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IMPROVING POST-THAW SEMEN QUALITY AND REDUCING OXIDATIVE STRESS IN BUFFALO BULL SEMEN VIA MELATONIN-ENHANCED CRYOPROTECTANTS

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ABTRACT

This study evaluated the effects of melatonin supplementation in semen extenders on post-thaw sperm quality and oxidative stress in frozen semen from Thai swamp buffalo (*Bubalus bubalis*) bulls. Semen samples were collected from 4 bulls and divided into 4 groups: a Control (0 mM melatonin) and three Treatment groups supplemented with 1, 2, or 3 mM melatonin in egg yolk-tris extender. Diluted semen was loaded into 0.25 ml French straws and then sealed. The straws were subsequently frozen and stored in liquid nitrogen. After 24 h of storage, semen samples were thawed, and assessments of sperm quality and lipid peroxidation. The results demonstrated that total sperm motility was significantly higher (P=0.017) in the 1 mM (49.20%) and 2 mM (48.73%) Melatonin groups compared to the control (40.71%), with no significant difference observed in the 3 mM group (43.15%). The proportion of static sperm was significantly reduced (P=0.018) in the 1 and 2 mM groups. Furthermore, malondialdehyde (MDA) concentrations, indicative of lipid peroxidation, were significantly lower (P<0.001) in all melatonin-treated groups (1.84, 1.17, and 1.10 μM for 1, 2, and 3 mM, respectively) compared to the control (2.91 μM). These findings indicate that supplementation of egg yolk-tris extender with 1 or 2 mM melatonin improves post-thaw sperm motility and reduces oxidative damage, thereby enhancing the cryopreservative quality of buffalo bull semen.

Keywords: *Bubalus bubalis*, buffaloes, melatonin, egg yolk-tris-fructose-glycerol, semen quality, lipid peroxidation, Thai swamp buffalo

INTRODUCTION

Optimizing the efficiency of semen utilized in artificial insemination is crucial for enhancing pregnancy rates and the overall success of animal reproduction programs. The formulation of effective semen cryopreservation media is therefore pivotal for maintaining the viability and functionality of high-quality sire spermatozoa over extended storage periods. Following thawing, sperm must exhibit vigorous motility, minimal immotility, and low rates of morphological abnormalities to ensure fertilization potential (Chenoweth, 2022). Nevertheless, the cryopreservation process encompasses a series of

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stress-inducing steps that can compromise sperm integrity and subsequently diminish fertilization outcomes. A primary challenge is the structural and functional damage to sperm cells incurred during freezing and thawing cycles, which can disrupt membrane architecture and lead to protein leakage. Additionally, oxidative stress-arising from the excessive generation of reactive oxygen species (ROS)-is a major contributor to cryodamage (Aitken *et al*., 2014). Factors such as abrupt temperature shifts during cryopreservation can exacerbate cellular stress, promoting ROS formation. Mitochondrial function is particularly vulnerable, with cryopreservation often resulting in mitochondrial structural alterations and changes in membrane potential (Gualtieri *et al*., 2021). Rapid cooling and osmotic fluctuations activate enzymes like NADPH oxidase (NOX), further increasing superoxide (O_2^-) production and promoting lipid peroxidation (LPO) in sperm membranes (Toor and Sikka, 2019; Yeste *et al*., 2015). As a result, ROS accumulation is almost inevitable during semen freezing and is closely linked to declines in post-thaw sperm quality (Len *et al*., 2019).

