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Melatonin supplementation improves the intactness of plasma membrane and acrosomal membrane of cryopreserved spermatozoa in Hariana bull

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Generation of reactive oxygen species (ROS) during freeze-thaw cycle reduces semen quality (Ashrafi *et al.* 2013). Extenders are supplemented with enzymatic and non-enzymatic additives to enhance the post-thawed semen quality. Melatonin (MT), secreted by the pineal gland in the brain (Awad *et al.* 2006), participates in control of seasonal reproduction (Reiter 1973), affects the immune system (Esquifino *et al.* 2004) and circadian rhythms (Reiter 1991). The protective effects of MT as an antioxidant are because of its high efficacy as a hydroxyl radical scavenger (El-Sokkary *et al.* 2003). MT has ability to detoxify reactive oxygen and nitrogen species (Kapadiya *et al.* 2016). MT also stimulates the activities of enzymes involved in metabolising ROS and preserves cell membrane fluidity. Melatonin has protective effect on sperm parameters in cryopreserved semen of boar (Jang *et al.* 2009), human (Du Plessis *et al.* 2010) and ram (Succu *et al.* 2011, Ashrafi *et al.* 2011). However, information on the use of MT to improve the post-thaw sperm quality in indigenous Hariana bull is lacking. Therefore, present study was designed to investigate the role of MT on intactness of plasma membrane and acrosome of Hariana bull spermatozoa after freezing-thawing.

The present study was conducted at the University Instructional Livestock Farm Complex during 2017–18. Apparently healthy breeding Hariana bulls (4), 7.5–8.5 year-old, weighing between 450–500 kg were used for semen collection and freeze-thaw evaluation. Total 32 ejaculates were collected (eight from each bull) using artificial vagina. The ejaculates having mass activity 3 and above, forward progressive motility 80% and above were selected for freezing with liquid nitrogen. Ejaculates were diluted in Tris diluent with concentration of 100×10^6 spermatozoa/mL and were divided equally into 4 groups (1 control and 3 treatments groups). Groups were Gr 1, control (without addition of MT); Gr 2, 0.5 mM MT; Gr 3, 1.0 mM MT and Gr 4, 2.0 mM MT. In all the experimental groups, MT was supplemented with EYTG in 100×10^6 spermatozoa/ml. Semen ejaculates were processed for equilibration, followed by their freezing and thawing in the semen biology laboratory as described earlier (Shah *et al.* 2017). Intactness of plasma membrane was evaluated using hypo-osmotic swelling test (HOST) using hypo-osmotic solution of 150 mOsm/L (Shah *et al.* 2017, Yadav *et al.* 2017) and sperm acrosomal status was evaluated using Giemsa stain (Watson 1975) after equilibration and freezing-thawing in all the groups. Data obtained from the study were statistically analysed using 2-way ANOVA and Post Hoc Tukey test was used to study the significance using SPSS 16.1 version (Chicago, USA).

Per cent spermatozoa showing intact acrosome in all the 4 groups are illustrated in Fig. 1. Results revealed nonsignificant ($P>0.05$) difference among the Gr 1, 3 and 4 in per cent of HOS positive response in pre-freeze stage (Fig. 1). However, significant ($P<0.05$) difference was observed between Gr 1 and 4. Post thaw HOS response was significantly higher ($P<0.05$) in Gr 4 as compared to Gr 1, 2 and 3 in freeze-thawed semen. Percentage of intact acrosome was significantly ($P>0.05$) higher in Gr 4 as compared to Gr 1 and 2, however no significant difference was observed between Gr 1, 2 and 3 in pre-freeze stage. In

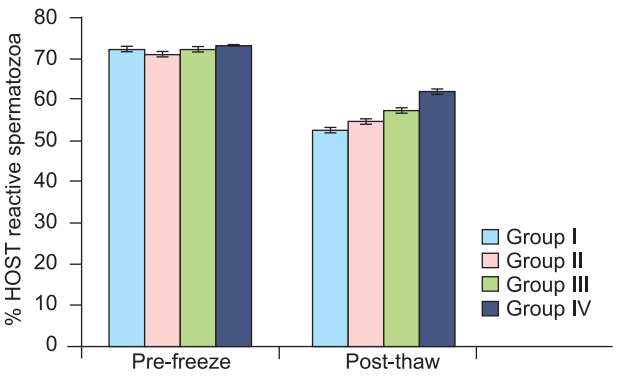

Fig. 1. Effect of melatonin on per cent HOST response of Hariana bull spermatozoa at pre-freeze and post-thaw stage (mean±SEM, bars indicate the standard error).

