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Evidences for the Role of Melatonin as a Protective Additive During Buffalo Semen Freezing

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Abstract: Till now, the exact role of melatonin in male infertility hasn't been fully discovered. Moreover, the intracellular signaling pathways activated by melatonin in buffalo spermatozoa especially on the level of cryopreservation process haven't been reported. The current study aimed simply to clarify the precise role of this hormone during buffalo semen cryopreservation. Semen samples were obtained randomly from 6 fertile buffalo bulls (aged 3 to 5 years). Weekly, two consecutive ejaculates were collected from each bull for successive six weeks duration using an artificial vagina. The ejaculates were pooled to eliminate variability between the evaluated samples. After that, semen samples were subjected to extension with Tris-based extender supplemented with different concentrations of melatonin (0.1, 0.25, 0.5, 0.75 and 1 mM) Vs. Tris-based extender only (control). Then they were processed to cryopreservation and thawing to assess the different semen characteristics. It had been found that 0.1 mM of melatonin significantly ($p < 0.05$) improved post thawing motility, viability index, acrosomal integrity, total antioxidant capacity, superoxide dismutase and glutathione reductase activity. On the other hand, it significantly ($p < 0.05$) decreased the rate of lipid peroxidation, aspartate amino transferase, alanine aminotransferase, alkaline phosphatase and DNA fragmentation. All of these previously enhanced semen characteristics were reflected positively on its *in vitro* fertilizing capacity, as well as the percent of harvested embryos. In conclusion melatonin supplementation to the extension media of buffalo semen during processing significantly enhanced its characteristics. Moreover, the preceding results focusing more light on the potential roles of melatonin in regulating male reproduction.

Keywords: Melatonin, Buffalo, Frozen, Semen, Quality

Introduction

The sperm processing procedures used for Assisted Reproductive Techniques (ART) are aim to improve sperm quality by increasing the number of progressive motile and morphologically normal sperm cells. Sperm membrane integrity and fertilizing capacity are two indicators that might be disturbed by the action of Reactive Oxygen Species (ROS) generated by the metabolism of sperm cellular components, likes superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2) and lipid hydroperoxides, formed via lipid peroxidation of the membrane lipids of the spermatozoa

(Del Maestro, 1980; Alvarez and Storey, 2005). Lipid peroxidation usually results in irreversible loss of sperm motility and DNA damage (De Lamirande and Gagnon, 1992; Aitken, 1994; Maxwell and Watson, 1996); Ruminant semen normally contains antioxidants, including taurine, hypotaurine, Catalase (CAT) and reduced glutathione (GSH), which can offset lipid peroxidation (Jeulin *et al.*, 1989; Griveau *et al.*, 1995; Kantola *et al.*, 1998). However, this endogenous antioxidative capacity may be insufficient to prevent lipid peroxidation during prolonged storage (Aurich *et al.*, 1997). Thus the addition of external antioxidants such as melatonin might protect the sperm cells against the

harmful effects of these oxidative stressors (ROS) and subsequently improve sperm motility during sperm storage (Ashrafi *et al.*, 2011). Ruminant sperm seems to be especially sensitive to Reactive Oxygen Species (ROS) damage due to the relative high content of unsaturated fatty acids in their phospholipids membrane. Fluidity is linked to the integrity of the membrane lipids (Stubbs and Smith, 1984) and any change in lipid composition of the plasma membrane might therefore be associated with the cooling and storage effects. Lipid peroxidation might be one of the mechanisms responsible for the negative biochemical and physiological changes during sperm storage (Cerolini *et al.*, 2000) and the relative low antioxidant capacity of seminal plasma (Brezinska-Slevbodzinska *et al.*, 1995).

Melatonin (*N*-acetyl-5-Methoxytryptamine) is an indole derivative secreted rhythmically from the pineal gland which plays a major role in regulating the circadian clock in mammals in general and regulating the reproductive functions in particular (Reiter, 1991).

More recent studies had demonstrated that beside its multiple actions on different physiological processes, melatonin as well as its metabolites are indirect antioxidants and powerful direct scavengers that protected the cells from the free radicals brought about by their metabolism (Ahn and Bae, 2004b; 2004a; Adriaens *et al.*, 2006; Kang *et al.*, 2009). Melatonin exerts its effects via two specific receptors termed as MT₁ and MT₂ (Reppert *et al.*, 1994; 1995).

