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Research Article

Melatonin Supplementation to the Freezing Extender Improves the Quality of Canine Epididymal Spermatozoa

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The present study aimed to determine the cryoprotective effect of melatonin supplements on the extender on the quality of canine epididymal spermatozoa. Epididymis from 20 castrated dogs were minced and incubated in Tris buffer. Spermatozoa were diluted in tris-citric acid-fructose (TCF) extender with two doses of melatonin (0.002 and 0.0035 mol) as well as a control (0.0 mol). The samples were packed in 0.25 ml straws after 2 hr of equilibration at 5°C and kept in liquid nitrogen (−196°C). Sperm parameters such as viability, motility, integrity of acrosome, sperm membrane functioning, and DNA integrity were assessed after thawing (37°C for 30 s). In addition, lipid peroxidation was assessed using the determination of malondialdehyde (MDA) levels. Results revealed that adding 0.0035 mol melatonin to the cryopreservation medium enhanced ($P < 0.05$) the measured parameters of spermatozoa compared to the control and 0.002 mol melatonin. Levels of MDA were obviously ($P < 0.05$) lower when 0.0035 mol melatonin was added in comparison to 0.002 mol melatonin and the control. Eventually, adding 0.0035 mol melatonin to the TCF extender significantly improved the percentage of motility, viability, and integrity of both plasma membrane and acrosome integrity of dog epididymal spermatozoa. In addition, melatonin supplementation improved DNA integrity and reduced membrane lipid peroxidation.

1. Introduction

Domestic animals, particularly dogs, have become increasingly crucial to human culture in recent years. Changes in the human–animal relationship have sparked a surge in dog breeding. The retrieval and cryopreservation of viable sperm from the epididymis of dead animals (postmortem retrieval) was an important approach for conserving male gametes from genetically treasured animals or endangered species [1]. Assisted reproductive technologies could benefit from the successful management of these cryopreserved gametes [2, 3]. Sperm stored in the cauda epididymis is normally of high quality and maturity, allowing it to fertilize oocytes [4, 5]. In several mammalian species, including carnivores, the fertilizing capacity of fresh and frozen epididymal sperm has been illustrated, and in dogs, intrauterine and intravaginal artificial insemination using fresh, cooled, and frozen epididymal spermatozoa yielded pregnancies and viable puppies [6, 7]. During cryopreservation, dog sperm cells are vulnerable to a variety of stressors, and many sperm cells suffer ultrastructural, biochemical, and functional damage because of the freezing–thawing process [8]. The motility, viability, and fertilizing capacity of cryopreserved dog sperm are all reduced because of cryoinjury [9].

Due to various intra- and extra-cellular transitions during various stages of sperm maturation [10, 11], such as the loss of a large amount of cytoplasm and a rise in the amount of plasma membrane polyunsaturated fatty acid [12, 13], epididymal spermatozoa are extremely vulnerable to oxidative imbalance. Furthermore, lipid peroxidation products like malondialdehyde (MDA) can be just as detrimental as reactive oxygen species (ROS), resulting in DNA fragmentation and lowered fertilization potential [14], indicating the need for additional research to reduce cryodamage, such as the inclusion of certain antioxidants. Melatonin (*N*-acetyl-5-methoxy tryptamine) is primarily produced by the pineal gland [15]. It has a variety of physiologic activities in mammals, including circadian cycle regulation, seasonal reproduction signaling, and immunomodulation [16, 17].

Due to its unique features, melatonin is recognized as an essential endogenous antioxidant agent [18]. These include its amphiphilic character, which allows it to pass through different barriers such as the blood–brain barrier or the placental barrier rapidly and readily [19], as well as its interactions with nuclear receptors [20]. Melatonin's antioxidant activities can be mediated by either a receptor-dependent mechanism [21] or by directly scavenging free radicals [22]. Melatonin may also have indirect effects by enhancing the action of some antioxidant enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSH-Px) [17, 23].

The cryoprotective efficacy of melatonin depends on its concentration in semen extenders as proposed by several reports. For example, in buffaloes [24] and horses [25], a supplement of melatonin with 1 and 100 μM concentrations, respectively improved sperm membrane integrity during the cryopreservation process. In rams, the concentration of 1 mM melatonin led to higher viability rates, higher percentages of total motile progressive ram spermatozoa, higher intracellular adenosine triphosphate (ATP) concentrations, and higher DNA integrity compared to other concentrations used [26]. Melatonin supplementation to the extender of ram semen enhanced the cryopreservation of ram sperm by restraining mitochondrial permeability and keeping its integrity [27]. Other investigations found various effective concentrations of melatonin (0.01 mM [28], 3 mM [29], and 0.1 mM melatonin concentration [30]) in protecting human spermatozoa from intracellular ROS and reducing MDA generation. In bulls, 2 and up to 3-mMol melatonin [31] supplementation to the semen extender enhanced the post-thawed semen parameters, while other studies found that 0.25 mM concentration of melatonin [32] could putative the cryopreserved spermatozoa. In mithun bull (*Bos frontalis*), administration of melatonin (in a slow-release form)reduced oxidative stress and enhanced the in vitro fertilizing capability of the cryopreserved spermatozoa [33]. In dogs, melatonin concentrations of 0.002 and 0.0035 mM improved the cryosurvival of ejaculated sperm from bulldogs [34]. In chicken sperm, melatonin improved quality at 0.25 mg/ml concentration [35] and 2 mM of melatonin in fish semen extender [36]. However, till now, the effect of melatonin concentrations on the epididymal canine spermatozoa has not been investigated. Therefore, and relying on these prior outcomes, the current study aimed to see how melatonin supplementation to the freezing extender affects the properties of post-thawed epididymal sperm in canine species. Parameters of sperm motility, viability, the integrity of acrosome, DNA, sperm membrane, and lipid peroxidation were specifically studied following the procedures of cryopreservation and thawing of canine epididymal spermatozoa.