To counteract these deleterious effects, the incorporation of antioxidants into cryopreservation extenders has been explored as a strategy to mitigate LPO and ROS-induced damage, thereby enhancing sperm resilience to cryoinjury (Amidi *et al*., 2016; Bansal and Bilaspuri, 2011). Various antioxidants have demonstrated positive effects on buffalo semen quality post-thaw, including L-proline (Lp) and fulvic acid (FA) supplementation at 40 mM Lp and 1.7% FA in egg yolk-tris extenders (Ramazani *et al*., 2023), garlic extract (*Allium sativum*) at 3, 6 and 9% in egg yolk-tris extenders (Eliraqy *et al*., 2022) and supplementation of 20 ng/ml leptin and 10^{-3} M melatonin in egg yolk-tris extender (Abdel-Khalek *et al*., 2016). Among these, melatonin stands out as a potent antioxidant, capable of scavenging free radicals and protecting spermatozoa from oxidative stress, as evidenced in human (Najafi *et al*., 2018) and animal studies. Melatonin supplementation has been shown to improve sperm viability under oxidative conditions in chilled ram semen (Bhalothia *et al*., 2022) and frozen bovine semen (Ashrafi *et al*., 2013). Furthermore, melatonin can enhance the activity of endogenous antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase, in post-thaw bovine sperm (Niu *et al*., 2024). These findings suggest that studying the properties and amounts of melatonin in semen diluents can guide efforts to maintain the quality of frozen bovine semen in egg yolk-tris-fructose-glycerol solutions using 8% glycerol, a context where melatonin supplementation has not been previously reported. Therefore, this study aims to investigate the effects of melatonin supplementation in egg yolk-tris-fructose-glycerol diluents at varying levels on post-thaw semen quality and the generation of free radicals in frozen buffalo semen.

MATERIALS AND METHODS

Location and animals

The study used 4 Thai swamp buffalo bulls selected based on semen quality and libido. Animals raised in pens (5x5 m), fed mainly with Para grass (*Brachiaria mutica*), and supplemented with concentrate (16.02% CP), to meet nutritional requirements according to 0.5% DM concentrate based on body weight, and fresh water is supplied *ad libitum*. This study was conducted at the buffalo and beef cattle farm within the Department of Animal Science, Faculty of Agriculture, at Kamphaeng Saen, Kasetsart University, located in

Nakhon Pathom, Thailand. The farm is situated at the coordinates 14.0200° N latitude and 99.9730° E longitude. The research took place during the summer season, from February to April, when the average annual temperature ranges from 25°C to 37°C. The present study was approved by the committee on animal experiments of the Kasetsart University, Thailand, following the guidelines of animal care and use under the ethical review board of the office of the national research council of Thailand (ACKU68-AGR-026).

Preparation of semen cryoprotectant and melatonin supplementation

The semen cryoprotectant solution, formulated with egg yolk-tris-fructose-glycerol (ETG), was supplemented with varying levels of melatonin (EMD Millipore Corp., USA). Preparation of fructose-tris-glycerol extender (8% glycerol), a clear solution consisting of fructose 1.25 g, citric acid 1.79 g, tris (hydroxymethyl) aminomethane 3.028 g, penicillin 0.21 g. Each component was weighed and added to a beaker. The mixture was dissolved in 92 mL of sterile distilled water. After complete dissolution, 8 mL of glycerol was added to achieve a final glycerol concentration of 8% (v/v). The solution was stirred continuously for 30 minutes. The prepared solution was combined with fresh, albumin-free chicken egg yolk at a volume ratio of 80:20 (solution:egg yolk, v/v) to produce the ETG solution. The mixture was homogenized using a magnetic stirrer at room temperature for 1 h to ensure thorough mixing. Subsequently, the ETG solution was supplemented with melatonin at specified levels (1, 2 and 3 mM). The mixture was stirred using a magnetic stirrer while the flask containing the solution was immersed in an ice-cold water bath to reduce the temperature of the ETG solution during the mixing process. This process was conducted for 1 additional hour. After preparation, the ETG solution with melatonin supplementation was stored in a refrigerator at 4°C. The ETG solution without melatonin supplementation was stored in the refrigerator immediately.

Collection and evaluation of semen samples

Semen is collected from 4 buffalo bulls, with each bull providing samples 4 times at one-week intervals. Semen was collected using artificial vagina and subjected to laboratory testing to study the effects of melatonin supplementation in semen cryoprotectants on the quality of frozen-thawed semen. The collected semen undergoes a preliminary quality assessment. The criteria for acceptable semen include cleanliness and absence of contaminants, motility of at least 75%, and concentration of at least 800 million sperm per ml. Semen that meets the quality standards is divided into 4 equal parts. Each part is diluted with a different concentration of melatonin supplementation in egg yolk-tris-fructose-glycerol (ETG), cryoprotectant formula as follows:

Formula 1: No melatonin supplementation in ETG, which serves as the control (M0).