In order to fertilize the egg, mammalian spermatozoa have to be capacitated and then undergo the acrosome reaction and hyperactivation. The acrosome reaction is a modified exocytotic event involving the acrosome (Yudin *et al.*, 1988); that is necessary for sperm-egg plasma membrane fusion and penetration of zona pellucida (Yanagimachi, 1994). Hyperactivation is a specialized movement of the sperm flagellum that creates the propulsive force for the penetration of the zona pellucida. Hyperactivated spermatozoa exhibit large bend amplitude, whiplash and frenzied flagellar movements (Yanagimachi, 1994; Morisawa, 1994; Suarez and Ho, 2003). It was reported that melatonin significantly induced sperm hyperactivation (Si and Olds-Clarke, 2000; Fujinoki, 2008). Recently, it had been demonstrated that melatonin has beneficial direct role on the other sperm characteristics like capacitation and fertilization rates (Casao *et al.*, 2010). Furthermore, melatonin and its metabolites potentially scavenge ROS (Tan *et al.*, 2007), thus altering redox-sensitive events and preventing oxidative damage. Although physiologic levels of ROS are required for normal sperm function (Agarwal and Prabakaran, 2005), the spermatozoa have limited scavenging enzymes, thus making them highly susceptible to ROS damage (Henkel, 2005). Additionally, melatonin might be involved in the protection of different cell types against damage-induced apoptosis (Mayo *et al.*, 1998).

So the current study aimed to clarify the role of melatonin in protection of buffalo semen from freeze-thawing hazards and its role in enhancing its *in vitro* fertilizing capacity.

Materials and Methods

Diluent Preparation

The cryoprotective extender used in the current study was composed of 2.42 g Tris, 1.48 g citric acid, 1.00 g fructose, 6.6 mL glycerol, 20 mL egg yolk, 25 mg gentamicin and 50,000 IU penicillin; all of these components were dissolved in 100 mL deionized water and supplemented with different concentrations of melatonin.

Semen Collection

Semen samples were obtained randomly from six fertile Egyptian buffalo bulls (aged 3 to 5 y) kept at the Animal Reproduction Research Institute farm (Cairo, Egypt). Two consecutive ejaculates were collected from each bull weekly for successive six weeks using an artificial vagina. The ejaculates were pooled to eliminate variability between the evaluated samples. The semen samples were assessed for volume, sperm concentration and percentage of motile spermatozoa. The ejaculates with at least 70% motility, 800×10^6 sperm cells/mL and >85% normal sperm morphology were used for the present study. All experiments were done with at least 3 replicates for each group.

Semen Processing

After the evaluation of semen quality, the fresh semen samples were pooled and then split into 6 equal portions and diluted at 30°C with Tris-based extender supplemented with different concentrations of melatonin (0.100, 0.250, 0.500, 0.750 and 1 mM) Vs. Tris-based extender only (control) to obtain 120×10^6 sperm/mL. Melatonin (M5250-250 mg, lot No. 068k1538; Sigma-Aldrich, St. Louis, MO) stock solution was prepared by dissolving powder in the diluent media according to its dissolving capability in solutions. The required concentrations were achieved using serial dilution manner; depending on published papers (Sliwa and Stochmal, 2001; Tanyildizi *et al.*, 2006; Di Francesco *et al.*, 2009; Succu *et al.*, 2011). The current work used a broad range of melatonin concentrations to determine the ideal dose that should be used for buffalo semen extension.

The fresh semen samples were transferred to pre warmed tubes. Semen was cooled from 37 to 5°C throughout 60 min in a cold cabinet. The cooled semen was loaded into 0.25 mL polyvinyl chloride straws (IMV, L'Aigle, France), horizontally placed in a refrigerator and kept at 4°C for 1 h. These straws were then placed 6 cm above the liquid nitrogen surface where the temperature was approximately -120°C. After 15

min, they were immersed directly into liquid nitrogen (-196°C) for storage. The straws were stored at least for 24 h before evaluation. Frozen semen straws were thawed in water bath at 37°C for 30 sec. Post-thawing sperm motility; viability and acrosomal integrity were assessed according to Mohammed *et al.* (1998).

Analysis of Sperm Motility

The percentage of linear motile sperm was examined visually. For each extender, 3 straws were thawed separately by immersion in a water bath at 37°C for 30 sec. The sperm samples were placed on glass slides, covered with a glass cover and then estimated at 37°C by phase contrast microscope equipped with a warm stage at $200\times$ magnifications. Sperm motility estimations were performed in 3 different microscopic fields and the mean of the 3 successive estimations was recorded as the final motility score.

Analysis of Acrosomal Membrane Integrity

Acrosomal integrity was assessed using silver nitrate stain in a procedure slightly modified from the method described by Chinoy *et al.* (1992). The sperm suspension was spread over slides and dried at room temperature. The preparations were fixed in ethyl alcohol 70% for 2 min and in ethyl alcohol 95% for another 2 min. The preparations were stained with the solution (AgNO_3) for 2 h in an incubator at 65°C , in complete humidity. After the preparations turned gold in colour, the chemical reaction was interrupted and the preparations were rinsed several times with distilled water. The preparations were dried at room temperature. The dried preparations were analyzed for acrosomal integrity using the Olympus BX50 light microscope with a 100-fold magnification. At least 300 sperm cells were counted per slide and the percent of acrosome intact spermatozoa was calculated.

