Original Research

Melatonin Has a Beneficial Effect on Stallion Sperm Quality in Cool Condition

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Abstract

The objective of this study was to evaluate the effects of melatonin on quality of Caspian stallion spermatozoa during cooling preservation. Semen of stallions were collected and diluted with Kenney’s extender that was supplemented with different antioxidant concentrations: no melatonin and dimethyl sulfoxide (DMSO) (negative control [NC]); 0.9% (vol/vol) DMSO (vehicle control [VC]); 1-mM melatonin (M-1); 1.5-mM melatonin (M-1.5); and 2-mM melatonin (M-2). Total and progressive motility, viability, plasma membrane integrity, and lipid peroxidation (LPO) were determined at 0 (fresh), 6, 24, and 48 hours after the start of cooling. Total motility was greater in M-1.5 extender (63.6 ± 3.12%) than in M-2, NC, and VC extenders (P < .05) during 48 hours of storage. Progressive motility was greater in M-1.5 extender (35.97 ± 2.79%) than in M-2 extender (P < .05) during 48 hours of storage. The percentage of plasma membrane integrity was greater in M-1.5 (57.58 ± 3%) than in M-2, NC, and VC during the 48-hour storage (P < .05). The percentage of sperm viability during the 48-hour storage was greater in M-1, M-1.5, and M-2 than in NC and VC extenders. The results showed that malondialdehyde concentration was lower in M-1.5 and M-2 (1.34 ± 0.2 and 1.36 ± 0.2 nm/mL, respectively) than in other extenders during 48 hours of storage. Also, all sperm parameters with exception of LPO were decreased with increasing storage time. In conclusion, the results showed that semen extender containing M-1.5 improved sperm quality of Caspian stallion during 48 hours of storage in cool condition.

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1. Introduction

Preservation of liquid stallion semen at 5°C is an important technique in the breeding management of horses. Oxidative damage to spermatozoa during storage is a potential cause of the decline in motility and fertility during hypothermic storage of liquid semen [1]. The use of cooled stallion semen is limited by its relatively short-term fertilizing capacity. An important reason for the decrease in fertility during storage is the peroxidation of sperm membrane lipids [2]. In other words, cooling and freezing is associated with the production of reactive oxygen species (ROS) which lead to lipid peroxidation (LPO) of sperm membranes, resulting in a loss of motility, viability, and fertility of sperm [3,4]. Numerous studies have shown that ROS play a significant role in male infertility and reduced sperm function [5,6]. However, physiological concentrations of ROS increase membrane fluidity, preserve the fertilizing ability of sperm, and also have some beneficial effects on sperm hyperactivation, capacitation, and acrosome reaction [7–9]. Sperm cells and seminal plasma contain ROS scavengers, including superoxide dismutase, glutathione peroxidase, and catalase. However, an improper balance between ROS production and antioxidant enzyme activities can cause
failure of sperm function [10]. Furthermore, the antioxidant system of seminal plasma and spermatozoa is compromised during semen cryopreservation [11]. In recent years, many studies investigated the effects of different antioxidants for improvement of sperm quality during semen storage and cryopreservation. Several studies have even shown that melatonin may have an effect on human and animal sperm [12–16]. However, the regulation of sperm function by melatonin is not well understood. In fact, reports on its antioxidant activities in semen are rather contradictory. Because of its antioxidant properties, melatonin could be added to a semen extender to improve semen characteristics during long-term in vitro storage [17]. It was shown to be effective in protecting mitochondria by scavenging toxic oxygen and nitrogen-based reactants, stimulating antioxidative enzymes, increasing the efficiency of the electron transport chain, and thereby limiting electron leakage, free radical generation, and promoting adenosine triphosphate (ATP) synthesis [1,18]. These features of melatonin would likely help the cell to maintain the integrity of the mitochondria and improve survival prospects. Also, melatonin is an efficient antioxidant that protects cells and tissues and was capable of directly scavenging the highly reactive hydroxyl radical [19]. Therefore, the aim of this study was to evaluate the effects of different concentrations of melatonin as antioxidant on in vitro quality (such as sperm viability, motility, membrane integrity, total abnormality, and LPO) of stallion spermatozoa during storage at 5°C in different times.

2. Materials and Methods

2.1. Chemicals

All chemicals used in this study were purchased from Sigma chemical Co (St. Louis, MO) and Merck (Darmstadt, Germany).

2.2. Animals

Semens samples were collected from three fertile Caspian stallions (aged 8–10 years) using artificial vagina (Missouri model; Minitüb, Tiefenbach, Germany) twice a week during the breeding season (five ejaculates for each stallion). The stallions were kept at the Animal Breeding Center (Karaj, Iran) under uniform nutritional conditions. After collection, the gel fraction of the ejaculates was filtered and then volume, concentration, motility, and morphology were assessed immediately. Ejaculates with standard criteria (volume: more than 20 mL; semen concentration: more than 500 × 10⁶ spermatozoa/mL; motility: more than 70%; and sperm abnormal forms: less than 10%) were selected for study.