Formula 2: Melatonin supplementation at a concentration of 1 mM in ETG (M1).

Formula 3: Melatonin supplementation at a concentration of 2 mM in ETG (M2).

Formula 4: Melatonin supplementation at a concentration of 3 mM in ETG (M3).

Buffalo semen cryopreservation

The fresh semen is initially evaluated for quality parameters, including color, volume, and pH. A Computer-Assisted Sperm Analysis (CASA; HTCasa II Motility Analyzer version, Hamilton-Thorne, Bioscience, MA, USA) is

employed to further assess the semen. Semen meeting the quality standards is then diluted with a cryoprotectant to achieve a final sperm concentration of 120 million sperm per ml. The diluted semen gradually cooled from 37°C to 4°C over 3 to 4 h. Subsequently, 0.25-milliliter straws (semen straw, IMV Technologies) are prepared and labeled with information regarding the name, number, and concentration level of melatonin supplementation. These straws are organized in a semen straw holder and refrigerated. The diluted semen is then dispensed into the 0.25 ml straws (30 million sperm per dose) using a straw filling and sealing machine (MRS1 Dual V2, IMV Technologies), which draws the semen to the midpoint of the sealing area. The straws are then sealed and arranged in a semen rack. Semen straws are exposed to liquid nitrogen vapor, approximately 2 inches above the liquid, at -120°C for 8 minutes. Following this exposure, the straws are submerged in liquid nitrogen. Finally, the frozen semen is stored in a liquid nitrogen tank at -196°C.

Analysis of post-thaw quality of frozen buffalo semen

To assess the quality of buffalo semen post-freezing, the semen is thawed using water at a temperature of 37°C for 1 minute. Following thawing, 3 µl of the semen is placed onto a counting chamber slide (20 micron to 4 chambers, Leja) that has been pre-warmed on a warm plate set to 37°C. The slide containing the semen sample is then analyzed using a CASA to evaluate the semen quality. The parameters assessed include sperm motility, progressive motility, sperm kinetic movement, sperm velocity, and tail abnormalities of the sperm.

Assessment of malondialdehyde (MDA) concentrations in thawed frozen buffalo semen

Prepare a 0.67% thiobarbituric acid (TBA) solution and a 10 to 15% trichloroacetic acid (TCA) solution. Mix TBA and TCA in a 1:1 ratio. Prepare a 1 Molar NaOH solution. Thaw the frozen semen in water at 37°C. Pipette 300 microliters of the thawed semen into a test tube, then add 300 microliters of NaOH. Mix using a vortex mixer and heat for 30 minutes at 60°C. Afterward, add 400 microliters of 15% TCA, then centrifuge for 15 minutes. Transfer 400 microliters of the supernatant into a new test tube and add 400 microliters of TBA. Mix using a vortex mixer and heat for 1 hour at 60°C. Allow the solution to cool to room temperature. Transfer 750 microliters of the solution into a cuvette, mixing the sample with butanol in a 750:750 microliter ratio to form the MDA-TBA complex. Analyze the MDA using a spectrophotometer at an absorbance of 532 nanometers (Helios Zeta UV-Vis Spectrophotometer, Thermo Scientific). Calculate the MDA concentrations in the semen samples using a standard MDA curve based on the absorbance values obtained.

Statistical analysis

Semen quality data obtained from assessments using CASA and the concentrations of MDA for each level of melatonin supplementation in ETG were analyzed for variance using Analysis of Variance (ANOVA) under a Completely Randomized Design (CRD). Subsequently, the differences in mean values were compared using Duncan's New Multiple Range Test (DMRT) at a 95% confidence level. The analyses were conducted using the SAS OnDemand for Academics (https://welcome.oda.sas.com/).