Assessment of Sperm DNA Integrity

DNA integrity and the incidence of DNA strand breaks or fragmentation were detected using the alkaline single cell gel electrophoresis (comet) assay according to Boe-Hansen (2005). Briefly, frozen-thawed spermatozoa were diluted in phosphate buffer saline (PPS), embedded in agarose, followed by cell lysis, DNA decondensation, electrophoresis and DNA staining with $50\ \mu\text{L}$ of $20\ \mu\text{g mL}^{-1}$ ethidium bromide (Sigma, St. Louis, MO). The cells were then visualized by fluorescent microscopy. Intact nuclei in the comet assay appeared to have compact and brightly fluorescent heads; in contrast, strand breaks in damaged cells allow DNA migration during electrophoresis and a tail of DNA could be seen behind the head, giving the appearance of a comet (Hughes *et al.*, 1996). After subjecting the spermatozoa to the comet assay, sperm nuclei were analyzed by computer software program (comet IV, 2001).

Biochemical Assays

Estimating Transaminases Enzymes

Semen straws from each group were centrifuged at $800\times g$ for 10 min and supernatant was collected for estimating the Aspartate-Aminotransferase (AST); Alanine-Aminotransferase (ALT) and Alkaline Phosphatase (AKP) enzymes spectrophotometrically in all groups. Reagent kits supplied by Span diagnostics, Ltd, Surat, India were used for estimating AST, ALT and AKP as described by Reitman and Frankel (1957) and Tietz (1976). A minimum of 6 observations were recorded for each enzyme in each group.

Superoxide Dismutase (SOD)

The SOD activity was measured according to Flohe and Otting (1984). Briefly, each semen sample was diluted 1:5 with PBS (pH 7.0). The assay solution contained sodium carbonate buffer (50 mM, pH 10.0), 0.1 mM xanthine, 0.025 mM nitroblue tetrazolium, 0.1 mM EDTA, xanthine oxidase and sample were mixed in a small cuvette. The SOD activity was measured at 560 nm on a spectrophotometer and expressed as units per milliliter.

Glutathione Reductase (GSH)

The GSH content of sperm was measured using the method of Sedlak and Lindsay (1968). The semen samples were precipitated with 50% trichloroacetic acid (vol/vol) and then centrifuged at $1,000\times g$ at 22°C for 5 min. The reaction mixture contained 0.5 mL supernatant, 2.0 mL Tris-EDTA buffer and 0.1 mL 5, 5'-dithiobis-2-nitrobenzoic acid. The solution was incubated at 22°C for 5 min and then read at 412 nm on a spectrophotometer. The values of GSH were expressed as units per liter for sperm samples.

Lipid Peroxidation (LPO)

An aliquot ($500\ \mu\text{L}$) of semen from each sample was centrifuged at $800\times g$ for 10 min, sperm pellets were separated and washed by resuspending in PBS and recentrifuging (three times). After the last centrifugation, 1 mL of deionized water was added to spermatozoa and they were snap-frozen and stored at -70°C till further analysis. The samples were thawed before the lipid peroxidation assay. The concentrations of Malondialdehyde (MDA), as indices of the LPO in the sperm samples, were measured using the thiobarbituric acid reaction according to the method of Placer *et al.* (1966). The quantification of thiobarbituric acid reactive substances was determined comparing the absorption with the standard curve of MDA equivalents generated by the acid catalyzed hydrolysis of 1, 1, 3, 3-tetramethoxypropane. The MDA concentrations were expressed in $\text{nmol}/10^9$.

Total Antioxidant Capacity (TAC)

Total antioxidant capacity: Total antioxidant capacity of the frozen-thawed semen was estimated using a commercial kit (Antioxidant Capacity Assay Kit, Cayman Chemical Co. Ann Arbor, MI, USA); according to Cortassa *et al.* (2004).

