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Melatonin Supplementation in Extender Enhances the Post Thaw Quality of Buffalo Bull Spermatozoa

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ABSTRACT

Oxidative damage to sperm during cryopreservation is one of the main causes of decline in fertilizing potential. Present study aimed at evaluating the role of melatonin in extender for its cryoprotective effect on post-thaw quality of buffalo bull semen. For this purpose, two consecutive ejaculates were collected from three Nili-Ravi buffalo bulls using artificial vagina at weekly intervals for a period of three weeks (three replicates). Qualifying semen ejaculates were diluted (50×10^6 motile spermatozoa ml^{-1}) in tris citric acid extender with melatonin at 0 (control), 0.1, 0.5, 1, and 1.5 mM. Diluted semen was cooled to 4°C for 2 h and equilibrated for 4 h at 4°C. Straws were then kept over liquid nitrogen vapours for 10 min and plunged in liquid nitrogen for storage. Thawing was performed after 24 h of storage, at 37°C for 30 seconds and post-thaw sperm quality parameters were assessed. Sperm progressive motility, plasma membrane integrity, acrosomal integrity, viability and chromatin integrity was higher ($P<0.05$) in extender containing melatonin 0.1 mM and 0.5 mM compared to control. In conclusion, the addition of melatonin 0.1-0.5 mM to extender improved the post-thaw quality of buffalo bull semen.

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Authors' Contributions
SA and MSA conceived and designed the study. A-H, RE and MSA performed experimental work. BAR and RE analyzed the data. AH, BAR, NU and SA participated in writing of manuscript.

Key words
Melatonin, Buffalo semen, Extender

INTRODUCTION

Semen cryopreservation allows the widespread dissemination of valuable genetic material through artificial insemination (AI), leading to an increased rate of genetic gain. However, to fully exploit the benefits of AI, successful freezing of semen is essential without any compromising fertility but so far this has been met with little success in buffalo (Anzar *et al*., 2003). Generation of reactive oxygen species (ROS) during freeze-thaw cycle accompanied by low antioxidant levels in seminal plasma and in extender induces a state of oxidative stress that cause lipid peroxidation of the bio-membrane system and resulted in reduced semen quality (Holt, 1997). High levels of ROS affect semen quality by deteriorating membrane lipids, proteins, and nuclear/mitochondrial DNA.

The semen antioxidant system comprising of enzymatic (reduced glutathione, glutathione peroxidase, catalase and superoxide dismutase) (Lasso *et al*., 1994; Gadea *et al*., 2004) and non enzymatic antioxidants (ascorbate, urate, tocopherol, pyruvate, glutathione, taurine, and hypotaurine) (Saleh and Agarwal, 2002; Ansari *et al*., 2011a, b) has defensive function against the lipid peroxidation of sperm membrane. Although bovine semen has natural defense system against the ROS, it is considered insufficient under cryopreservation-mediated stress (Nichi *et al*., 2006). Supplementation of extender with suitable antioxidant is suggested to reduce oxidative damage during freeze-thawing of buffalo bull spermatozoa.

Melatonin is an intracellular antioxidant which protects the cells from ROS-mediated damages under oxidative stress both *in vivo* and *in vitro*. Melatonin is reported to have 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 melatonin to improve post-thaw quality of Nili-Ravi buffalo bull spermatozoa is lacking. Therefore, in this study, melatonin in extender was investigated for cryopreserved buffalo bull semen.

MATERIALS AND METHODS

Preparation of extenders

Tris-citric acid was used as a buffer for the

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experimental extenders. It consisted of 1.56 g citric acid (Fisher Scientific, UK) and 3.0 g tris–(hydroxymethyl)-aminomethane (Research Organics, USA) in 73 mL distilled water. The pH of buffer was 7.0 and the osmotic pressure was 320 mOsmol Kg^{-1}. Fructose (Scharlau, Spain) 0.2% wt/vol; glycerol (Riedel-deHaen, Germany) 7%; egg yolk 20% v/v: antibiotics combination; streptomycin sulphate @ 1mg/mL, procaine penicillin@300IU/mL, benzyl penicillin@ 100IU/mL available as Sinbiotic® (China) were added. Five experimental extenders were prepared by adding melatonin at the rate of 0.0 (control), 0.1, 0.5, 1.0 and 1.5 mM.