RESULT AND DISCUSSION

Sperm motility and progressive motility of buffalo semen post-thawing

The study investigated the effects of supplementing melatonin at various concentrations in a semen cryoprotectant based on an egg yolk tris-fructose-glycerol extender. The results showed that the percentage of total sperm motility was significantly higher (P=0.017) in the groups supplemented with 1 mM (49.20%) and 2 mM (48.73%) melatonin compared to the Control group (40.71%). Though, these values were not significantly different from the 3 mM melatonin group (43.15%). In contrast, there were no significant differences in progressive motility rates among all 4 groups (P=0.099), as shown in Figure 2. This finding is consistent with the reported by Len *et al.* (2019), which stated that melatonin has the ability to protect the mitochondrial membrane potential of sperm, which is essential for the ATP energy production process required for sperm motility. When energy levels decrease, sperm motility also declines. Melatonin helps sperm maintain efficient motility. Similarly, the study by Inyawilert *et al.* (2021) demonstrated that supplementation with melatonin in a diluted semen solution of Thai buffalo using a tris-citrate egg yolk formula at a concentration of 1 mM increased the motility rate of frozen-thawed sperm after 1, 7, 15, and 30 days.

The study on the supplementation of melatonin in diluted egg yolk-tris semen extenders at varying concentrations in frozen-thawed bovine semen revealed that melatonin enhances sperm motility. This is evidenced by an increase in the percentage of motile sperm and a decrease in the percentage of immotile sperm. These findings align with studies on melatonin supplementation in frozen bovine semen using egg yolk-citrate at a concentration of 0.3 mg/ml (Niu *et al*., 2024) and in frozen canine semen at concentrations of 1 and 2 mM (Divar *et al*., 2022). The improvement in sperm motility post-thaw due to melatonin may result from its ability to protect sperm from oxidative stress, thereby reducing sperm damage, as confirmed by decreased levels of MDA following melatonin supplementation in ETG. This is supported by Su et al. (2021), reported that bovine sperm responded to melatonin supplementation at a concentration of 2 mM in frozen semen extenders, resulting in the lowest percentage of immotile sperm. Melatonin aids in mitochondrial protection, which is crucial for efficient ATP production, thus enhancing sperm motility. Furthermore, melatonin maintains cell membrane integrity, allowing sperm with intact membranes to survive and move effectively (Ofosu *et al*., 2021).

The velocity and kinetic movement of sperm

The effects of varying levels of melatonin supplementation in sperm diluent solutions on progressive sperm kinetics were investigated. It was found that the curvilinear velocity (VCL), average path velocity (VAP), and straight-line velocity (VSL) of sperm in all four melatonin-supplemented groups did not differ significantly (P>0.05). The straightness (STR) and linearly motile spermatozoa (LIN) in all four melatonin supplementation groups did not differ significantly (P>0.05). The slow-moving spermatozoa in all four melatonin supplementation groups also showed no significant differences (P>0.05). However, regarding static spermatozoa, it was observed that the group without melatonin supplementation and the group supplemented with melatonin at a concentration of 3 mM exhibited higher immobility compared to the groups supplemented with melatonin at concentrations of 1 and 2 mM

(P=0.018), as shown in Table 1. It mentions that the sperm kinetic does not differ significantly, possibly due to the use of tris and the addition of egg yolk in the diluent, which may increase viscosity and hinder sperm movement (Monteiro *et al*., 2022). Melatonin did not affect sperm movement and linear motility. This is consistent with the study by Makris *et al*. (2023), which reported that the addition of melatonin along with antioxidants significantly reduced the rate of immotile sperm. However, the study by Su *et al*. (2021) reported that supplementing melatonin at a level of 2 mM resulted in the lowest rate of immotility. This is attributed to melatonin's role in protecting mitochondria, enhancing energy production, and improving sperm motility. Additionally, melatonin helps maintain cell membrane integrity, which is crucial for sperm viability and motility.

The malondialdehyde (MDA) concentrations of post thawed cryopreserved buffalo bull semen

The study on the effects of supplementing melatonin at varying concentrations in semen extender solutions on the levels of MDA. MDA concentrations, indicative of lipid peroxidation, were significantly lower (P<0.001) in 1, 2 and 3 mM melatonin-treated groups (1.84, 1.17, and 1.10 µM, respectively) compared to the Control group (2.91 µM) (Figure 3).