The assay measures the capacity of the cryopreserved semen to inhibit the production of TBA Reactive Substances (TBARS) from sodium benzoate under the influence of the reactive oxygen free radicals derived from the Fenton's reaction. The reaction was measured spectrophotometrically at 532 nm. Antioxidants from the added sample cause suppression of the production of TBARS and the inhibition of the colour development is defined as AOA. A solution of 1 mmol L⁻¹ uric acid was used as standard.

in vitro Maturation

Immature buffalo oocytes were collected from fresh ovaries just after slaughter at a local abattoir. Cumulus-Oocyte Complexes (COCs) were collected by aspiration of medium-sized (2-8 mm) ovarian follicles using 18-gauge needle attached to a disposable 10 mL syringe. Evenly granulated oocytes surrounded with multi-layered compact cumulus cells were selected for the experiments. Selected COCs were firstly washed three times in sterile D-PBS and then washed three times in IVM medium; IVM was performed in TCM-199 medium (Earl's salt, Sigma Chemical CO., St. Louis, Mo., USA) supplemented with 10% heat inactivated fetal calf serum (FCS, Gibco, 30 K-0351), 10 µg mL⁻¹ Luteinizing hormone, 5 µg mL⁻¹ follicle stimulating hormone and 1 µg mL⁻¹ estradiol-17β. The oocytes were cultured for 24 h at 39°C in an atmosphere of 5% CO₂ in air with maximum humidity.

in vitro Fertilization

Three straws from each melatonin treatment were thawed in a water bath at 37°C for 30 sec. The most motile spermatozoa were separated by swim up technique in the fertilization medium, modified Tyrode's Albumin-Lactate-Pyruvate (TALP) containing 6 mg mL⁻¹ Bovine Serum Albumin (BSA), for 1 h as recorded by Parrish *et al.* (1988). The uppermost layer of the medium containing the most motile spermatozoa was collected and washed twice by centrifugation at 800 xg for 10 min. The sperm pellet was resuspended in the fertilization TALP medium containing 10 µg mL⁻¹ heparin. After appropriate dilution, 2 µL (final concentration 2×10⁶ sperm cell/mL) of sperm suspension was added to the fertilization drops, containing *in vitro* matured oocytes. Gametes were co-incubated in the fertilization drops under sterile mineral oil for 18 h at 39°C in an atmosphere of 5% CO₂ in air with complete humidity.

Evaluation of *in vitro* Fertilizing Potential

The oocyte fertilization rate was examined on the base of the pronuclei number found in the oocyte after fertilization. In brief, the previously matured oocytes were incubated with the sperms for 6 h at 5% CO₂ and 20% O₂ in humid incubator; after that the oocytes were subjected to denudation with perfect handmade pipette. Then they were cultured for 10~12 h in CR1aa medium. They were fixed for 24~48 h in ethanol: Acetic acid (3:1 v/v), then stained with 1% Orcein dissolved in 45% acetic acid according to Marei *et al.* (2009). The oocytes were evaluated under a phase-contrast microscope (Nikon, Tokyo, Japan). Oocytes were evaluated for normal fertilization according to Martino *et al.* (1994), on basis of oocyte that had set of male and female pronuclei in the ooplasm were considered to be fertilized normally, while the penetration rate was evaluated by the presence of sperm tail only in the ooplasm.

in vitro Culture

Denuded zygotes were transferred into SOFaa culture medium (SOF with 1 mM glutamine, 1% MEM nonessential amino acids and 1% MEM essential amino acids) according to Badr (2009) and then covered with mineral oil for 7- days at 38.5°C in an atmosphere of 5% CO₂ in air with maximum humidity. The cleaved oocytes were recorded after 48 h of insemination and those developed to the morula and blastocyst stages were recorded at 5-7 days post-insemination according to Totey *et al.* (1992).

Statistical Analysis

All data were analyzed by using Costat Computer Program (1986), Version 3.03 copyright Cottort Software and were compared by the Least Significant Difference least (LSD) at 5% levels of probability. The results were expressed as means ±SE. The mean values of the percentages of motile sperm, acrosome-intact sperm, enzyme activity and embryo development were compared using Duncan's multiple range test by one-way ANOVA procedure, when the F-value was significant ($p < 0.05$). Sperm fertilizing capacity and zygotes developmental competences were assessed using Chi-square at ($p < 0.01$ and 0.05 , respectively).

Results

As shown in Table 1, there was a significant ($p < 0.05$) increase in post thawing motility of buffalo semen treated by 0.1 and 0.25 mM of melatonin (60.00±2.9 and 55.00±5.01, respectively). Moreover, there was a significant ($p < 0.05$) increase in the viability index of buffalo semen treated with 0.1 and 0.25 mM of melatonin (157.5±11.8 and 150.83±8.21, respectively); reflecting that these two concentrations have a beneficial protecting effect on buffalo semen post thawing characteristics than other melatonin concentrations (0.5, 0.75 and 1 mM).