Semen collection and initial evaluation

Semen was collected with artificial vagina at 42°C from three adult Nili-Ravi buffalo bulls at weekly intervals for 3 weeks (replicates). After collection, semen samples were assessed for volume, motility, concentration. Sperm progressive motility (%) was assessed (X 200) with phase contrast microscope. Sperm concentration was measured with Neubauer haemocytometer. The neat semen sample having 1 ml volume, 60% motility and 0.5 billion spermatozoa ml^{-1} of semen were selected for further processing. The qualifying ejaculates were pooled and held for 15 min at 37°C in a water bath before dilution. Pooled semen was split into five aliquots for dilution in experimental extenders.

Semen processing

Semen aliquots were diluted in a single step at 37°C with one of the five experimental extenders at 50×10^6 motile spermatozoa ml^{-1}. Diluted semen was cooled to 4°C in 2 h and equilibrated for 4 h at 4°C. Semen was then filled in 0.5 ml French straws (IMV, France) with suction pump at 4°C in the cold cabinet unit (Minitub, Germany) and kept over liquid nitrogen vapours for 10 min. Straws were then plunged into liquid nitrogen (-196°C) and stored. After 24 h of cryopreservation, the semen straws were thawed at 37°C for 30 seconds in water bath and then incubated for assessment of post-thaw semen quality.

Post-thaw sperm functional assays

Semen quality assays (motility, plasma membrane integrity, viability and DNA integrity) were performed immediately after thawing.

Sperm progressive motility

10 µl of thawed semen sample was placed on a pre-warmed glass slide and covered with a cover slip and assessed for progressive motility under phase contrast microscope at X 400 at 37°C (Akhter *et al*., 2010).

Sperm plasma membrane integrity

Sperm plasma membrane integrity of buffalo bull spermatozoa was assessed by hyposmotic swelling (HOS) assay (Ansari *et al*., 2011). Solution for HOS assay consisted of sodium citrate 0.73 g and fructose 1.35 g in 100 ml distilled water (osmotic pressure ~190 mOsmol Kg^{-1}). For this purpose, 50 µl of frozen thawed semen was mixed with 500 µl of HOS solution and incubated for 30-40 min at 37°C. 10 µl of semen sample was placed on a glass slide and covered with cover-slip to examine under phase contrast microscope (X 400). Two hundred spermatozoa per experimental extender per replicate were counted for their response characterized by coiled tail indicating intact sperm plasma membrane (Akhter *et al*., 2008).

Sperm viability

Sperm viability was assessed using 0.4% Trypan blue stain. For this purpose, 5µl semen sample and equal amount of trypan blue solution was mixed and air dried for 10 min. The dried slides were placed in formaldehyde-neutral red and fixed for 5 min. The fixed microscopic slides were washed with distilled water and kept in Giemsa stain (7.5%) for 4 h. Air dried slides were examined under phase contrast microscope (X 1000; oil immersion). Spermatozoa stained blue were considered as non viable; while unstained as viable. A total of two hundred spermatozoa were counted for each sample.

Sperm chromatin integrity

Sperm chromatin integrity was studied by toluidine blue as described by Mello (1982). Smears of semen samples were air dried and fixed in 96% ethanol-acetone (1:1) at 4ºC for 30 min. Acid hydrolysis with 4N HCl was carried out at 25ºC for 10-30 min. The smears were then rapidly rinsed in distilled water three times for every two min. The preparations were stained with toluidine blue for 10 min and evaluated under a light microscope (1000X). The lightly stained sperms were taken as having functional chromatin, while stained dark were considered as fragmented chromatin structure. A total of 200 spermatozoa were assessed for each experimental extender.

Statistical analysis

The data on semen quality parameters are presented as means ± SD. Data were analyzed using analysis of variance (ANOVA). When F-ratio was found significant, LSD test was applied to compare the treatment means.

RESULTS AND DISCUSSION

Sperm progressive motility

Cryopreservation directly damages sperm membrane, resulting in the loss of membrane permeability, absence or limited number of motile sperms and subsequent cell death (Cross and Hanks, 1991). The data on the effect of

melatonin in extender on the post-thaw motility (%) of buffalo bull spermatozoa are presented in Figure 1.