2.3. Semen Dilution and Processing

All collected ejaculates (n = 3) in each replicate (r = 5) were pooled to eliminate variability between stallions. The pooled ejaculates were diluted ratio to 1:1 (vol/vol) with Kenney extender (a skim milk–based extender). Then, diluted semen was centrifuged with 600g for 10 minutes. After centrifuge, supernatant was removed, the sperm-rich pellet was resuspended and divided into five equal aliquots. Each sperm aliquot was diluted with extenders containing different levels of melatonin (0 [containing no melatonin and no DMSO, negative control (NC)]; 0 [containing DMSO (as melatonin solvent) and no melatonin, 0.9% (vol/vol) vehicle control (VC)]; 1–mM melatonin [M-1]; 1.5-mM melatonin [M-1.5]; and 2–mM melatonin [M-2]) to a final concentration of 50 × 10⁶ spermatozoa/mL. The diluted samples were gradually cooled to 5°C for 2 hours.

2.4. Semen Evaluation

2.4.1. Sperm Motility and Velocity Parameters

The sperm motility and velocity parameters were evaluated using a computer-assisted sperm analysis system (CEROS version 12.3; Hamilton-Thorne Biosciences, Beverly, MA). Semen sample was placed in a chamber, and the loaded chamber was placed on the thermal plate of the microscope (37°C). Three randomly selected microscopic fields were scanned five times each. The mean of these 15 scans was used for statistical analysis.

2.4.2. Plasma Membrane Integrity

Integrity of plasma membrane was assessed by means of the hypoosmotic swelling test as described by Nie and Winzel [20]. Briefly, 10 μL of semen was mixed with 100 μL of a hypotonic solution (1.712-g sucrose dissolved in 50-mL distilled water [osmolarity: 100 mOsm/kg]) in a 1.5-mL test tube and incubated at 37°C for 30 minutes. After incubation, smear was prepared and 300 sperm were counted by phase-contrast microscope (CKX41; Olympus, Tokyo, Japan) at ×400 magnification. Sperm with swollen and coiled tails were recorded as intact and damaged membrane, respectively.

2.4.3. Sperm Viability

Viable and nonviable spermatozoa were determined by the eosin–nigrosin staining [21]. Sperm suspension smears were prepared by mixing a drop of sperm sample with two drops of stain on a warm slide and spreading the stain with a second slide. Viability was assessed by counting 200 cells at ×400 magnification (CKX41; Olympus). Sperm displaying partial or complete purple staining were considered nonviable; only sperm showing strict exclusion of stain were counted as viable.

2.4.4. Malondialdehyde Concentration Assay

Malondialdehyde concentrations, as an index for LPO in the semen samples, were measured using the thio-barbituric acid reaction [22]. Briefly, 1 mL of the diluted semen (250 × 10⁶ spermatozoa/mL) was mixed with 1 mL of cold 20% (wt/vol) trichloroacetic acid to precipitate protein. The precipitate was pelleted by centrifuging (960g for 15 minutes), and 1 mL of the supernatant was incubated with 1 mL of 0.67% (wt/vol) thiobarbituric acid in a boiling water bath at 100°C for 10 minutes. After cooling, the absorbance was determined by a spectrophotometer (Rochester, NY) at 532 nm. All malondialdehyde (MDA) concentrations were expressed as nmol/mL.
2.5. Statistical Analysis

The MIXED procedure of the SAS System (SAS version 9.12; SAS Institute Inc, Cary, NC) was used to perform a repeated-measures analysis of covariance to test for the effects of treatments, times of storage (0, 6, 24, and 48 hours), and their interactions on the parameters. The significance level was set at $P < .05$. All values given are least squares means ± standard error of the mean.

3. Results

The results showed that sperm parameter evaluations were decreased with increasing storage time, with exception of MDA. Malondialdehyde concentration in all extenders was increased with increasing storage time. The results (Table 1) showed that total motility (87.58 ± 3.12%) was significantly ($P < .05$) greater in M-2 extender than in M-1 and VC during the 24-hour storage. Furthermore, during the 48-hour storage, total motility (63.60 ± 3.12%) was greater in M-1.5 than in M-2, NC, and VC extenders ($P < .05$). Progressive motility (Table 2) was greater in M-2 (51.45 ± 2.79%) than in other all extenders during at 24 hours after cooling ($P < .05$), but more than 48 hours, M-1.5 (35.97 ± 2.79%) was greater than M-2 (25.51 ± 2.79%). As shown in Table 3, the percentage of plasma membrane integrity during the 48 hours of storage was significantly ($P < .05$) greater in M-1.5 (57.42 ± 3%) than in NC (46.92 ± 3%), VC (46.28 ± 3%), and M-2 (44.92 ± 3%). The percentage of sperm viability was increased in extenders containing different concentrations of melatonin (M-1, M-1.5, and M-2 extenders) compared with that in NC and VC extenders during the 48-hour storage (Table 4). Finally, the results showed (Table 5) that MDA concentration was lower in M-1.5 and M-2 extenders (1.34 ± 0.2 and 1.36 ± 0.2 nmol/mL, respectively) than in other extenders during 48 hours of storages ($P < .05$).