Table 1. The effect of melatonin supplementation to buffalo semen diluent medium on sperms characteristics

Treatment	Dilution motility (%)	Cooling motility (%)	Post-thawing motility (%)	Viability Index*	Acrosomal integrity (%)
Control	71.67±4.42 ^a	65.00±2.90 ^a	45.00±2.90 ^{bc}	97.00±9.09 ^b	17.33±0.88 ^{bc}
Melatonin 0.1mM	80.00±2.89 ^a	71.66±1.67 ^a	60.00±2.9 ^a	157.5±11.8 ^a	13.67±1.76 ^c
Melatonin 0.25 M	75.00±2.90 ^a	75.00±2.88 ^a	55.00±5.01 ^{ab}	150.83±8.21 ^a	15.00±1.73 ^{bc}
Melatonin 0.5 mM	68.33±6.02 ^a	66.67±3.34 ^a	43.33±4.41 ^{bc}	102.50±6.29 ^b	15.67±1.86 ^{bc}
Melatonin 0.75 M	70.00±5.78 ^a	65.00±7.64 ^a	38.66±3.34 ^c	95.83±4.65 ^b	21.33±2.03 ^{ab}
Melatonin 1 mM	70.00±2.89 ^a	63.33±6.02 ^a	36.66±3.34 ^c	66.66±4.42 ^c	24.66±2.96 ^a

Three replications of the experiment were conducted. Results are presented as mean ± SEM

^{a,b}Values with different letters in the same column are significantly different ($p < 0.05$)

*Viability index = post-thawing motility/2 +motility after 1 h +motility after 2 h +motility after 3 h

Table 2. The effect of melatonin supplementation to buffalo semen diluent medium on its antioxidant capacity

Treatment	TAC (μm/mL)	SOD (U/mL)	GSH (U/L)	MALON) (nmol/mL)
Control	0.17±0.2 ^b	27.33±3.49 ^c	52.33±4.09	26.00±4.04 ^a
Melatonin 0.1 mM	0.58±0.08 ^a	73.67±5.37 ^a	106.66±12.03 ^a	11.00±1.73 ^b
Melatonin 0.25 mM	0.35±0.03 ^b	52.66±6.07 ^b	83.33±4.48 ^{ab}	16.67±2.60 ^{ab}
Melatonin 0.5 mM	0.31±0.06 ^b	36.33±4.64 ^c	87.67±13.06 ^{ab}	16.00±2.51 ^{ab}
Melatonin 0.75 mM	0.25±0.04 ^b	24.33±4.38 ^c	73.00±7.24 ^{bc}	21.00±3.61 ^{ab}
Melatonin 1 mM	0.19±0.05 ^b	31.00±5.51 ^c	59.67±10.67 ^{bc}	20.67±4.49 ^{ab}

Three replications of the experiment were conducted. Results are presented as mean ± SEM

^{a,b}Values with different letters in the same column are significantly different ($p < 0.05$).

Abbreviations: TAC: Total Antioxidant Capacity; SOD: Superoxide Dismutase; GSH: Glutathione Reductase; Malon: malondialdehyde

Table 3. The effect of melatonin supplementation to buffalo semen diluent medium on its enzymatic activity

Treatment	AST (U/L)	ALT (U/L)	AKP (U/L)
Control	104.67±10.42 ^a	28.66±3.84 ^a	25.33±4.09 ^a
Melatonin 0.1 mM	65.33±5.90 ^b	14.33±2.40 ^b	11.33±2.40 ^b
Melatonin 0.25 mM	68.33±5.79 ^b	19.67±2.73 ^{ab}	15.67±2.02 ^{ab}
Melatonin 0.5 mM	80.33±8.3 ^{ab}	23.33±2.61 ^{ab}	19.33±4.67 ^{ab}
Melatonin 0.75 mM	83.67±4.06 ^{ab}	27.67±3.53 ^a	18.66±3.84 ^{ab}
Melatonin 1 mM	94.00±8.32 ^a	28.00±4.04 ^a	22.66±3.53 ^{ab}

Three replications of the experiment were conducted. Results are presented as mean ± SEM

^{a,b}Values with different letters in the same column are significantly different ($p < 0.05$).

Abbreviations: AST: Aspartate Amino Transferase; ALT: Alanine Aminotransferase; AKP: Alkaline Phosphatase

Table 4. The effect of melatonin supplementation to buffalo semen diluent medium on its DNA integrity

Treatment	DNA Fragmentation (%)	Tail length (μm)	Tail moment*
Control	5.22±2.74 ^a	3.45±0.75 ^{ab}	17.43±8.03 ^a
Melatonin 0.1 mM	2.14±0.85 ^b	2.12±1.01 ^b	4.76±3.78 ^b
Melatonin 0.25 mM	2.78±0.85 ^{ab}	3.18±0.90 ^{ab}	8.67±2.92 ^{ab}
Melatonin 0.5 mM	2.64±0.59 ^{ab}	2.18±0.88 ^{ab}	6.10±3.68 ^b
Melatonin 0.75 mM	2.94±1.19 ^{ab}	4.16±1.01 ^a	11.44±1.99 ^{ab}
Melatonin 1 mM	4.02±1.56 ^{ab}	4.62±1.37 ^a	18.12±7.9 ^a

Three replications of the experiment were conducted. Results are presented as mean ± SEM

^{a,b}Values with different letters in the same column are significantly different ($p < 0.05$).

