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Anti-apoptotic effect of melatonin in sperm of mithun

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ABSTRACT

The present study was designed to measure the effect of melatonin (MT) on apoptosis of sperm in mithun. Ejaculates (20) were collected from mithun bulls and were split into five equal aliquots, diluted with the TEYC extender. (Gr 1: semen without additives (control), Gr 2 to Gr 6: semen was diluted with 1, 2, 3, 4 and 5 mM of MT respectively). Apoptotic sperm percentage was estimated using commercially available apoptotic assessment kit at immediately after dilution, equilibration and post-thaw. Inclusion of MT into diluent resulted in a significant decrease in apoptotic sperm percentage at different stage of semen preservation as compared to untreated control group. Moreover, MT at 3 mM had significantly lower apoptotic sperm percentage than MT at 1 mM, 2 mM or 4 mM or 5 mM stored sperm at different stage. It was concluded that MT at 3 mM is suitable to minimize apoptosis in mithun sperm to pursuit future sperm preservation protocols.

Key words: Apoptosis, Melatonin, Mithun, Sperm

Apoptosis is a physiologically programmed cell death (Martin *et al.* 2004) and a significant negative correlation was reported between the proportion of apoptotic cells and sperm viability, motility and fertility in ejaculated semen. Evaluation of apoptosis in sperm cells would be a good indicative of the semen quality (Bora *et al.* 2015). Apoptosis is a normal phenomenon in the freshly ejaculated sperm cells, but this becomes abnormal when the sperm cells are exposed to low temperature. Liquid preservation acts as an apoptotic mechanism inducer in bovine sperm cells (Khan *et al.* 2009). Melatonin as well as its metabolites are indirect antioxidants and powerful direct scavengers of free radicals and it is a multifunctional and universal scavenger (Reiter *et al.* 1998). Indeed, Melatonin was reported to be twice as potent as vitamin E in eliminating the peroxyl radicals and it is more effective in scavenging hydroxyl radicals than glutathione and mannitol (Hardeland *et al.* 1993). However, it has been reported recently that melatonin prevents *in vitro* sperm capacitation and apoptotic like changes (Casao *et al.* 2009). The effect of melatonin in preventing apoptotic like changes may be related to its antioxidant and free radical scavenging activities. The addition of anti-apoptotic/ antioxidant such as melatonin to ram sperm (Casao *et al.* 2009), boar sperm (Hyun-Yong *et al.* 2006) and bull sperm (Ashrafi *et al.* 2013) has been shown to protect sperm against the harmful effects of ROS and improve sperm motility and membrane integrity during sperm storage. Further, perusal of literatures revealed no information on anti-apoptotic effect of melatonin in sperm of mithun. Therefore, a study was designed to assess the effect of melatonin on the anti-apoptotic effect on sperm in mithun to pursuit future sperm preservation protocols.

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MATERIALS AND METHODS

Eight apparently healthy mithun bulls of ~4 to 6 yr of age (body weight: 490 to 510 kg) with good body condition (score 5–6) were selected from the herd, ICAR-National Research Centre on Mithun, Medziphema, Nagaland, India. They were maintained under uniform feeding, housing and managemental conditions. Experimental animals were fed as per the farm schedule. During the study, all the experimental protocols met the Institutional Animal Care and Use Committee regulations. A total numbers of 20 ejaculates were collected from the mithun bulls twice a week through rectal massage method. After the initial evaluations, samples were subjected to the initial dilution with pre-warmed (37°C) standard Tris egg yolk citrate extender (TEYC) for bovine species. The partially diluted samples were then brought to the laboratory for further processing. The ejaculates were evaluated and accepted for evaluation if the following criteria were met: volume: 1–2 ml, concentration: >500 million/ml; mass activity >3+, individual motility: >70% and total abnormality: <10%.

Melatonin (Sigma-Aldrich, St. Louis, Mo, USA) was dissolved in a small volume (0.04 ml) of absolute ethanol and diluted with isotonic sodium citrate solution (3% in distilled water) upto concentrations of 1, 2, 3, 4 and 5 mM. The control group contained only diluted solution of ethanol.

Each ejaculate was split into five equal aliquots and diluted with the TEYC extender with melatonin (Gr 1: semen without additives (control), Gr 2 to Gr 6: semen with 1, 2, 3, 4 and 5 mM of melatonin, respectively) and semen samples were processed as per the standard protocol for freezing and stored in liquid nitrogen. The percentage of apoptosis was studied with commercially available apoptotic measurement kit (Apo Detect, Annexin V-FITC kit, Invitrogen Corporation, USA) according to manufacturer's instructions. A total of 300 cells were counted per slide and the percentage of apoptotic cells was calculated.

The results were analysed statistically and expressed as the mean±S.E.M. Means were analyzed by one way analysis of variance (ANOVA), followed by the Tukey's post hoc test to determine significant differences among the five experimental groups at different stage of cryopreservation on the apoptotic sperm% using the SPSS/PC computer program (version 15.0; SPSS, Chicago, IL). Differences with values of $P<0.05$ were considered to be statistically significant after arcsine transformation of percentage data by using SPSS 15.

RESULTS AND DISCUSSION

Effects of various doses of MT on apoptosis in different stages of cryopreservation revealed that inclusion of MT into diluent significantly decreased ($P<0.05$) percentage of apoptosis when examined at different stages as compared to the control group. Additionally, MT at 1, 2, 4 and 5 mM showed higher percentage of apoptosis than MT at 3 mM and significant ($P<0.05$) difference was observed among the treatment groups for this response. It was obvious from the data of this study that inclusion of MT, particularly at 3 mM to the diluent resulted in significant decrease in apoptotic sperm percentage of semen stored in ultra low temperature preservation at -196°C (Table 1).