4. Discussion

Providing a suitable condition using semen extender for decreasing lipid peroxide damage of spermatozoa and maintaining sperm motility, viability, and membrane integrity during long-term storage is of practical technique to improving the stallion sperm quality. In recent years, enzymatic (such as vitamins E and C) and synthetic antioxidants (such as melatonin, glutathione, glutathione peroxidase, coenzyme Q10, and ells) were used in semen extender as a defense mechanism against LPO and play a major role in improving sperm quality [13,19,21,23–25]. The present study demonstrated the in vitro quality of stallion sperm with different concentrations (M-1, M-1.5, and M-2) of melatonin as antioxidant in semen extender during 48 hours of storage. Many studies reported that melatonin has beneficial effects on preservation of mammalian sperm function and improves the microscopic parameters of spermatozoa [13,14,26–30].

It has been reported that the axoneme and mitochondria in sperms may be damaged by a high level of ROS [31]. The studies have shown that melatonin can stabilize and protect mitochondria via several mechanisms [32–35]. Melatonin is a highly lipophilic molecule that crosses cell membranes to easily reach subcellular compartments. Melatonin appears to accumulate at high concentrations in mitochondria in which it interacts with enzyme complexes I and IV [33]. The consequence of its actions on mitochondria is an increase in ATP production [35]. Moreover, melatonin interacts with lipid bilayers and stabilizes...
Mitochondrial inner membranes, an effect that may improve the electron transport chain activity and thus increasing motility [33]. Our study agreed with other studies showing increases in total and progressive stallion sperm using semen extender supplementation with M-1 and M-1.5. Of course, there were publications that reported melatonin can lead to a decrease in sperm motility. For example, Gwayi and Bernard [15] reported that melatonin had negative effects on sperm forward progression and the quality of sperm motility in rats. It has been reported that stallion sperm motility and kinematics were not affected by melatonin during in vitro incubation [36]. This discrepancy can be attributed to differences in the processing procedure and different species.

The results of study indicated that plasma membrane integrity was significantly greater in M-1 and M-1.5 treatments. Oxidative stress can induce an increase in oxidation of plasma membrane polyunsaturated fatty acids that in turn result in disruption of plasma membrane integrity during the long-term storage [37]. Therefore, destruction of membrane integrity causes a rise in the membrane permeability and a decrease in the ability of sperm to control the intracellular concentrations of ions that in turn are involved in reduced sperm functionality [36,38]. Many studies have confirmed the ability of melatonin and its metabolites to reduce oxidative stress [39]. Also, some studies such as our study reported positive effects of melatonin supplementation on sperm membranes permeability and fluidity [34,36]. It has been shown that melatonin administration increased plasminogen activity in ram spermatozoa [40]. In our study, the extenders contained different concentrations of melatonin caused greater percentage of viable spermatozoa. Also, in agreement with our result, Martín-Hidalgo et al [17] reported that 1-μM melatonin increased the proportion of live boar sperm. Melatonin may stimulate the activities of enzymes involved in metabolizing ROS and preserve cell membrane integrity. The previous studies reported that melatonin has anti-apoptotic effects on a wide range of cell types [27,41], prevents capacitation and apoptotic-like changes of ram spermatozoa, and also increases fertility rate [42]. Also, it has been reported that melatonin has beneficial effects for human spermatozoa as indicated by the enhanced sperm motility parameters and increased sperm viability [13].

A routine method to evaluate LPO in sperm is the assay of sperm MDA, a thiobarbituric acid-reactive substance that is a stable LPO product. Malondialdehyde concentration was lower in M-1.5 and M-2 in the 48 hours of storage. Melatonin is an efficient antioxidant that protects cells and tissues, and it was capable of directly scavenging the highly reactive hydroxyl radical [19,43]. Also, reported that melatonin has a potent inhibitor of LPO on stallion spermatozoa because dramatic reductions in the percentage of peroxidized cells were rapidly observed [36].

### 5. Conclusions

The results of the present study showed that melatonin in Kenney extender could maintain the quality of cooled and stored stallion semen by decreasing lipids peroxidation during the 48-hour storage. Supplementation of M-1 and M-1.5 groups in semen extender improved total and progressive motility during 48 hours of storage. However, melatonin at the level of 2 mM improved motility of stallion spermatozoa at 5°C until 24 hours of storage. All concentrations of melatonin in this study have positive effects on sperm viability during liquid preservation of semen for 48 hours. Therefore, between previously mentioned concentrations of melatonin, it suggests that the level of 1.5 mM of melatonin can be used in semen extender for improvement of the in vitro quality of Caspian stallion sperm during cool condition.

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### References