*tail moment is the result of multiplying the percent of DNA fragmentation by Tail length

Table 5. The effect of melatonin supplementation to buffalo semen diluent medium on the sperm fertilizing capacity

Treatment	No. oocytes	Penetration rate (%)	Fertilization rate (%)
Control	59	30 (50.84 ^{ab})	14 (23.72 ^c)
Melatonin 0.1 mM	57	36 (63.15 ^a)	33 (57.89 ^a)
Melatonin 0.25 mM	65	42 (65.07 ^a)	27 (41.53 ^{ab})
Melatonin 0.5 mM	60	22 (36.66 ^b)	16 (26.66 ^{cb})
Melatonin 0.75 mM	63	24 (38.09 ^b)	15 (23.80 ^c)
Melatonin 1 mM	56	23 (41.07 ^b)	13(23.21 ^c)

Three replications of the experiment were conducted. Results are presented as mean ± SEM

Percentages are based on the number of oocytes examined

^{a,b}Values with different letters in the same column are significantly different ($p < 0.01$)

Table 6. The effect of melatonin supplementation to buffalo semen diluent medium on resultant zygotes developmental competences

Treatment	No. oocytes	Cleavage rate (%)	Morula stage (%)	Blastocyst stage (%)
Control	49	12 (24.48 ^{ab})	3 (6.12b)	1 (2.04 ^b)
Melatonin 0.1 mM	48	20 (41.66 ^a)	11 (22.92a)	6 (12.50 ^b)
Melatonin 0.25 mM	46	18 (39.13 ^{ab})	9 (19.56a)	5 (10.86 ^{ab})
Melatonin 0.5 mM	49	11 (22.44 ^{bc})	3 (6.12b)	2 (4.08 ^{ab})
Melatonin 0.75 mM	42	11 (26.19 ^{ab})	3 (7.14 b)	1 (2.38 ^{ab})
Melatonin 1 mM	46	8 (17.39 ^b)	2 (4.35b)	1 (2.17 ^b)

Five replications of the experiment were conducted

Results are presented as mean ± SEM Percentages are based on the number of oocytes examined

^{a,b}Values with different letters in the same column are significantly different ($p < 0.05$)

*Table (5 and 6) not the same set of experiment. In Table 5 we stained most of oocytes by aceto-orcein stain to evaluate both penetration and fertilization rate. The remaining oocytes are few-(normally the harvested oocyte number from buffaloes ovary are few) -so not cultured for embryo development, otherwise our results in such condition will not be reliable. So Table 6 is a new experiment in which all oocytes after insemination were cultured (without staining) to evaluate embryo development.

Additionally there was a significant ($p < 0.05$) significant improvement (13.67 ± 1.76) in the sperm acrosomal integrity in the semen sample treated with 0.1 mM of melatonin if it was compared with control and other melatonin treated semen samples.

Moreover, there was a significant increase ($p < 0.05$) in TAC, SOD and GSH activity in semen samples treated with 0.1 mM of melatonin (0.58 ± 0.08 , 73.67 ± 5.37 and 106.66 ± 12.03 , respectively). Furthermore, there was a significant ($p < 0.05$) decrease in the level of lipid peroxidation in the membrane of the cryopreserved buffalo spermatozoa treated with 0.1 mM of melatonin (11.00 ± 1.73), compared with control (Table 2). These results indicate that there was a significant enhancement in the antioxidant activity of the cryopreserved buffalo semen treated with melatonin by increasing the active enzymes that decrease the level of its lipid peroxidation; which in turn decrease the rate of its damage. Studying the effect of melatonin supplementation to buffalo semen diluent medium on its enzymatic activity reflecting that there were significant decreases ($p < 0.05$) in AST, ALT and AKP levels in melatonin treated buffalo sperm cells especially those treated with 0.1 mM (65.33 ± 5.90 , 14.33 ± 2.40 and 11.33 ± 2.40 , respectively) if they were compared with the results of control (104.67 ± 010.42 , 28.66 ± 3.84 and 25.33 ± 4.09 , respectively) (Table 3). This indicates that melatonin addition to buffalo semen resulting in obvious decrease in the rate of the sperm degradation during cryopreservation process, consequently, enhancing the sperm viability and activity. Additionally, there was a significant decrease ($p < 0.05$) in the DNA fragmentation, tail length and tail moment of buffalo semen samples treated with 0.1 mM of melatonin (2.14 ± 0.85 , 2.12 ± 1.01 and 4.76 ± 3.78 , respectively), especially if it was compared with the result of control and 1mM melatonin treated semen samples (Table 4). This indicates that buffalo semen treatment with melatonin (0.1 mM) during cryopreservation process resulting in DNA protection which consequently protected the fertilization process and the resultant embryos. Looking through Table 5 of the effect of melatonin supplementation to buffalo semen