2. Materials and Methods

The Ethics Board for Animal Use at the Faculty of Veterinary Medicine, Cairo University, Egypt, examined and approved all experimental protocols (approval code: Vet CU28/04/2021/300). This research was performed at Cairo University's Theriogenology Department, Faculty of Veterinary Medicine in collaboration with the Artificial Insemination Department, Animal Reproduction Research Institute, and Agricultural Research Centre. Unless otherwise specified, all compounds were purchased from Sigma (Sigma–Aldrich Corp., St. Louis, MO, USA).

2.1. Animals. Following typical procedures of castration at the Egyptian Society of Mercy to Animals dog shelter, testes, and epididymides were extracted from 20 clinically sound dogs, representing different breeds, ranging in age from 2 to 5 years, and weighing 25–45 kg. A clinical examination of their male reproductive organs revealed no obvious abnormalities and verified that they were healthy. Dogs were kept in separate tangible-floored enclosures with an exterior and covered shelter to keep them out of direct sunshine. Dogs were fed dry food (Royal Canin®) once daily and had free access to water. We did five replicates using the same experimental conditions. In this regard, we followed the methods of Fayez et al. [37] to perform our study (as appended below).

2.2. Collecting Epididymal Sperm. Each dog was castrated, and its testicles, epididymis, and surrounding tunics were placed in a glass thermos flask filled with sterile 0.9% NaCl, stored at 4°C, and brought as quickly as possible (within 12 hr) to the laboratory for further procedures [38]. Each testis' tunic was gently removed, being careful not to damage the epididymis where it connects to the testis. A curved scissor was used to cut the blood supply to the testis and the vas deferens alongside the testicle's natural curvature. Sperm cells were collected from the cauda epididymis. On a hygienic, dry Petri dish, the cauda epididymis was removed from each testis, sliced using a surgical blade, and washed in 4 ml of Tris buffer extender (Tris (3.025 g), citric acid (1.7 g), fructose (1.25 g), yolk of eggs (20%, v/v), glycerol (7%, v/v), Na-benzyl-penicillin (100,000 IU), and dihydrostreptomycin sulfate (100 mg) in 100 ml ultrapure purified water) as previously proposed [28]. After incubation in Tris buffer for 30 min at 37°C [39], tissue pieces were removed by filtration through an 80 μm metal mesh, and the suspension of sperm was harvested in a standardized plastic tube. The motility and percentage of alive spermatozoa were determined in retrieved samples from the right and left cauda (tail) epididymis. For subsequent processing, samples with greater than 70% motility and 75% living sperm were chosen.

2.3. Sperm Extending and Cryopreservation. The obtained sperm suspension was diluted with tris-citric acid-fructose (TCF) to a final concentration that was approximately 300×10^6 sperm/ml [40]. Melatonin was given to the diluents of sperm in three aliquots: 0.0 mol (control), 0.002 mol, and 0.0035 mol according to a previously published report [34]. To prepare cryopreserved media containing 0.0, 0.002, and 0.0035 mol of melatonin concentrations, we did the following: On the day of the experiment and just before use, the melatonin powder was weighed (according to the required concentrations), dissolved in ethanol (99.9%), and then added to the prepared media in each group. Because the solution is light sensitive and could be destroyed by light,

the extender containing melatonin solution was prepared just before use and kept in a dark tube.

The diluted spermatozoa were then gradually chilled to 5°C and held there for about 120 min for the equilibration purpose [41], packed in 0.25 ml straws (IMV, L'Aigle, France), and frozen in a horizontal position at 5 cm over the liquid nitrogen vapor for 10 min in a Styrofoam box filled with liquid nitrogen according to Silva et al. [42]. Subsequently, straws were promptly submerged in a liquid nitrogen tank and stored for at least 7 days then thawed. The samples were thawed at 37°C for 30 s [43].

2.4. Assessment of Frozen-Thawed Canine Epididymal Spermatozoa. Various methods were performed to properly assess the epididymal spermatozoa, and the obtained results were validated by different authors who participated in the current study.

2.4.1. Progressive Motility of Spermatozoa. A small drop of frozen-thawed spermatozoa was placed onto a prewarmed microscopic glass slide, overlaid by a prewarmed coverslip (37°C), and observed under a microscope using a heated stage and 40x objective lens in order to test sperm motility in all extended samples. The proportion of progressively motile spermatozoa was evaluated in at least five microscope fields subjectively.

2.4.2. Viability of Frozen-Thawed Spermatozoa. To test sperm viability (percentage of live spermatozoa), the eosin–nigrosin staining method was used [44]. To prepare the stain 1.67 g of eosin and 10 g of nigrosin were mixed in 100 ml of a buffering medium (sodium citrate solution 2.90%). Just a little drop (30 μl) of frozen-thawed spermatozoa was put on a glass slide, and an equal quantity of eosin–nigrosin stain was applied to prepare the slide. A thin smear of the mixture was created and air-dried, and the sperm viability percentage was assessed by observing 200 spermatozoa in different microscopic areas using a 40X objective. Dead sperm is visualized by the red or pink head, while alive sperm is nonstained.

2.4.3. Integrity of Sperm Plasma Membrane. The integrity of the plasma membrane of dog epididymal sperm was assessed using the hypo-osmotic swelling test (HOST) [45]. This test is created by dissolving sodium citrate (0.73 g) and fructose (1.35 g) in 100 ml of purified water. HOST was carried out by incubating 50 μl of frozen-thawed dog epididymal sperm sample in HOST solution (500 μl) for 40 min at 37°C. Following that, a drop of the sample was put on the glass side, covered with a coverslip, and viewed with a light microscope equipped with a 40X objective. The percentage of HOST reacting sperm cells (coiled and swollen tails) was determined after 200 spermatozoa were tested in five different microscopic fields.