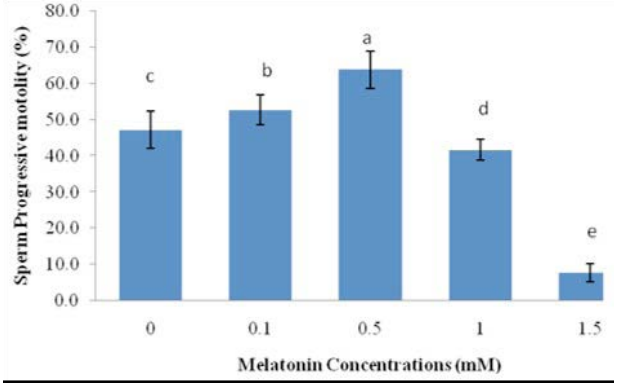

Fig. 1. Effect of different concentration of melatonin on post thaw sperm progressive motility (Mean ± SE) in buffalo bull. Bars showing different letters differ significantly (P<0.05).

Higher (P<0.05) sperm motility was observed in extender containing melatonin 0.1 mM and 0.5 mM compared to control. It is reported that melatonin supplementation improved motility in boar (Jang *et al*., 2009), human (Du Plessis *et al*., 2010), red deer (Dominguez-Rebolledo, 2010) and ram (Succu *et al*., 2011) semen. It is to believe that lower motility in extender containing higher concentrations of melatonin might be due to its relation with Ca^{+2} influx and decreased level of cyclic AMP (Slanar *et al*., 2000).

Sperm plasma membrane integrity

During cryopreservation, sperm undergo dramatic changes in their intracellular and extracellular environment due to exposure to cryoprotectives, cooling, freezing, storage and thawing. The chemical and physical effects of these reagents/processes may cause extensive cryodamage to plasma membranes with resultant changes in their normal functions (Keel and Webster, 1993). The data on the effect of melatonin in extender on post-thaw sperm plasma membrane integrity (%) of buffalo spermatozoa are presented in Figure 2. The percentage sperm plasma membrane integrity was higher (P<0.05) in extender containing melatonin 0.5 mM compared to control. At 1 mM of melatonin, sperm plasma membrane integrity remained similar (P>0.05) compared to control, while, a significant decrease in sperm plasma membrane integrity was observed when melatonin was supplemented at 1.5 mM. The melatonin supplementation at 0.5 mM might have eliminated the toxic ROS, whereas sperm plasmalemma saturated with melatonin at 1.5 mM concentration may have depleted all ROS/NOS and thereby had a depressing effect on sperm plasma membrane integrity (Martin-Hidalgo *et al*., 2011).

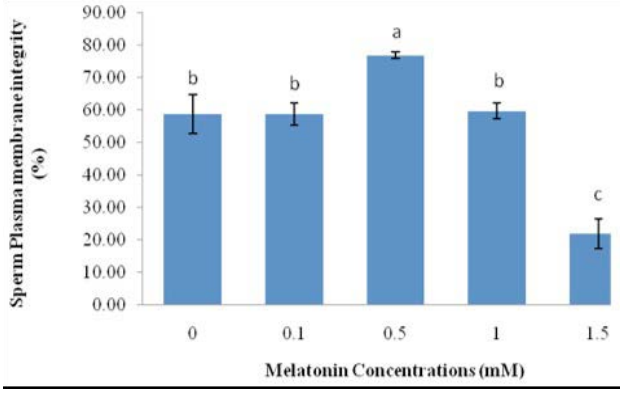

Fig. 2. Effect of different concentrations of melatonin on post thaw plasma membrane integrity (Mean ± SE) of buffalo bulls spermatozoa. Bars showing different letters differ significantly (P<0.05).

Sperm viability

Cryopreservation causes permanent functional damage to sperm viability that can be explained partially by the reduction in the percentage of normal intact acrosomes and in total acrosin activity. Although, damage to the acrosome after cryopreservation may also be secondary to cell death, an increase in the proportion of viable acrosome reacted spermatozoa after cryopreservation has been reported (Cross and Hanks, 1991). The data on effect of melatonin supplementation of extender on post-thaw viability of buffalo bull spermatozoa are presented in Figure 3. Higher (P < 0.05) post-thaw sperm viability was observed in extender containing melatonin (0.5mM) compared to control. Higher concentrations of melatonin supplementation resulted in dose dependent decrease of sperm viability. The improvement in viability of buffalo bull spermatozoa with melatonin supplementation might be due to increased ATP synthesis by mitochondria as was reported in ram semen (Succu *et al*., 2011). Improvement in post thaw sperm viability by supplementation of melatonin has also been reported in boar semen stored at 17°C (Martin-Hidalgo *et al*., 2011).

