±0.22	16.66^{c}±0.25	17.18^{b}±0.20	16.81^{c}±0.21	16.52^{c}±0.13
T1	Head (%)	2.79^{c}±0.13	2.85^{a}±0.18	3.40^{a}±0.14	3.54^{ab}±0.16	3.29^{a}±0.10	2.99^{a}±0.10	3.14^{a}±0.07
	Mid-piece (%)	5.95^{ab}±0.26	5.76^{a}±0.08	5.39^{ab}±0.09	5.35^{a}±0.15	5.90^{ab}±0.09	5.91^{a}±0.07	5.71^{ab}±0.07
	Tail (%)	6.17±0.38	5.66^{a}±0.14	5.42^{a}±0.11	5.77^{ab}±0.07	6.11^{ab}±0.15	6.09^{a}±0.10	5.87^{a}±0.08
	Total (%)	14.92^{b}±0.51	14.26^{a}±0.28	14.22^{a}±0.21	14.65^{a}±0.12	15.30^{a}±0.23	14.99^{ab}±0.18	14.72^{ab}±0.12
T2	Head (%)	2.28^{a}±0.11	3.48^{bc}±0.19	4.28^{b}±0.08	4.25^{c}±0.15	4.76^{c}±0.35	3.63^{b}±0.11	3.78^{b}±0.14
	Mid-piece (%)	5.29^{a}±0.26	6.98^{d}±0.15	6.14^{c}±0.16	6.35^{cd}±0.12	6.44^{bc}±0.28	6.53^{b}±0.14	6.29^{c}±0.11
	Tail (%)	5.41±0.36	6.61^{c}±0.10	6.350^{c}±0.09	6.49^{d}±0.10	6.38^{ab}±0.14	6.68^{b}±0.12	6.32^{b}±0.10
	Total (%)	12.97^{a}±0.32	17.07^{c}±0.31	16.77^{c}±0.24	17.09^{c}±0.08	17.58^{b}±0.58	16.84^{c}±0.19	16.39^{c}±0.27

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

In the current study, the protective effect of trehalose was identified at a concentration of 50 mM, and at a much-reduced rate at 150 mM as compared to 50 mM trehalose. The later concentration might result in a high osmolarity of the extender, which is deleterious to the sperm cells.

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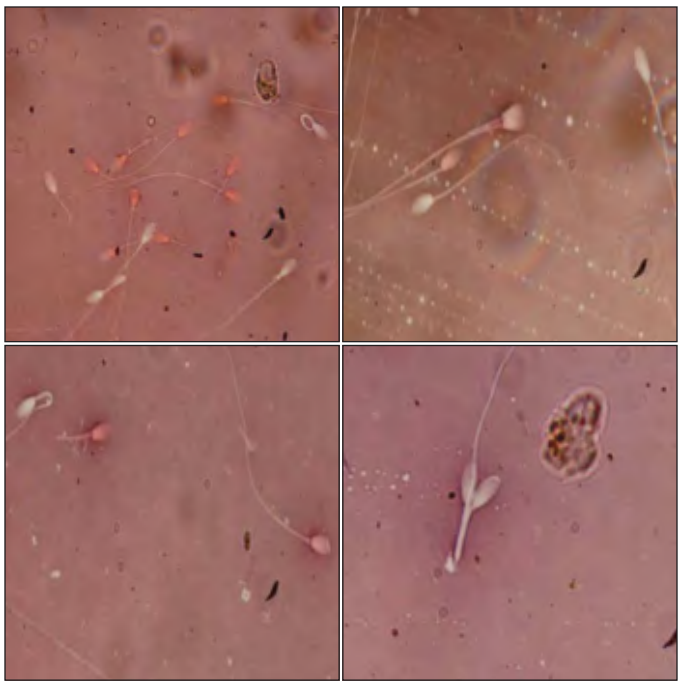

Fig. 1. sperm abnormalities

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