2.4.4. Sperm Acrosome Integrity. The integrity of the acrosome of spermatozoa was evaluated by a Trypan Blue-Giemsa stain [46]. A drop of trypan blue (0.27%) and another drop of diluted sperm sample were placed on a glass slide, mixed, and distributed over the whole surface using another slide. After vertical air drying, the slides were immersed in a fixative solution containing 1N HCL (86 ml) and 37% formaldehyde solution (14 ml) with Neutral red (0.2 g) for 2 min before being rinsed with tap and purified water. Giemsa staining solution coupling flasks were used to hold the slides overnight at room temperature. Giemsa stock solution 7.5% (v/v) and 100 ml of distilled water were combined to create the fresh Giemsa staining solution. Slides were rinsed in both tap and purified water for 2 min before being air-dried upright. Using a light microscope with a 100X oil immersion magnification, sperm cells were investigated. An intact acrosome (appeared as light purple) and a damaged acrosome (nonstained or blue color) were standardized to assess the spermatozoa.

2.4.5. Assessment of Peroxidation of Lipids. MDA assay, a lipid peroxidation biomarker, was measured in the extender after thawing, as described [47]. Thiobarbituric acid (TBA) reacts with MDA in an acidic medium for 30 min at 95°C to produce a TBA reactive product utilizing commercial kits (Biodiagnostic, Egypt). The absorption value of the pink output was measured at 534 nm using a spectrophotometer (UV-1800PC; Shanghai, Mapada Co., Ltd., China). MDA concentrations in the samples were estimated in nmol/ml.

2.4.6. Integrity of Sperm DNA (Comet Assay). According to Codrington et al. [48], single-cell gel electrophoresis, known as the alkaline comet test, was performed to assess the DNA quality of frozen-thawed canine epididymal sperm. Sperm samples were rinsed with PBS and 50 μl of 1% low-melt point agarose before being placed on frosted microscope slides precoated with 50 μl of 1% normal melting point agarose. The slides were then lysed for 1 hr in a lysis buffer. Electrophoresis was performed on the lysed cells. Following neutralization in Tris solution, the slides were stained with ethidium bromide (pH 7). A fluorescence microscope (Olympus, Japan) was used to study 200 sperm cells (Figure 1). The amount of DNA present was expected to be proportional to the stain intensity in the comet tail area. Image analysis software (Comet-Score program) was used to evaluate DNA damage based on measurements of the DNA% in the tail, tail length, and olive tail moment. Spermatozoa with damaged DNA migrated from the nucleus to the anode at a faster rate, but spermatozoa with intact DNA did not create a "comet" [49].

2.5. Statistical Analysis. To figure out the type of data, data were subjected to a normality test using the Kolmogorov–Smirnov test. All values are presented as the mean $\pm$ *SEM* of five replicates. SPSS (version 25.0; SPSS Inc., Chicago, IL) was used for statistical analysis. The sperm characteristics were investigated using one-way ANOVA and Duncan's comparison test. The significance level was chosen to be at least $P < 0.05$. All statistical analyses were carried out in accordance with Snedecor and Cochran [50].

3. Results

3.1. Sperm Properties and MDA Content in Frozen-Thawed Epididymal Canine Spermatozoa. Table 1 showed the studied parameters of post-thawed epididymal dog sperm (motility,

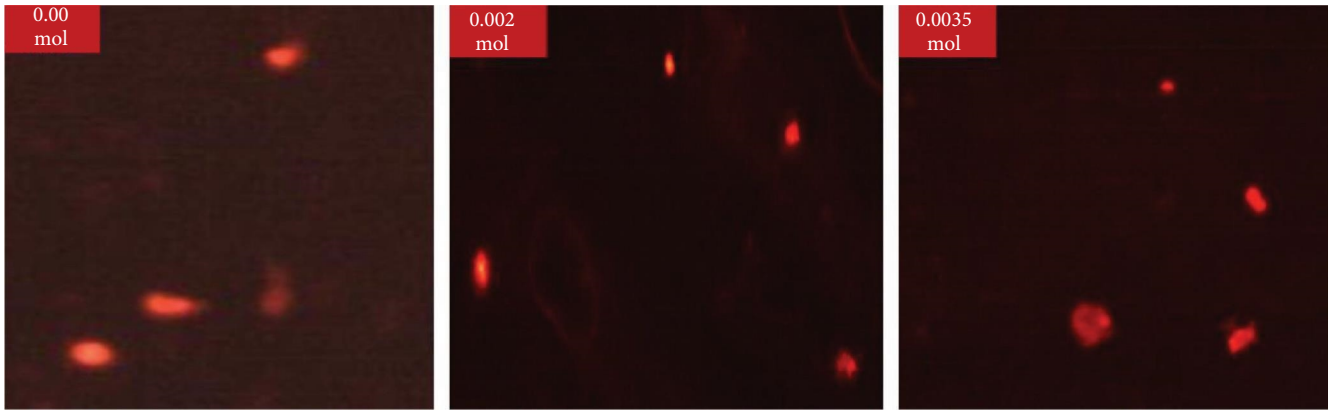

Figure 1: Imaging canine epididymal spermatozoa for assessment of the integrity of DNA using the technique of alkaline comet. The amount of DNA present was expected to be proportional to the staining intensity in the comet tail area. Image analysis software was utilized to evaluate DNA damage (Comet-Score program). Spermatozoa with damaged DNA had a higher proportion of DNA migration from the nucleus to the anode, but spermatozoa with intact DNA did not produce a "comet." The addition of 0.0035 mol melatonin to the TCF extender considerably ($P<0.05$) reduced DNA damage percentage in comparison to 0.002 mol melatonin and the control one (0.0 mol).

Table 1: Melatonin's effects on epididymal sperm's progressive motility, viability, integrity of membranes, acrosome integrity, and MDA content.