Sperm chromatin integrity

Sperm DNA damage during cryopreservation process has been reported in buffalo (Kumar *et al*., 2011). Levels of DNA fragmentation in buffalo semen has been correlated with ROS molecules (Kadirvel *et al*., 2009). In present study, post thaw chromatin integrity of buffalo spermatozoa was improved (P<0.05) in extender containing melatonin 0.1 mM and 0.5 mM compared

to control (Fig. 4). Melatonin is reported to protect the oxidative and nitrosative changes in DNA (Reiter *et al*., 2009; Hardeland *et al*., 2009).

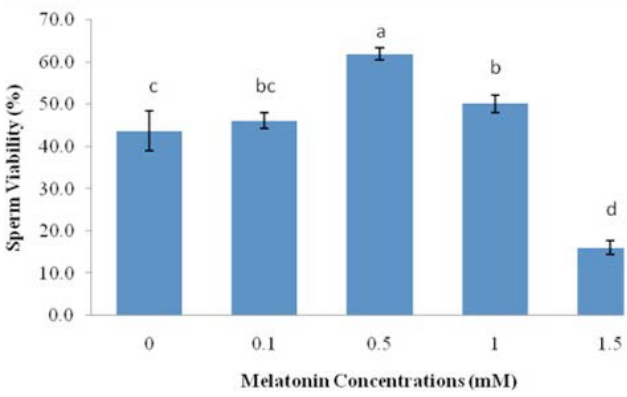

Fig. 3. Effect of different concentrations of melatonin on post thaw viability (Mean ± SE) of buffalo bull spermatozoa. The bars with different letters are significantly different (P<0.05).

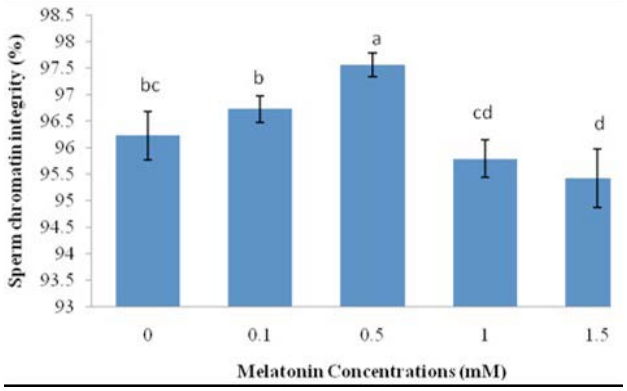

Fig. 4. Effect of different concentrations of melatonin on post thaw sperm chromatin integrity (Mean ± SE) in buffalo bulls. Bars showing different letters differ significantly (P<0.05).

In present study, sperm motility (%), plasma lemma integrity (%), viability (%) and chromatin integrity (%) was improved in extender containing 0.5 mM of melatonin while higher concentrations of melatonin were detrimental for the aforementioned semen quality parameters. The precise mechanism still remain unclear, however, its protective effect on semen quality parameters might be due to (i); direct scavenging of NO *i.e.,* in cell free systems, direct detoxification of $ONOO^-$ or Peroxinitrous (Reiter *et al*., 2007); (ii) activation of antioxidant enzymes that scavenge NO by suppressing cGMP production or (iii) inhibition of eNOS (The enzyme responsible for NO synthesis). Melatonin addition in semen extender is reported to increase the ATP synthesis that favors sperm quality parameters i.e. motility, sperm plasmalemma integrity, acrosomal integrity, viability, livability and chromatin integrity (Miki, 2007). Deterioration of all semen quality parameters at higher concentrations of melatonin may be attributed to depletion of all ROS/NOS and thereby having a negative effect on the functional parameters of spermatozoa (Martin-Hidalgo *et al*., 2011). In conclusion, the addition of melatonin to buffalo bull semen extender at 0.1-0.5 mM improved the post- thaw quality of buffalo bull semen.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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