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Melatonin in cryodiluent improves post-thaw quality and fertility of Murrah buffalo (*Bubalus bubalis*) bull spermatozoa

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ABSTRACT

The current research aimed to analyze the role of melatonin supplementation (0, 0.25, 0.5, 0.75 and 1.0 mM) on the freezability of buffalo bull spermatozoa. Thirty-six ejaculates were collected from six healthy bulls using artificial vagina. Only ejaculates with ≥70% progressive motility, ≤20% morphological defects and >500 million/mL sperm concentration were selected for the study. Each ejaculate was diluted in Tris-egg yolk cryodiluent with selected concentrations of melatonin along with control and cryopreserved following established protocol. The post-thaw semen was assessed for sperm attributes and lipid peroxidation (LPO). IBM SPSS 22.0 software was used for statistical evaluations. The sperm total and progressive motility in post-thaw semen were evaluated using computer assisted semen analyzer (CASA) and were higher (P<0.05) in freezing media having 0.25 mM melatonin in comparison to other concentrations. The sperm viability and acrosome integrity were higher (P<0.05) at 0.25 mM melatonin concentration than at 0.75 mM, 1.0 mM and control. The sperm plasma membrane integrity among the experimental extenders supplemented with melatonin and control did not differ (P>0.05). All melatonin concentrations exhibited lower (P<0.05) MDA levels compared to control. The first service conception rate (FSCR) was higher (P>0.05) following inseminations using semen containing 0.25 mM melatonin than in control. Overall, the addition of 0.25 mM melatonin to the cryodiluent improved post-thaw semen quality of Murrah buffalo bulls.

Keywords: Buffalo bull semen, Cryopreservation, FSCR, Lipid peroxidation, Melatonin, Sperm attributes

Artificial insemination using frozen-thawed semen is an extremely useful tool that promotes rapid genetic progress across species, leading to improved herd efficiency and higher productivity. However, the use of cryopreservation is restricted because spermatozoa show relatively poor tolerance to freezing in buffaloes (Andrabi 2008). The high level of polyunsaturated fatty acids in buffalo sperm membranes makes them highly vulnerable to lipid peroxidation (LPO), leading to higher oxidative stress in comparison to cattle spermatozoa (Nair *et al*. 2006). During freeze-thaw processes, oxidative stress impairs cell membrane, proteins and nuclear material, damages DNA and induces apoptosis-like alterations in sperm, thus affecting sperm viability and fertility (Yadav *et al.* 2017). Bovine semen possesses natural defenses against reactive oxygen species (ROS); which however, are not adequate during cryopreservation (Nichi *et al.* 2006), and may decrease semen quality by more than 50 percent (Hu *et al.* 2011). Complementing semen with suitable additives can enhance the post-thaw semen quality (Memon *et al.* 2012). Studies on various additives highlight the need to select suitable one in order to minimize cryodamage and improve the fertilizing potential of buffalo bull spermatozoa.

Melatonin has recently gained attention for its beneficial role in improving the semen quality (Fayez *et al*. 2023). Melatonin is an amphiphilic antioxidant (García *et al.* 2014) that protects cells against oxidative stress through its free radical-scavenging activity (Deng *et al.* 2017). Compared to vitamin E, melatonin is twice effective in neutralizing peroxyl radicals (Reiter *et al.* 2000). Additionally, melatonin increases the activity of antioxidant enzymes and regulates cellular redox balance, thus maintaining membrane fluidity (Rodriguez *et al*. 2004). Such effects of melatonin have been documented in several species, including goat (Ramadan *et al.* 2009), bull (Ashrafi *et al.* 2013) and mithun (Perumal *et al.* 2013). Thus, melatonin plays an important role in maintaining sperm quality and reproductive efficiency across different mammalian

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species. Yet, beneficial effects of melatonin on preserving semen quality of buffalo bulls are sparse and limited. Hence, the current research was undertaken to evaluate the influence of melatonin supplementation in the cryodiluent on frozen-thawed semen quality of buffalo bulls.

MATERIALS AND METHODS

The *in vitro* studies on cryopreserved buffalo bull semen were approved by the Institutional Animal Ethics Committee (IAEC) of the University (GADVASU/2024/IAEC/74/04). For conducting fertility trials, the study was approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (Regn No. 497/Go/Re-S/ReRc-L/02/CPCSEA dated 24/05/2024).