Parameters of epididymal spermatozoa	Control (0.0 mol)	Melatonin (0.002 mol)	Melatonin (0.0035 mol)
Post-thaw motility (%)	21.00 ± 2.91^{b}	19.00 ± 1.87^{b}	33.00 ± 2.54^{a}
Viability (%)	37.40 ± 1.02^{b}	34.80 ± 1.07^{b}	49.22 ± 1.36^{a}
Membrane integrity (%)	37.20 ± 1.65^{b}	30.60 ± 1.80^{b}	48.00 ± 2.64^{a}
Acrosome integrity (%)	43.40 ± 1.56^{b}	42.20 ± 1.59^{b}	50.40 ± 1.63^{a}
MDA (nmol/ml)	3.68 ± 0.086^{b}	3.80 ± 0.12^{b}	3.22 ± 0.19^{a}

Note: The means with distinct superscripts (a, b) throughout the same rows are substantially varied ($P<0.05$). Data are displayed as mean $\pm$ *SEM* and five replicates of sperm were analyzed. MDA, malondialdehyde.

Table 2: Impact of melatonin on the DNA integrity of frozen-thawed canine epididymal spermatozoa.

Epididymal sperm parameters	Control (0.0 mol)	Melatonin (0.002 mol)	Melatonin (0.0035 mol)
DNA damage (%)	20.30 ± 0.76^{a}	19.00 ± 0.80^{a}	14.60 ± 0.89^{b}
DNA in tail (%)	10.37 ± 0.45^{a}	7.87 ± 0.55^{b}	6.18 ± 0.61^{b}
Tail length (px)	11.00 ± 0.57^{b}	16.00 ± 0.80^{a}	8.80 ± 0.49^{c}
Tail moment	1.13 ± 0.05^{a}	1.08 ± 0.06^{a}	0.61 ± 0.04^{b}
Olive tail moment	1.68 ± 0.08^{a}	1.71 ± 0.06^{a}	1.12 ± 0.07^{b}

Note: The means with distinct superscripts (a, b, and c) within the same row are substantially different ($P<0.05$). Data are presented as mean $\pm$ *SEM*. Five replicates of sperm were analyzed.

viability, membrane integrity, and acrosome integrity, as well as MDA content) using different melatonin concentrations (0.0, 0.002, and 0.0035 mol) in TCF extender. Post-thaw sperm motility, viability, percentages of the membrane, and acrosome integrities were significantly ($P<0.05$) higher in 0.0035 mol melatonin-supplemented extenders than in the control one. There were no significant differences in post-thaw motility, viability, membrane integrity, and acrosome integrity between the 0.002 mol melatonin and the control concentrations (Table 1). Table 1 shows the effect of melatonin concentrations on the amount of MDA generated as a biomarker for lipid peroxidation. MDA levels were significantly ($P<0.05$) reduced at 0.0035 mol melatonin compared to 0.0 and 0.002 mol melatonin. suggesting that lipid peroxidation could be prevented by melatonin at the membrane level (Table 1).

3.2. DNA Integrity. Figure 1 illustrates the imaging of canine epididymal spermatozoa that were analyzed for DNA integrity using the alkaline comet technique. The results of the comet assay data analysis (percentage of DNA damage, DNA in tail, tail length, tail moment, and olive tail moment) were presented in Table 2. The addition of 0.0035 mol melatonin to the TCF extender considerably ($P<0.05$) reduced the percentages of DNA damage compared to 0.002 mol melatonin and the control (0.0 mol). Tail and olive tail moments

were obviously ($P<0.05$) lower at 0.0035 mol melatonin than at 0.002 mol melatonin and the control.

4. Discussion

Melatonin administration is a necessity to lessen intracellular ice damage and mitigate the phase transition brought on by temperature variations during sperm cryopreservation. In living things, the ability of the antioxidant defense system, composed of a combination of enzymatic and nonenzymatic elements, to effectively combat ROS generation and maintain homeostasis depends on a healthy balance between these two factors. The quality of thawed spermatozoa is diminished by excessive ROS generation during cryopreservation [51]. Dog cauda epididymal spermatozoa had the highest concentration of polyunsaturated fatty acids in their plasma membranes [12], making them more susceptible to free radicals [52]. Furthermore, both the head and tail portions of the epididymis contain a high percentage of cytoplasmic droplets (proximal and distal ones) [10], which makes sperm more vulnerable to free radicals because of an increase in the functioning of the glucose-6-phosphate dehydrogenase enzyme with coparticipation from nicotinamide adenine dinucleotide phosphate (NADPH) [53, 54]. NADPH, according to Aitken et al. [55], promotes the formation of free radicals. Thus, the presence of cytoplasmic droplets in cauda epididymal sperm may be the primary cause of decreased sperm motility, increasing sensitivity to oxidative agents such as hydrogen peroxide, hydroxyl radicals, and MDA. As antioxidants are the main line of defense against oxidative stress, adding one to the freezing extender of epididymal dog sperm may increase its quality.

The present work revealed the cryoputative impacts of melatonin supplementation on the extender of canine epididymal spermatozoa. Melatonin preserves spermatozoa by neutralizing ROS, reducing oxidative stress, and guarding against free radical damage, allowing spermatozoa to maintain their self-life [28, 56]. It may also boost the activity of antioxidant enzymes such as GSH-Px and SOD [17, 57, 58]. Melatonin's antioxidative activity has been shown to preserve spermatozoa from ischemia and minimize sperm abnormalities [59]. In humans, a high-melatonin sperm maturation medium has been demonstrated to increase sperm mitochondrial activity, accelerate sperm progressive motility, and increase the number of motile spermatozoa while decreasing endogenous nitric oxide levels [60].