diluent medium on the sperm *in vitro* fertilizing capacity, finding that there was a significant increase ($p < 0.01$) in the fertilization rate of buffalo semen samples treated with 0.1 mM of melatonin (57.89), compared with the result of control and 1mM melatonin treated semen samples (23.72 and 23.21, respectively). The results of the current study indicate that, 0.1 mM of melatonin is an ideal melatonin concentration enhancing sperm *in vitro* fertilization potentials. Also, there was a significant increase ($p < 0.05$) in the cleavage rate, morula stage and blastocyst rate resulting from fertilization of fresh qualified buffalo oocytes with buffalo sperms cells treated with 0.1 and 0.25 mM of melatonin compared with control and 1 mM melatonin.

In conclusion, the role of melatonin in enhancing the antioxidant activity of buffalo sperm cells, decreasing the rate of lipid peroxidation and DNA fragmentation was reflected in enhancement of the sperm *in vitro* fertilization potentials and the rate of blastocyst harvesting.

Discussion

Naturally, seminal fluid contains melatonin (Bornman *et al.*, 1989) and the spermatozoa reportedly possess membrane melatonin receptors (Bornman *et al.*, 1989; Van Vuuren *et al.*, 1992). Removing of buffalo seminal plasma during the cryopreservation process deprives the sperm cells from important elements in between melatonin. The current study clarified that melatonin has some fantastic protecting and enhancing functions on the post thawing buffalo semen characteristic in a dose dependent trend. Addition of 0.1 mM melatonin to the semen diluent is sufficiently effective to improve buffalo spermatozoa fertilizing ability and *in vitro* fertilizing potentials. These results came in agreement with (Fujinoki, 2008; Du Plessis *et al.*, 2010; Succu *et al.*, 2011) who reported that *in vitro* melatonin addition to hamster, human and ram spermatozoa, respectively had beneficial effects on their total progress motility. Moreover, Succu *et al.* (2011) reported that those effects of melatonin might be built on its role in enhancing the total ATP level in the sperm cells after treatment.

While our results disagreed with Bornman *et al.* (1989); Sliwa and Stochmal (2001) and Gwayi and Bernard (2002) who reported that melatonin played no important role in the sperm motility. This disagreement might be attributed to species difference or differences in treatment doses and methodology or due to the inhibitory effect of ethanol that was used in melatonin dissolving through pH changing which directly affected not only the sperm motility but also the other aspects of sperm characteristics (Gwayi and Bernard, 2002). Where, the high concentrations of melatonin inhibited the quality of sperm motility in rats (Gwayi and Bernard, 2002). Additionally, melatonin at 0.1 mM enhanced the acrosomal integrity and viability index of buffalo sperm cells. This melatonin improving effect might be due to a potent scavenging effect of melatonin on NO and Reactive Oxygen Species (ROS) that directly and indirectly affect the sperm viability by triggering the sperm apoptosis (Du Plessis *et al.*, 2010; Espino *et al.*, 2011). Therefore, the beneficial effect of melatonin on the freezability and *in vitro* fertilizing potentials may be attributed to its positive effect on antioxidant enzymes. Melatonin addition at 0.1mM augments the activities of GSH, superoxide dismutase antioxidant enzymes. GSH was very abundant intracellular free radical scavenger and antioxidant. It had been reported that melatonin stimulates the rate limiting enzyme γ -glutamylcysteine synthase, thereby increasing intracellular GSH concentrations (Urata *et al.*, 1999). The results of the current study as shown in Table 2 throw an illuminating proof on these previous facts where it had been observed that melatonin addition to buffalo semen at 0.1mM enhanced the level of GSH (106.66 ± 12.03). Till the current study the relationship between melatonin and glutathione level in buffalo semen hasn't been detected yet; although, this relationship had been discovered in other cell types and tissues like brain tissues (Floreani *et al.*, 1997; Martin *et al.*, 2000). Furthermore, melatonin treatments augment the activities of other antioxidants (Gitto *et al.*, 2001), like Superoxide Dismutase (SOD) (Reiter *et al.*, 1998). The results of the current study came in harmony with the previous reports, where it has been observed that melatonin treatment to buffalo semen at 0.1mM augment the level of SOD (73.67 ± 5.37) (Table 2). So, melatonin is an effective factor in lowering molecular damage under conditions of elevated oxidative stress (Reiter, 1998; Reiter and Tan, 2003). Moreover, as shown in Table 2 there was a significant ($p < 0.05$) decrease in the level of lipid peroxidation in the membrane of the cryopreserved buffalo sperms treated with 0.1 mM of melatonin (11.00 ± 1.73). Indicating that there was a significant enhancement in the Total Antioxidant Activity (TAC) of the cryopreserved buffalo semen treated with 0.1mM of melatonin by increasing its active enzymes that compete the free