Collection, evaluation and cryopreservation of semen: Thirty six semen ejaculates from six apparently healthy breeding Murrah buffalo bulls (six ejaculates per bull), having age 5.3±0.2 years (4-6 years) and weighing 712.6±22.3 kg (638-762 kg) were collected with the help of artificial vagina. Qualified ejaculates having ≥70% sperm motility and ≤20% morphological defects assessed with a phase-contrast microscope subjectively and >500 million/ml sperm concentration measured using Accucell spectrophotometer were used in this study. Each ejaculate was split in five equal portions and diluted to obtain a concentration of 80×10^6 sperm/ml in a Tris-egg yolk extender containing different melatonin concentrations viz. 0.0 mM (control), 0.25 mM, 0.50 mM, 0.75 mM, and 1.0 mM. After extended semen was suspended into 0.25 ml straws which were kept for 4 h equilibration period at 4 °C prior to freezing. The straws were first exposed to liquid nitrogen vapors at a height of 4 cm from the surface for eight minutes and then immersed into liquid nitrogen. The frozen-thawed semen samples were assessed after 24 h of cryopreservation for sperm motility and kinetics, sperm viability and acrosome integrity using computer assisted semen analyzer (CASA), plasma membrane integrity and LPO to determine the most effective concentration of melatonin.

Sperm motility and kinetics: A calibrated CASA system (Androvision, Minitube, Germany) was used to assess various sperm motility and kinematics. In brief, the post-thaw semen (10 µl) was placed on a glass slide to determine CASA based sperm motility traits. An average of five optical fields in each semen sample was used to count sperm. The software settings of the CASA used in the present study include resolution (h/v)=2048 pixels x 2048 pixels, frame rate=75 fps, digital input=1, digital output=1, lens mount=C-mount, sensor type=CMOS, ALH cut-off=≥3 µm, VCL cut-off=48 µm/s, VSL cut-off=24 µm/s and BCF cut-off=≥20 Hz.

Sperm viability: Briefly, the thawing of straw was done horizontally by completely submerging it in thawing kit (IMV Technologies, L'Aigle, France) at 35-37°C for 30 seconds.The frozen-thawed semen (50 µl) was treated with 1.5 µl of Hoechst 33342/PI (5 µg/ml+3 µg/ml) and kept in a 2 ml Eppendorf tube at 38 °C for 15 minutes in dark. Then, 10 µl of semen stain mixture was pipetted on a slide, coverslip was placed over it and viewed under microscope (20x, Androvision). In each slide, a total of 200 sperm were counted. Sperm with red fluorescence were considered dead while those with blue fluorescence were considered live. Thereafter, module "viability" of Androvision was selected to record the percentage of viable sperm objectively.

Acrosome integrity: In brief, frozen-thawed semen (50 µl) was mixed with 27 µl Hoechst 33342/FITC-PNA (5 µg/ml+50 µg/ml) solution and kept in a 2 ml microcentrifuge tube at 38 °C for 15 minutes in dark. Thereafter, 10 µl of semen stain mixture was pipetted on a slide, coverslip was placed over it and viewed under microscope (20x, Androvision). In each slide, atleast 200 sperm were counted. All the sperm within green box exhibited intact acrosome and those within red box had damaged acrosome. Finally, module "acrosome integrity" of Androvision was selected to record the percentage of sperm with intact acrosomes objectively.

Plasma membrane integrity: A hypo-osmotic solution (HOS, 150 mOsmol/L) was used to evaluate sperm plasma membrane integrity (Prasad *et al.* 1999). The post-thaw semen (100 µl) was mixed in HOS (1.0 ml) for 1 h at 37°C. Then, 10 µl of the semen mixture was pipetted on a microscopic slide, covered with coverslip and observed under bright-field microscope (40x). About 200 sperm were counted and those exhibiting coiled tails were expressed in percentage

Estimation of lipid peroxidation: Lipid peroxidation (LPO) of spermatozoa was assessed by determining malondialdehyde (MDA) concentration in the post-thaw semen (Buege and Steven, 1978). The sperm pellet was immersed in phosphate buffer saline (pH 7.2) to attain 20 million/ml concentration. Out of this, 1 ml was mixed with 2 ml of TBA-TCA reagent and the solution was heated for 1 h in water during which the color changed from transparent to yellowish. Thereafter, the solution was cooled at room temperature, centrifuged for 10 minutes at 3000 rpm and the supernatant was separated. The absorbance of supernatant was recorded at 535 nm in a bio-spectrophotometer (Eppendorf, Hamburg, Germany). Using specific absorbance coefficient of 1.56×10^6 cm³, the concentrations of MDA (µmol/20 million sperm/ml) according to the following formula:

$$\text{MDA (µmole/20 million sperm/ml)} = \frac{\text{O.D} \times 30}{1.56}$$

Fertility trial: The *in vivo* fertility trial was performed on 60 buffaloes maintained at university dairy farm (n=20) and private organized dairy farm (n=40) located in the surrounding area of Ludhiana. All the buffaloes selected for inseminations were apparently healthy, had calved 60-90 days back, with 2nd to 5th parity and were free from physical and reproductive problems. The buffaloes were inseminated using frozen-thawed semen of control (0.0 mM) and best evolved melatonin concentration (0.25 mM, n=30 each). Pregnancy diagnosis was conducted

using ultrasound machine (Exago, IMV Technologies, L'Aigle, France) at university dairy farm and with rectal examination at private dairy farm on day 60 following insemination. The first service conception rate (FSCR) was calculated and expressed in percentage.

Statistical analysis: IBM SPSS 22.0 software was used for statistical evaluations. The proportionality data pertaining to sperm characteristics were normalized following arcsine transformation. One way Analysis of Variance was carried out for sperm attributes and LPO among treatments and the means were compared with the Duncan Multiple Range test. The Chi-square test was used to analyze *in vivo* fertility. For all analyses, a confidence level of $P<0.05$ was considered to be significant. The results are expressed as Mean±SEM.

RESULTS AND DISCUSSION

The results of the *in vitro* trial indicated that total and progressive motility were higher ($P<0.05$) at 0.25 mM melatonin concentration than in other concentrations and control (Table 1). These findings are in consonance with the observations of earlier studies, who showed higher ($P<0.05$) sperm motility in semen extended with 0.25 mM melatonin than in control ones in buffalo bulls (El-Raey *et al.* 2015), bulls (Chaithrashree *et al.* 2020) and bucks (Widyastuti *et al.* 2024). Previous studies (Reiter *et al.* 2009, Medeiros *et al.* 2002) have shown that melatonin plays a pivotal role in reinforcement of sperm plasma membrane, protection against cold shock and decline in LPO. Melatonin, enhances the activity of electron transport chain complexes, stimulates ATP production and promotes flagellar motility of spermatozoa (Fujinoki 2008). Moreover, melatonin stabilizes and exerts a protective effect on mitochondrial membranes which may enhance sperm motility (Dai *et al.* 2019, Jang *et al*. 2010). Incubation of buffalo bull semen containing 0.1 mM to 0.5 mM melatonin concentrations in comparison to control resulted in higher sperm motility (Asma-ul-Husna *et al.* 2017). Supplementation of melatonin in the extender led to an increase in membrane fluidity and consequently improved sperm motility (Okatani *et al.* 2000) although higher doses, melatonin may reduce sperm motility by affecting calcium influx and decreasing cyclic AMP levels (Slanar *et al.* 2000).

Regarding various sperm kinematic traits, supplementation of 0.25 mM melatonin ($P<0.05$) improved VSL and VAP in comparison to other tested concentrations and control (Table 1). No difference was observed in VCL, ALH and BCF between different concentrations of melatonin and control. Although supplementation of 0.25 mM melatonin significantly increased VSL and VAP compared to control, these changes do not suggest the induction of premature capacitation or detrimental hyperactivation. Both VSL and VAP are considered indicators of the forward progressive movement and fertilizing competence of spermatozoa and higher values generally reflect improved sperm vigor and transport efficiency within the female reproductive tract (Kathiravan *et al.* 2011). Alternatively, capacitation-associated hyperactivation is characterized by vigorous non-linear movement, typically manifested by increased VCL and ALH along with reduced trajectory linearity and straightness (Suarez and Ho 2003). A similar increase ($P<0.05$) in VSL and VAP following addition of melatonin (0.25 mM) in extender was also noticed by Chaithrashree *et al*. (2020) in the cryopreserved sperm of bull. Likewise, Ozgür *et al.* (2020) in *Capoeta trutta* (fish) also noticed higher ($P<0.05$) VSL and VAP at 0.1 mM and 1.0 mM melatonin concentrations. Melatonin, together with these sperm velocities stimulates mitochondrial complexes I and IV, increases ATP production and thus, protect the sperm from cryo-damage (Inyawilert *et al.* 2020). In the present study, the increase in VSL and VAP was not accompanied by significant changes in VCL, ALH or BCF, indicating that melatonin supplementation enhanced progressive sperm motility without inducing a hyperactivated motility pattern. Therefore, improved kinematic parameters observed in the melatonin-treated group are more likely to be attributed to enhanced sperm functionality and preservation of cellular integrity rather than premature capacitation. This is further supported by

Table 1. CASA-based sperm motility traits (Mean±SEM) in post-thaw semen of buffalo bulls cryopreserved in freezing media supplemented with different concentrations of melatonin (n=36 ejaculates).