Melatonin's anticryopreservation damage effects are dose dependent [26, 31]. The dose of melatonin needed to have antioxidant effects on preserved sperm differs by animal species [61]. The most effective melatonin concentrations for dog sperm cryopreservation are unknown. In the current study, we assessed the effect of different melatonin concentrations (0.0, 0.002, and 0.0035 mol) on dog epididymal sperm parameters and discovered that post-thaw motility, viability, the percentage of epididymal spermatozoa with intact membranes, and the percentage of spermatozoa with intact acrosomes were all significantly higher ($P<0.05$) in the 0.0035 melatonin-supplemented extenders compared to the control (0.0 mol melatonin) and the 0.002 mol of melatonin. These findings were consistent with those of Martinez-Rodriguez et al. [34], who found that 0.0035 mol melatonin enhanced the cryosurvival of male bulldog spermatozoa. When 0.001 mol of melatonin was supplemented with the cryoextender [62], they found no differences in the canine sperm's cryosurvival parameters (motility, acrosome integrity). This might be connected to melatonin levels in dog spermatozoa being low. In bulls, melatonin concentrations of 0.002 and 0.003 mol boosted spermatozoa cryosurvival characteristics in terms of motility, viability, membrane integrity, normal spermatozoa, catalase, and SOD activity [31]. After freeze–thawing, buffalo extenders treated with 0.0001 and 0.00025 mol melatonin showed better sperm motility, membrane integrity, acrosome integrity, and conception rate than nonsupplemented sperm [24]. In rams, sperm motility, intracellular ATP concentration, DNA integrity, and fertilization rate were higher in the extender treated with 0.001 mol melatonin than in sperm supplemented with lower or higher doses. In stallions, mitochondrial activity and integrity of both plasma membrane and acrosome were enhanced when 0.001 mol melatonin was added to frozen-thawed sperm [25]. Human frozen-thawed sperm treated with 0.00001 mol melatonin demonstrated greater motility, viability, and decreased intracellular ROS concentrations as compared to those supplemented with lower or higher quantities [28]. As a result, depending on the species, melatonin may be advantageous in a dose-dependent manner.

In the current research, we also indirectly assessed the antioxidant efficiency of melatonin in protecting spermatozoa from the detrimental effects of ROS by evaluating MDA levels. MDA is a lipid peroxidation byproduct that is used in spermatozoa as an indication of ROS-induced lipid peroxidative damage [63]. MDA can react with glycosyl amino acid and glycation intermediates to form extremely toxic chemicals that are connected to cell aging and protein cross-linking [54, 64]. MDA and hydrogen peroxide, on the other hand, had a stronger deleterious impact on sperm motility in the cauda portion of the epididymis [20]. In the present work, MDA concentrations were significantly lower ($P<0.05$) in the 0.0035 mol melatonin-supplemented groups compared to the control and 0.002 mol groups. Melatonin maintains the properties of bull sperm by lowering MDA production and maintaining intracellular enzymes during cryopreservation, according to Perumal et al. [33]. In rams [24, 31], bulls [24, 31, 65, 66], boar [4, 17], rats [67], and humans [30], melatonin was found to protect sperm cells from the damaging effects of ROS. Our study is the first that addresses the antioxidant effect of melatonin on MDA during the cryopreservation of dog epididymal spermatozoa.

Owing to the existence of antioxidants in the seminal plasma of dogs' semen, prostatic fluid-frozen samples had higher DNA integrity-protective functions [68–70]. Therefore, it is not advisable to remove seminal plasma from the second portion of the ejaculate before sperm cryopreservation in dogs. Canine epididymal spermatozoa, on the other hand, lack seminal plasma, which protects epididymal spermatozoa from oxidative stress and DNA damage during

cryopreservation procedures. As a result, melatonin was used as a substitute for the deficit of antioxidants in the current investigation. Melatonin (0.0035 mol) increased the DNA integrity of epididymal dog sperm substantially ($P < 0.05$). Melatonin may inhibit sperm DNA breakage and apoptosis caused by hydrogen peroxide via MT1 and extracellular signal-regulated kinases [56].

In conclusion, the addition of 0.0035 mol melatonin to the TCF extender enhanced the proportion of viability, motility, membrane integrity, and acrosome integrity of epididymal sperm in dogs. By lowering MDA levels, it preserved the integrity of DNA and prevented peroxidation of lipids at the cellular level. Melatonin concentrations of 0.0035 mol can thus be used as a protective agent for cryopreservation of dog epididymal spermatozoa.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors state that there is no conflict of interest.

Authors' Contributions

Eman Fayez contributed in the conceptualization, experimental methodology, data visualization and curation, formal analysis, validation, writing-original draft. Haney Samir contributed in the conceptualization, investigation, methodology, analysis, validation, and writing-review and editing, and the correspondence. Mohamed A. I. El Sayed contributed in the supervision, visualization, critical writing-review and editing. Zaher M. Rawash contributed in the methodology, resources, and editing of the manuscript. Ali Salama contributed in the visualization, conceptualization, methodology, investigation validation, writing-review and editing.

References

[1] C. Kirchhoff, "The dog as a model to study human epididymal function at a molecular level," *Molecular Human Reproduction*, vol. 8, no. 8, pp. 695–701, 2002.

[2] W. Farstad, "Cryopreservation of canine semen-new challenges," *Reproduction in Domestic Animals*, vol. 44, no. s2, pp. 336–341, 2009.

[3] D. A. Hewitt, R. Leahy, I. M. Sheldon, and G. C. W. England, "Cryopreservation of epididymal dog sperm," *Animal Reproduction Science*, vol. 67, no. 1-2, pp. 101–111, 2001.

[4] C. Matás, M. Sansegundo, S. Ruiz et al., "Sperm treatment affects capacitation parameters and penetration ability of ejaculated and epididymal boar spermatozoa," *Theriogenology*, vol. 74, no. 8, pp. 1327–1340, 2010.

[5] F. Pamungkas, M. A. Setiadi, and N. W. K. Karja, "Characteristics and in vitro fertilization ability of ram spermatozoa: comparison of epididymal and ejaculated spermatozoa," *Media Peternakan*, vol. 35, pp. 38–38, 2012.