radicles (ROS) and this was reflected by decreasing the level of lipid peroxidation in buffalo sperm cells that was characterized naturally by high level of unsaturated fatty acids. This result came in agreement with Sarabia *et al.* (2011) and Hardeland *et al.* (2011) who reported that melatonin has powerful ability to purify peroxide radicals (ROO-) and consequently lipid peroxidation.

In Summary, melatonin addition to buffalo semen can improve its rate of cryopreservation by decreasing the rate of its destruction. In addition, there was a significant increase ($p < 0.01$) in fertilization rate of buffalo semen samples treated with 0.1 mM of melatonin (57.89). The significant effect of melatonin on IVF outcomes (Table 5) might be explained firstly depending upon its effects in lowering the higher free radicals level that resulting in damaging and aging of the mitochondria (Bize *et al.*, 1980). Moreover, melatonin via modulating the glutathione activity has the potency to improve mitochondrial health state and functions (Table 2). Active and healthy mitochondria have essential roles during fertilization process (Van Blerkom and Davis, 2007). It had been reported that healthy condition of mitochondria was important for several cellular activities; like ATP production (Mtango *et al.*, 2008), regulation of cell survival (Dumollard *et al.*, 2007). In addition, mitochondria play a vital role in signal transduction, oxygen sensing and maintenance of the cellular homeostasis especially calcium oscillations (Gutierrez *et al.*, 2006; Quintero *et al.*, 2006). Calcium oscillations are very important for fertilization, embryonic cell-cycle transition and embryonic axis establishment (Whitaker, 2008). So, melatonin by enhancing the mitochondrial healthy condition might share indirectly in all of these processes. Secondly, the improving effect of melatonin on IVF outcomes might be attributed depending upon melatonin antioxidant activity. Where, it was stated that the balance between ROS production and scavenging was an important factor for acquisition of *in vitro* fertilizing ability (De Lamirande *et al.*, 1997), as it had been observed that high level of ROS had the capability to inhibit sperm-oocyte fusion (Aitken *et al.*, 1993). Moreover, Ishizuka *et al.* (2000) concluded that melatonin significantly stimulated and supported fertilization and early mouse embryo development at concentration between 10^{-6} and 10^{-4} M through its ROS scavenging action. So, in harmony with the previous studies and according to the current results, melatonin has a powerful antioxidant capacity (Table 2 and 6). Thirdly, the augmenting effect of melatonin on IVF outcomes might be attributed to the effects of melatonin on glutathione manipulation. In this area, glutathione was considered as the major intracellular free thiol that had important biological functions during cellular proliferation, amino acid transport, protein and DNA synthesis, reduction of disulfides; as well as it protects

cells against oxidation (Gasparrini *et al.*, 2000; Luberda, 2005). In fact, several investigators reported that the reduction of sperm nuclear disulfide bonds was required to induce sperm nuclear decondensation as the first step of Male Pronuclear (MPN) formation (Perreault, 1990) and glutathione was an important factor for this reduction (Sutovsky and Schatten, 1997). Consequently, it promotes MPN formation following sperm *in vitro* penetration (Zuelke *et al.*, 2003), as shown in Table 2 and 5. Moreover, it promotes the development of preimplantation-stage of mouse embryos (Gardiner and Reed, 1994), which came in harmony with the results of the current study (Table 6). Moreover, Fernandez *et al.* (1995) and Ishizuka *et al.* (2000) noted that melatonin affect the oocytes development; sustain fertilization and early embryo development after IVF in rats and mice.

Conclusion

The present data demonstrated that the presence of melatonin in the freezing extender at concentrations of 0.1 mM improved the freezability and *in vitro* fertilizing potentials of buffalo spermatozoa. The current results also suggested that the protective effect of melatonin on the cryopreserved spermatozoa is directly related to the elevated antioxidant capacity, reduction of lipid peroxidation and protection of sperm DNA from deterioration.

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Author's Contributions

Mohamed El-Raey and M.R. Badr: Performed the chemical analysis and IVEP procedures and manuscript publishing.

Z.M. Rawash: Collected the semen sample and prepared it and performed the ordinary semen analysis procedures and semen extension.

G.M. Darwish: Achieved manuscript writing and statistics besides sharing in IVEP procedures.

Ethics

Authors should address any ethical issues that may arise after the publication of this manuscript (There is no any ethical issues that may arise after publication of this manuscript).

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