Parameters	Melatonin (mM)				
	0.0 (Control)	0.25	0.50	0.75	1.0
TM (%)	48.9±1.8^{a}	58.6±1.9^{b}	52.4±2.1^{a}	48.7±2.3^{a}	51.3±2.0^{a}
PM (%)	33.7±1.2^{a}	39.4±1.4^{b}	34.7±1.6^{a}	31.7±1.9^{a}	34.3±1.5^{a}
VCL (µm/s)	68.4±3.8	68.0±3.1	70.5±5.3	64.1±3.3	67.6±4.1
VAP (µm/s)	40.2±1.8^{a}	47.7±.2.6^{b}	40.0±2.7^{a}	36.5±2.4^{a}	38.2±2.2^{a}
VSL (µm/s)	26.6±1.4^{a}	33.6±1.6^{b}	25.9±1.1^{a}	22.8±1.3^{a}	26.3±1.8^{a}
ALH (µm)	1.0±0.1	0.9±0.2	1.1±0.3	1.0±0.1	0.9±0.1
BCF (Hz)	10.0±0.8	8.8±0.7	9.2±0.9	10.1±1.0	8.0±0.8

Mean values with different superscripts within the row differ significantly ($P < 0.05$).

Abbreviations: TM = Total motility; PM = Progressive motility; VSL = Velocity straight line; VAP = velocity average path; VCL = Velocity curvilinear; ALH: Amplitude of lateral head displacement; BCF = Beat cross frequency.

Table 2. Sperm function tests (Mean±SEM) in post-thaw buffalo bull semen cryopreserved in freezing media supplemented with different concentrations of melatonin (n=36 ejaculates).

Parameters	Melatonin (mM)				
	0.0 (Control)	0.25	0.5	0.75	1.0
Viability (%)	52.1±2.5[a]	64.6±2.3[b]	61.9±1.8[b]	55.0±2.4[a]	53.9±2.3[a]
PMI (%)	45.9±1.5	44.1±1.7	47.5±1.9	45.1±1.7	43.0±2.0
Acrosome integrity (%)	50.5±1.7[a]	58.9±1.5[b]	55.4±1.9[ab]	51.2±1.8[a]	50.2±2.3[a]

Mean values with different superscripts within the row differ significantly (P < 0.05). Abbreviations: PMI = Plasma membrane integrity

the fertility results obtained under field conditions, wherein spermatozoa supplemented with melatonin achieved a higher pregnancy rate than the control group.

A reverse dose-dependent response was observed between different concentrations of melatonin and control vis-à-vis sperm viability. Among the tested concentrations, the extender supplemented with 0.25 mM melatonin exhibited significantly (P<0.05) higher sperm viability in comparison to that supplemented with 0.75 mM, 1.0 mM and control and non-significantly (P>0.05) higher than with 0.5 mM. Similarly, the sperm viability at 0.5 mM concentration was better (P<0.05) than in remaining concentrations and control (Table 2). Our findings are in agreement to an earlier report by El-Raey *et al.* (2015) that supplementing 0.25 mM melatonin in freezing extender led to higher (P<0.05) sperm viability index as compared to control in buffalo bulls. The percentage of intact plasma membrane did not seem to be affected following addition of melatonin since no difference (P>0.05) were noted across all treatments (Table 2), which is in accordance with the observations of Shahandeh *et al.* (2022) that supplementation of melatonin in extender at different concentrations did not affect (P>0.05) the plasma membrane integrity in Wistar Albino rats. The acrosome integrity was non-significantly higher (P>0.05) at 0.25 mM than at 0.5 mM melatonin concentration but was significantly higher (P<0.05) than in remaining concentrations and control (Table 2). Previous investigations in Holstein Friesian bulls (Chaithrashree *et al*. 2020) and bucks (Widyastuti *et al.* 2024) have depicted that supplementation of

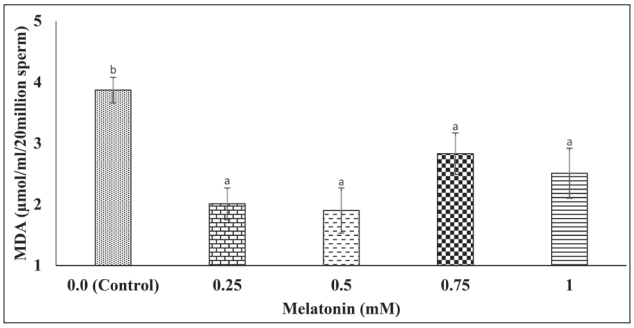

Fig. 1. Oxidative stress (MDA, µmole/20 million sperm/ml) in frozen-thawed semen of buffalo bulls cryopreserved in freezing media supplemented with different concentrations of melatonin (n=36 ejaculates). Values with different superscripts differ significantly (P<0.05).