[6] G. C. Luvoni and M. G. Morselli, "Canine epididymal spermatozoa: a hidden treasure with great potential," *Reproduction in Domestic Animals*, vol. 52, no. S2, pp. 197–201, 2017.

[7] S. L. Marks, J. Dupuis, W. D. Mickelsen, M. A. Memon, and C. C. Platz Jr, "Conception by use of postmortem epididymal semen extraction in a dog," *Journal of Veterinary Internal Medicine*, vol. 204, pp. 1639-1640, 1994.

[8] S.-H. Kim, D.-H. Yu, and Y.-J. Kim, "Effects of cryopreservation on phosphatidylserine translocation, intracellular hydrogen peroxide, and DNA integrity in canine sperm," *Theriogenology*, vol. 73, no. 3, pp. 282–292, 2010.

[9] C. C. Sicherle, F. F. D. Souza, C. D. P. Freitas-Dell'Aqua et al., "Effects of the cryopreservation process on dog sperm integrity," *Animal Reproduction*, vol. 17, no. 1, pp. 1–10, 2020.

[10] D. S. R. Angrimani, J. D. A. Losano, C. F. Lucio et al., "Role of residual cytoplasm on oxidative status during sperm maturation in dogs," *Animal Reproduction Science*, vol. 151, no. 3-4, pp. 256–261, 2014.

[11] F. Tramer, F. Rocco, F. Micali, G. Sandri, and E. Panfili, "Antioxidant systems in rat epididymal spermatozoa," *Biology of Reproduction*, vol. 59, no. 4, pp. 753–758, 1998.

[12] D. S. R. Angrimani, J. D. A. Losano, C. F. Lucio et al., "Cytoplasmic droplet acting as a mitochondrial modulator during sperm maturation in dogs," *Animal Reproduction Science*, vol. 181, pp. 50–56, 2017.

[13] A. K. Bansal and G. S. Bilaspuri, "Impacts of oxidative stress and antioxidants on semen functions," *Veterinary Medicine International*, vol. 2011, pp. 1–7, 2011.

[14] G. K. V. Kawai, J. R. C. Gurgel, J. D. de Agostini Losano et al., "Susceptibility of stallion spermatozoa to different oxidative challenges: role of seminal plasma," *Journal of Equine Veterinary Science*, vol. 55, pp. 76–83, 2017.

[15] H. Awad, F. Halawa, T. Mostafa, and H. Atta, "Melatonin hormone profile in infertile males," *International Journal of Andrology*, vol. 29, no. 3, pp. 409–413, 2006.

[16] D.-X. Tan, L. C. Manchester, M. P. Terron, L. J. Flores, and R. J. Reiter, "One molecule, many derivatives: a never-ending interaction of melatonin with reactive oxygen and nitrogen species," *Journal of Pineal Research*, vol. 42, no. 1, pp. 28–42, 2007.

[17] H. Y. Jang, Y. H. Kim, B. W. Kim et al., "Ameliorative effects of melatonin against hydrogen peroxide-induced oxidative stress on boar sperm characteristics and subsequent in vitro embryo development," *Reproduction in Domestic Animals*, vol. 45, no. 6, pp. 943–950, 2010.

[18] S. Dragojevic Dikic, A. M. Jovanovic, S. Dikic, T. Jovanovic, A. Jurisic, and A. Dobrosavljevic, "Melatonin: a, "Higgs boson" in human reproduction," *Gynecological Endocrinology*, vol. 31, no. 2, pp. 92–101, 2015.

[19] A. Tarocco, N. Caroccia, G. Morciano et al., "Melatonin as a master regulator of cell death and inflammation: molecular mechanisms and clinical implications for newborn care," *Cell Death & Disease*, vol. 10, no. 4, pp. 1–12, 2019.

[20] R. R. da Rosa Filho, D. S. R. Angrimani, M. M. Brito, M. Nichi, C. I. Vannucchi, and C. F. Lucio, "Susceptibility of epididymal sperm against reactive oxygen species in dogs," *Animal Biotechnology*, vol. 32, no. 1, pp. 92–99, 2021.

[21] M. L. Dubocovich and M. Markowska, "Functional MT_1 and MT_2 melatonin receptors in mammals," *Endocrine*, vol. 27, no. 2, pp. 101–110, 2005.

[22] H. Tamura, A. Takasaki, T. Taketani et al., "The role of melatonin as an antioxidant in the follicle," *Journal of Ovarian Research*, vol. 5, no. 1, pp. 1–9, 2012.

[23] C. Rodriguez, J. C. Mayo, R. M. Sainz et al., "Regulation of antioxidant enzymes: a significant role for melatonin," *Journal of Pineal Research*, vol. 36, no. 1, pp. 1–9, 2004.

[24] M. El-Raey, M. Badr, M. M. Assi, and Z. M. Rawash, "Effect of melatonin on buffalo bull sperm freezability, ultrastructure changes and fertilizing potentials," *Assiut Veterinary Medical Journal*, vol. 61, no. 144, pp. 201–208, 2015.

[25] R. Lançoni, E. Celeghini, M. Bianchi-Alves et al., "Use of melatonin and ferulic acid as promoters of cryopreserved equine sperm," *Animal Reproduction*, vol. 12, pp. 559–566, 2015.

[26] S. Succu, F. Berlinguer, V. Pasciu, V. Satta, G. G. Leoni, and S. Naitana, "Melatonin protects ram spermatozoa from cryopreservation injuries in a dose-dependent manner," *Journal of Pineal Research*, vol. 50, no. 3, pp. 310–318, 2011.

[27] Y. Fang, C. Zhao, H. Xiang, G. X. Jia, and R. Zhong, "Melatonin improves cryopreservation of ram sperm by inhibiting mitochondrial permeability transition pore opening," *Reproduction in Domestic Animals*, vol. 55, no. 9, pp. 1240–1249, 2020.