Studies have demonstrated that the supplementation of melatonin in frozen semen solutions can reduce the levels of MDA in buffalo semen post-thawing. This aligns with research on the effects of melatonin supplementation in preventing oxidative stress damage during the freezing and thawing of animal semen, including cattle (Ashrafi *et al*., 2013), buffalo (Luo *et al*., 2023; Inyawilert *et al*., 2021) and pigs (Lee and Lee, 2023). Additionally, the study by Kumar *et al*. (2022) investigated the supplementation of melatonin in tris-citric fructose egg yolk solutions at concentrations of 1 and 2 mM in frozen sheep semen, which was able to reduce MDA levels throughout the storage period during freezing. Sönmez *et al*. (2007) reported that melatonin protects sperm from the detrimental effects of peroxidative agents by enhancing the activity of the enzymes SOD and GPx, which are key antioxidants. This results in reduced damage to mitochondria and cell membranes. During semen freezing, and as sperm cells encounter rapid temperature decreases and osmotic pressure changes, these factors stimulate NOX enzymes to produce superoxide (O_2^-), and sperm cell membranes are susceptible to lipid peroxidation (LPO), leading to increased reactive oxygen species (ROS) (Toor and Sikka, 2019; Yeste *et al*., 2015). The formation of free radicals results in elevated MDA levels, which is an end product of LPO and serves as an indicator of oxidative stress (Jadoon and Malik, 2017). Melatonin supplementation in semen extenders has been shown to effectively reduce ROS levels (Lee and Lee, 2023; Su *et al*., 2021). Specifically, the incorporation of melatonin at a concentration of 0.3 mg/ml in egg yolk-citrate-based extenders for bovine semen cryopreservation has been reported to enhance the activity of key antioxidant enzymes, including catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) (Niu *et al*., 2024). As a result, post-thaw buffalo semen treated with melatonin exhibits significantly lower MDA concentrations, which is indicative of reduced lipid peroxidation. This protective effect is attributed to melatonin's ability to upregulate antioxidant enzyme activity and the expression of genes associated with antioxidant defense (Niu *et al*., 2024), thereby facilitating the

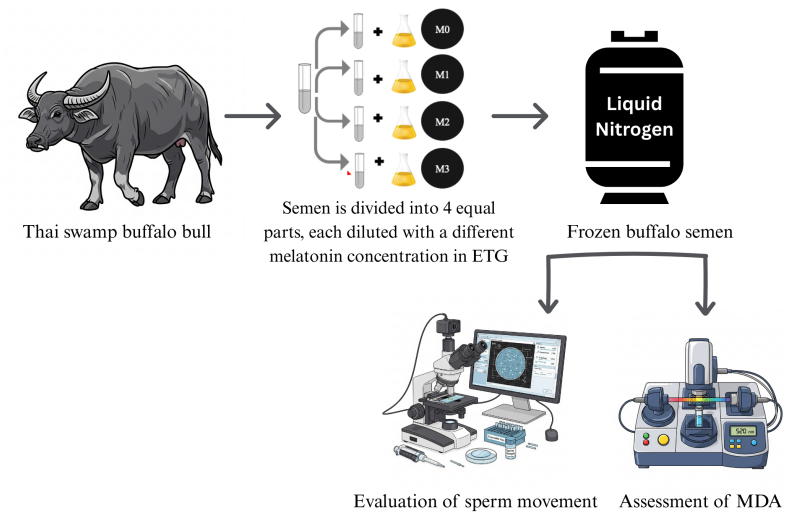

Figure 1. Schematic representation of the experimental procedure.