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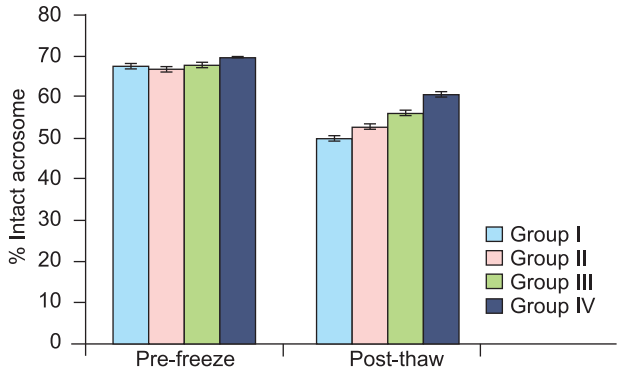

Fig. 2. Effect of melatonin on per cent intact acrosome of Hariana bull spermatozoa at pre-freeze and post-thaw stage (mean±SEM, n=32). Bars indicate the standard error of mean.

post thawed stage, the percentage of intact acrosome was significantly (P>0.05) higher in Gr 4 as compared to other three groups and significant difference was observed in percentage of intact acrosome among Gr 1, 2, 3 and 4 being lowest was in Gr 1 and highest in Gr 4 (Fig. 2).

Melatonin was used for semen cryopreservation in different animal species such as bull (Ashrafia *et al.* 2013), he-buffalo (Asma-ul-Husna *et al.* 2017), ram (Succu *et al.* 2011), stallion (Izadpanah *et al.* 2015), boar (Martin *et al.* 2011) and mithun (Perumal *et al.* 2016) with varying degree of beneficial effects. It was further postulated that MT can be useful to certain extent for cryopreservation of bull spermatozoa (Ashrafia *et al.* 2013). Such study is lacking in Hariana bulls. Therefore, the present study was conducted.

Our findings are in accordance with Kapadiya *et al.* (2016) who reported that 2 mM MT had significantly higher per cent of HOS positive spermatozoa at post-dilution, post-equilibration and post-thaw stages of cryopreservation as compared to control in Kankrej semen. Similarly, Ashrafi *et al.* (2013) reported significantly greater HOST reactive sperm per cent for 2 mM MT as compared to control group in Holstein bulls at post-thaw stage of the cryopreservation. However, Perumal *et al.* (2013, 2015) found significantly higher HOST reactive spermatozoa at 3 mM MT concentration for liquid storage of mithun semen. Chankitisakul (2014) also observed significantly higher HOST reactive spermatozoa at 1 mM MT during cooling storage of boar semen; while Jang *et al.* (2007) in boar reported a nonsignificant effect on per cent HOST reactive spermatozoa in MT added semen extender during incubation. This might be owing to variation in animal species.

Melatonin maintains plasma and mitochondrial membrane integrity and cytoskeleton structure of flagella of sperm as cell protecting effects (Perumal *et al.* 2013). MT also protects and stimulates the activities of antioxidant enzymes such as superoxide dismutase (SOD), reduced glutathione (GSH) and catalase (CAT), which help to maintain the membrane transportation and fertility of the spermatozoa (Perumal *et al.* 2013). This might be the mechanism implicated in increasing the HOST positive sperm per cent in the present study.

The physiologic acrosomal reaction is a well-coordinated process that can occur only in a living spermatozoon in response to natural inducers. Acrosomal integrity of mammalian spermatozoa is the prerequisite for capacitation, normal acrosome reaction and successful fertilization *in vivo.* In contrast, loss of acrosomal content can occur with the breakdown of the membranes during cell death, cryopreservation and during addition of oxidants which showed similar acrosomal changes (Perumal *et al.* 2009).

Percentage of spermatozoa with intact acrosome was significantly (P≤0.05) higher in Gr 4 as compared to Gr 1, 2 and 3 at post-thawed stage. However, no significant difference was observed between Gr 1, 2 and 3 at pre-freeze stage. Gr 2 and 3 also showed significantly (P≤0.05) higher percentage acrosomal integrity as compared to Gr 1 at post-thawed stage. Our findings were in concurrence with Kapadiya *et al.* (2016), who reported 2 mM MT had significantly higher percentage of sperm with acrosomal intactness at post-dilution, equilibration and post-thaw stages of cryopreservation as compared to control in Kankrej semen. Similarly, Ashrafi *et al.* (2013) reported significantly lower acrosomal abnormality in 2 mM MT at post-thawed stage of cryopreservation in Holstein bulls. El-Raey *et al.* (2014) reported a significant improvement in post-thawed acrosomal integrity by using lower concentration of MT in Egyptian buffalo bulls. However, Perumal *et al.* (2013) and Perumal *et al.* (2015) observed a significant rise in intact acrosome sperm per cent using 3 mM MT in liquid storage of mithun semen.

Melatonin, in sperm cells is able to react with many ROS directly for protecting mammalian cells against oxidative stress (Perumal *et al.* 2013, 2015). Beneficial effect of addition of MT on acrosomal integrity in the present study may be attributed to its stabilizing combat for the integrity of plasmalemma of spermatozoa.

SUMMARY

Supplementation of MT @ 2 mM was more beneficial in cryopreservation of Hariana bull spermatozoa as evidenced from post-thawed sperm membrane integrity and acrosomal intactness. Melatonin can be recommended to be used @ 2 mM concentration into the semen extender to increase the post thaw sperm functional attributes in Hariana bull.

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