[28] M. H. Karimfar, F. Niazvand, K. Haghani, S. Ghafourian, R. Shirazi, and S. Bakhtiyari, "The protective effects of melatonin against cryopreservation-induced oxidative stress in human sperm," *International Journal of Immunopathology and Pharmacology*, vol. 28, no. 1, pp. 69–76, 2015.

[29] A. Najafi, E. Adutwum, A. Yari et al., "Melatonin affects membrane integrity, intracellular reactive oxygen species, caspase3 activity and AKT phosphorylation in frozen thawed human sperm," *Cell and Tissue Research*, vol. 372, no. 1, pp. 149–159, 2018.

[30] S.-L. Deng, T.-C. Sun, K. Yu et al., "Melatonin reduces oxidative damage and upregulates heat shock protein 90 expression in cryopreserved human semen," *Free Radical Biology and Medicine*, vol. 113, pp. 347–354, 2017.

[31] I. Ashrafi, H. Kohram, and F. F. Ardabili, "Antioxidative effects of melatonin on kinetics, microscopic and oxidative parameters of cryopreserved bull spermatozoa," *Animal Reproduction Science*, vol. 139, no. 1-4, pp. 25–30, 2013.

[32] A. R. ChaithraShree, S. D. Ingole, V. D. Dighe et al., "Effect of melatonin on bovine sperm characteristics and ultrastructure changes following cryopreservation," *Veterinary Medicine and Science*, vol. 6, no. 2, pp. 177–186, 2020.

[33] P. Perumal, S. Chang, K. K. Baruah, and N. Srivastava, "Administration of slow release exogenous melatonin modulates oxidative stress profiles and in vitro fertilizing ability of the cryopreserved mithun (*Bos frontalis*) spermatozoa," *Theriogenology*, vol. 120, pp. 79–90, 2018.

[34] J. A. Martinez-Rodriguez, F. J. Carbajal, R. Martinez-De-Anda, A. Alcantar-Rodriguez, and A. Medrano, "Melatonin added to freezing diluent improves canine (bulldog) sperm cryosurvival," *Reproduction and Fertility*, vol. 1, no. 1, pp. 11–19, 2020.

[35] M. O. Appiah, B. He, W. Lu, and J. Wang, "Antioxidative effect of melatonin on cryopreserved chicken semen," *Cryobiology*, vol. 89, pp. 90–95, 2019.

[36] A. I. Alevra, A. Exadactylos, E. Mente, and S. Papadopoulos, "The protective role of melatonin in sperm cryopreservation of farm animals and human: lessons for male fish cryopreservation," *Animals*, vol. 12, no. 6, Article ID 791, 2022.

[37] E. Fayez, M. A. I. El Sayed, Z. M. Rawash, and A. Salama, "Influence of the addition of zinc oxide nanoparticles to cryopreservation medium for dog epididymal spermatozoa," *Topics in Companion Animal Medicine*, vol. 52, Article ID 100736, 2023.

[38] N. Simons, A. Van Soom, E. Domain, and E. Wydooghe, "Collection and cryopreservation of epididymal spermatozoa in dogs," *Vlaams Diergeneeskundig Tijdschrift*, vol. 88, no. 2, pp. 77–82, 2019.

[39] I. Ortiz, M. Urbano, J. Dorado et al., "Comparison of DNA fragmentation of frozen-thawed epididymal sperm of dogs using sperm chromatin structure analysis and sperm chromatin dispersion test," *Animal Reproduction Science*, vol. 187, pp. 74–78, 2017.

[40] A. Rota, B. Ström, and C. Linde-Forsberg, "Effects of seminal plasma and three extenders on canine semen stored at 4°C," *Theriogenology*, vol. 44, no. 6, pp. 885–900, 1995.

[41] N. Songsasen, I. Yu, S. Murton et al., "Osmotic sensitivity of canine spermatozoa," *Cryobiology*, vol. 44, no. 1, pp. 79–90, 2002.

[42] A. R. Silva, R. C. S. Cardoso, and L. D. M. Silva, "Influence of temperature during glycerol addition and post-thaw dilution on the quality of canine frozen semen," *Reproduction in Domestic Animals*, vol. 41, no. 1, pp. 74–78, 2006.

[43] M. M. Brito, C. F. Lúcio, D. S. R. Angrimani et al., "Comparison of cryopreservation protocols (single and two-steps) and thawing (fast and slow) for canine sperm," *Animal Biotechnology*, vol. 28, no. 1, pp. 67–73, 2017.

[44] R. C. Campbell, H. M. Dott, and T. D. Glover, "Nigrosin eosin as a stain for differentiating live and dead spermatozoa," *The Journal of Agricultural Science*, vol. 48, no. 1, pp. 1–8, 1956.

[45] C. R. F. Pinto and D. M. Kozink, "Simplified hypoosmotic swelling testing (HOST) of fresh and frozen-thawed canine spermatozoa," *Animal Reproduction Science*, vol. 104, no. 2-4, pp. 450–455, 2008.

[46] A. Kovács and R. H. Foote, "Viability and acrosome staining of bull, boar and rabbit spermatozoa," *Biotechnic & Histochemistry*, vol. 67, no. 3, pp. 119–124, 1992.

[47] H. Ohkawa, N. Ohishi, and K. Yagi, "Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction," *Analytical Biochemistry*, vol. 95, no. 2, pp. 351–358, 1979.

[48] A. M. Codrington, B. F. Hales, and B. Robaire, "Spermiogenic germ cell phase-specific DNA damage following cyclophosphamide exposure," *Journal of Andrology*, vol. 25, no. 3, pp. 354–362, 2004.

[49] L. Fraser, "Structural damage to nuclear DNA in mammalian spermatozoa: its evaluation techniques and relationship with male infertility," *Polish Journal of Veterinary Sciences*, vol. 7, no. 4, pp. 311–321, 2004.