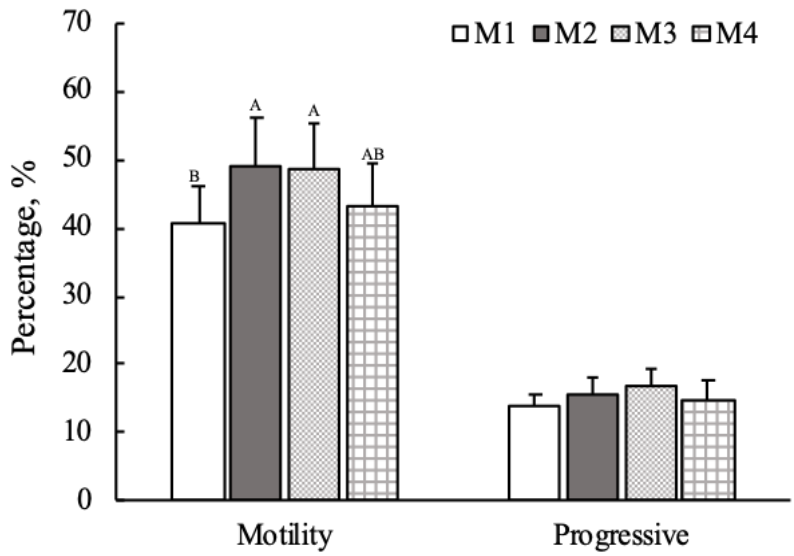

Figure 2. The supplementation of melatonin in the semen cryoprotectant affects the motility characteristics of sperm in cryopreserved buffalo semen post-thawing. A, B different letters above the bar, indicate a statistically significant difference (P=0.017).

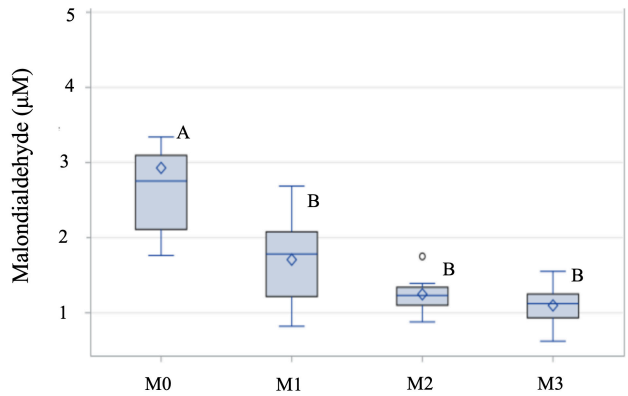

Figure 3. The supplementation of melatonin in the semen cryoprotectant on the levels of malondialdehyde (MDA) in the culture medium of frozen-thawed bovine and buffalo semen. A, B different letters above the bar, indicate a statistically significant difference (P<0.001).

Table 1. The supplementation of melatonin in the semen cryoprotectant on the velocity and kinetic movement of sperm in cryopreserved buffalo semen post-thawing.

Parameters	M0	M1	M2	M3	SEM	P-value
Velocity						
VCL, um/s	85.15	87.67	87.71	85.93	0.73	0.571
VAP, um/s	48.13	48.98	49.19	48.46	0.38	0.761
VSL, um/s	40.01	40.41	40.63	40.20	0.34	0.927
Kinetic movement						
STR, %	83.10	82.24	82.58	82.94	0.41	0.896
LIN, %	40.87	40.76	40.91	40.88	0.20	0.995
Slow, %	16.94	21.23	21.32	18.27	0.77	0.102
Static, %	59.29^{A}	50.65^{B}	51.27^{B}	56.85^{AB}	1.18	0.018

^{A,B} difference superscript letters within row are significantly different (P<0.05).

efficient scavenging of free radicals and improving buffalo semen quality following cryopreservation.

The supplementation of melatonin in ETG cryoprotectant demonstrated beneficial effects on the quality of frozen-thawed buffalo semen. Specifically, the addition of melatonin at concentrations of 1 and 2 mM significantly enhanced post-thaw sperm motility and reduced the proportion of static spermatozoa. Moreover, melatonin supplementation at all tested concentrations effectively decreased malondialdehyde (MDA) levels, indicating a reduction in lipid peroxidation and oxidative stress in buffalo frozen semen. These findings suggest that melatonin supplementation, particularly at 1 and 2 mM, can be considered a promising strategy to enhance the post-thaw quality of buffalo semen by improving sperm functional parameters and reducing oxidative stress.

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