There was no report on effect of exogenous MT on apoptotic sperm percentage in mithun and to the best of our knowledge this is the first report of the effect of melatonin on this parameter. But many authors reported that MT has beneficial effects on prevention of apoptosis in mammalian sperm and improves the functional parameters of spermatozoa. The beneficial effects of MT in prevention of apoptosis in semen preservation is due to the fact that it is a very potent anti-apoptotic and antioxidant (Casao *et al.* 2009, Ashrafi *et al.* 2013, Hyun-Yong *et al.* 2006).

Mammalian sperm membrane has high polyunsatured fatty acids and renders the sperm very susceptible to lipid peroxidation (LPO) which leads to the impairment of sperm function, damage to sperm DNA and apoptosis (Gavella *et al.* 1996). In addition, the antioxidant system of seminal plasma and spermatozoa is compromised during semen processing and the levels of antioxidant decreased during the preservation process leads to excessive generation of ROS and apoptosis (Alvarez *et al.* 1992). Therefore, exogenous antioxidants need to be included with natural antioxidants to reduce the ROS and apoptosis in chilled semen (Ashrafi *et al.* 2013). Results of the present study showed that inclusion of 3 mM of MT reduced apoptosis of mithun semen preserved at -196°C. Effect of different levels of melatonin might be explained according to the report of Ashrafi *et al.* (2013) who showed that the higher amount of antioxidants than optimum caused high fluidity of plasma membrane above the desired point, making sperm more prone to acrosomal damages and apoptotic. In addition, the concentration of antioxidants added to extender should be considered since high dosage of antioxidants may be harmful to spermatozoa due to the change in physiological condition of semen extender. In mithun, survival of spermatozoa will increase when the dosage of antioxidant added to extender increases. However, the antioxidant dosage higher than required amount was toxic to spermatozoa (Maxwell and Stojanov 1996). Similarly, in the present study, increasing dosage of melatonin, at 4 or 5 mM increased the percentage of apoptotic sperm in mithun semen TEYC extender. At the same time, less dosage rate also affected the sperm apoptotic percentage. But as per the dosage, the apoptotic percentage decreased upto 3 mM then increased to 5 mM. The reduction of apoptotic sperm percentage due to exogenous MT was recorded in the earlier study in ram (Casao *et al.* 2009), bull (Ashrafi *et al.* 2013) and boar sperm (Hyun-Yong *et al.* 2006).

Moreover, it maintains plasma and mitochondrial membrane integrity and cytoskeleton structure of flagella of sperm as cell protecting effects (Lopez *et al.* 2009). Melatonin also protects and stimulates the activities of antioxidant enzymes such as SOD, GSH and CAT (Karbownik and Reiter 2000), which helps to maintain membrane transportation, anti-apoptotic status and fertility of the spermatozoa (Alvarez and Storey 1992). It results indirectly reduces the number of LPO, ROS and also may increase the production of antioxidant molecules protecting

Table 1. Mean (±SE) apoptotic sperm percentage in semen samples treated with melatonin in mithun semen cryopreservation

Stages of cryopreservation	Control	Treatment groups				
		MT (1 mM)	MT (2 mM)	MT (3 mM)	MT (4 mM)	MT (5 mM)
After initial dilution	12.89 ± 1.25^{aA}	12.55 ± 1.26^{aA}	12.10 ± 1.31^{aA}	11.80 ± 1.01^{aA}	12.60 ± 0.76^{aA}	12.75 ± 0.54^{aA}
After equilibration	43.86 ± 1.43^{dB}	28.23 ± 1.63^{abB}	27.31 ± 1.36^{aB}	24.18 ± 1.46^{aB}	34.33 ± 1.39^{bcB}	35.26 ± 1.21^{cB}
After freezing and thawing	52.39 ± 2.21^{dC}	38.97 ± 2.78^{abC}	37.41 ± 1.78^{abC}	34.86 ± 2.41^{aC}	41.75 ± 2.12^{bC}	45.66 ± 1.98^{cC}

^{a,b,c,d}Means with different superscripts within rows differ significantly ($P<0.05$). $^{(A, B \text{ and } C)}$Means with different superscripts within columns differ significantly ($P<0.05$).

the sperm cells against oxidative stress that enhances anti-apoptotic effect of melatonin. Indeed, melatonin was reported be twice as potent as vitamin E in eliminating the peroxyl radicals and it is more effective in scavenging hydroxyl radicals than glutathione or mannitol (Hardeland *et al.* 1993). The effect of melatonin in preventing apoptotic like changes is related to its antioxidant and free radical scavenging activities also increases fertility rate (Casao *et al.* 2009). Therefore the semen samples treated with MT will have high cryoresistance power than untreated control group. In the present study, it was observed that apoptosis of samples that received 3 mM of melatonin were significantly lower than those of the other treatment and control groups. These results were basically consistent with the results previously reported (Gavella *et al.* 1996). In the present study, based on the result, the effect of MT on the apoptosis is dose dependent (Casao *et al.* 2009, Ashrafi *et al.* 2013) and 3 mM melatonin is optimum dose for mithun semen preservation. Moreover, 1 and 2 mM of MT were low and 4–5 mM of MT was over dosage for mithun semen preservation.

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