[50] G. W. Snedecor and W. G. Cochran, *Statistical Methods*, vol. 54, pp. 71–82, Iowa State University Press, Ames, 8th edition, 1989.

[51] G. Stradaioli, T. Noro, L. Sylla, and M. Monaci, "Decrease in glutathione (GSH) content in bovine sperm after cryopreservation: comparison between two extenders," *Theriogenology*, vol. 67, no. 7, pp. 1249–1255, 2007.

[52] R. P. Amann, R. H. Hammerstedt, and D. N. Veeramachaneni, "The epididymis and sperm maturation: a perspective," *Reproduction, Fertility and Development*, vol. 5, no. 4, pp. 361–381, 1993.

[53] V. Otasevic, A. Korac, M. Vucetic et al., "Is manganese (II) pentaazamacrocyclic superoxide dismutase mimic beneficial for human sperm mitochondria function and motility?" *Antioxidants & Redox Signaling*, vol. 18, no. 2, pp. 170–178, 2013.

[54] E. Gomez, D. W. Buckingham, J. Brindle, F. Lanzafame, D. S. Irvine, and R. J. Aitken, "Development of an image analysis system to monitor the retention of residual cytoplasm by human spermatozoa: correlation with biochemical markers of the cytoplasmic space, oxidative stress, and sperm function," *Journal of Andrology*, vol. 17, no. 3, pp. 276–287, 1996.

[55] R. J. Aitken, M. Paterson, H. Fisher, D. W. Buckingham, and M. Van Duin, "Redox regulation of tyrosine phosphorylation in human spermatozoa and its role in the control of human sperm function," *Journal of Cell Science*, vol. 108, no. 5, pp. 2017–2025, 1995.

[56] J. Espino, Á. Ortiz, I. Bejarano et al., "Melatonin protects human spermatozoa from apoptosis via melatonin receptor- and extracellular signal-regulated kinase-mediated pathways," *Fertility and Sterility*, vol. 95, no. 7, pp. 2290–2296, 2011.

[57] M. Karbownik and R. J. Reiter, "Antioxidative effects of melatonin in protection against cellular damage caused by ionizing radiation," *Proceedings of the Society for Experimental Biology and Medicine*, vol. 225, no. 1, pp. 9–22, 2000.

[58] R. J. Reiter, D.-X. Tan, L. C. Manchester, S. D. Paredes, J. C. Mayo, and R. M. Sainz, "Melatonin and reproduction revisited," *Biology of Reproduction*, vol. 81, no. 3, pp. 445–456, 2009.

[59] M. Koksal, E. Oğuz, F. Baba et al., "Effects of melatonin on testis histology, oxidative stress and spermatogenesis after experimental testis ischemia-reperfusion in rats," *European Review for Medical and Pharmacological Sciences*, vol. 16, no. 5, pp. 582–588, 2012.

[60] S. S. Du Plessis, K. Hagenaar, and F. Lampiao, "The in vitro effects of melatonin on human sperm function and its scavenging activities on NO and ROS," *Andrologia*, vol. 42, no. 2, pp. 112–116, 2010.

[61] A. Medrano, C. F. B. Contreras, F. M. Herrera, and A. M. Alcantar-Rodriguez, "Melatonin as an antioxidant preserving sperm from domestic animals," *Asian Pacific Journal of Reproduction*, vol. 6, pp. 241–246, 2017.

[62] S. Varesi, V. Vernocchi, M. G. Morselli, and G. C. Luvoni, "DNA integrity of fresh and frozen canine epididymal spermatozoa," *Reproductive Biology*, vol. 14, no. 4, pp. 257–261, 2014.

[63] K. Makker, A. Agarwal, and R. Sharma, "Oxidative stress & male infertility," *The Indian Journal of Medical Research*, vol. 129, no. 4, pp. 357–367, 2009.

[64] A. R. Hipkiss, V. C. Worthington, D. T. J. Himsworth, and W. Herwig, "Protective effects of carnosine against protein modification mediated by malondialdehyde and hypochlorite," *Biochimica et Biophysica Acta (BBA)—General Subjects*, vol. 1380, no. 1, pp. 46–54, 1998.

[65] P. Perumal, K. Vupru, and K. Khate, "Effect of addition of melatonin on the liquid storage (5°C) of mithun (*Bos frontalis*) semen," *International Journal of Zoology*, vol. 2013, Article ID 642632, 10 pages, 2013.

[66] V. Brazão, F. H. Santello, R. P. Colato et al., "Melatonin: antioxidant and modulatory properties in age-related changes during *Trypanosoma cruzi* infection," *Journal of Pineal Research*, vol. 63, no. 1, Article ID e12409, 2017.

[67] N. Zhang, B. Zhao, H.-J. Zhang et al., "Melatonin promotes the in vitro development of pronuclear embryos and increases the efficiency of blastocyst implantation in murine," *Journal of Pineal Research*, vol. 55, no. 3, pp. 267–274, 2013.

[68] M. Koderle, C. Aurich, and S. Schäfer-Somi, "The influence of cryopreservation and seminal plasma on the chromatin structure of dog spermatozoa," *Theriogenology*, vol. 72, no. 9, pp. 1215–1220, 2009.

[69] H. J. Kim, C. H. Kim, J. Y. Lee, J. H. Kwon, D. Y. Hwang, and K. C. Kim, "Effect of cryopreservation day on pregnancy outcomes in frozen-thawed blastocyst transfer," *Clinical and Experimental Reproductive Medicine*, vol. 37, pp. 57–64, 2010.

[70] M. Di Santo, N. Tarozzi, M. Nadalini, and A. Borini, "Human sperm cryopreservation: update on techniques, effect on DNA integrity, and implications for ART," *Advances in Urology*, vol. 2012, Article ID 854837, 12 pages, 2011.