melatonin in extender at 0.25 mM resulted in enhanced (P<0.05) percentage of intact acrosomes than in other tested concentrations. Melatonin binds directly to specific melatonin receptors especially MT1 and MT2 in sperm acrosome and upregulates functioning of endogenous antioxidant enzymes and, therefore, leads to enhancement of sperm functions (Medrano *et al*. 2017). In addition, incorporation of melatonin at optimal concentrations might lead to high percentage of viable acrosomes due to less damage of membrane and reduced ATP depletion from H_2O_2 (Roca *et al.* 2005).

The MDA levels were lower (P<0.05) at all melatonin concentrations compared to control. Among different

Table 3. Effect of melatonin supplementation in freezing media on first service conception rate using post-thaw buffalo bull semen (n=30 each)

Extender	Dairy Farm	Number of buffaloes inseminated	Number of buffaloes conceived	FSCR (%)	Chi- square	P-value
Control (0.0 mM)	University	10	4	40.0	0.625	0.429[NS]
	Private organized	20	6	30.0		
	Overall University and Private organized	30	10	33.3		
Melatonin supplemented (0.25 mM)	University	10	6	60.0		
	Private organized	20	8	40.0		
	Overall University and Private organized	30	14	46.7		

melatonin concentrations, the MDA levels at 0.25 mM and 0.5 mM were non-significantly (P>0.05) lower than that at 0.75 mM and 1.0 mM (Fig 1). Literature on the impact of melatonin supplementation in semen on MDA profiles in buffalo semen is not available. However, similar studies in other species (Holstein Friesian bulls) showed that supplementing melatonin at different concentrations (0.1 mM to 4.0 mM) exhibited lower MDA concentrations compared to control (Ashrafi *et al.* 2013). Melatonin is known to be a potent scavenger of peroxynitrite decomposition products, hydroxyl and peroxyl radicals, superoxide anion, hydrogen peroxide, hypochlorous acid and nitric oxide, thus, preventing the sperm function impairment (Allegra *et al.* 2002). Further, melatonin is reported to protect the oxidative and nitrosative changes in DNA (Hardeland *et al.* 2009). In the current study, melatonin might have lowered MDA production leading to reduced oxidative stress to spermatozoa in the freezing media supplemented with melatonin.

Although non-significant (P>0.05), the first service conception rate (FSCR) was higher following inseminations with semen having most effective concentration of melatonin (0.25 mM) than in control (Table 3) both at university dairy farm and private organized farms, which may partially be due to smaller number of animals inseminated in each group. Nevertheless, in the present study, melatonin in freezing media tended to increase (P>0.05) FSCR (by 13.4 percent) than in control ones. Reproductive efficiency is the primary driver of dairy farm profitability. Agricultural and veterinary universities continuously study the intersection of nutrition and reproduction to maximize dairy herd economics. Moreover, organized farms in Punjab actively employ modern reproduction-management tools to overcome physiological inefficiencies like nutritional balancing through academic advisory push for targeted supplementation of mineral mixtures, urea molasses licks and common salt to maintain optimal body condition scores, use of ultrasonography to closely monitor uterine involution and improved semen technology for optimal breeding. Furthermore, FSCR in buffaloes also depends upon semen handling and quality, site of deposition, season of breeding, fertilization status, bull effect and time of AI (Dalton *et al.* 2010). In the current study, efforts were ensured to minimize the variations due to these variables at both types of farms. Hence, the difference in the FSCR might probably be due to difference in the semen quality owing to supplementation of melatonin. Limited studies (EL-Raey *et al.* 2015) in buffalo bulls have also depicted an improved (P<0.01) fertility rates following insemination with semen having 0.25 mM melatonin as compared to control ones (60.43% vs 45.65%). The damage during cryopreservation includes changes in enzyme activity and disruption of plasma and acrosome membranes (Guthrie and Welch 2005). Thus, supplementation of melatonin in extender might have counteracted peroxidation and stabilized membranes that consequently improved the survival of post-thaw sperm in the female reproductive tract leading to better fertility rates than control in the present study.

It may be concluded that, supplementation of extender with 0.25 mM melatonin prior to freezing improves post-thaw sperm attributes of buffalo bulls.

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