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Improving cryopreservation capacity of ram spermatozoa by supplementing the diluent with melatonin

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ABSTRACT

The aim of this investigation was to evaluate the efficiency of supplementing ram semen extender with melatonin on cryopreservation capacity of spermatozoa. A total of 80 ejaculates were collected from 5 Barki rams, 16 ejaculates each, by an artificial vagina throughout the period from January – February, 2017. Ejaculates of each collection session were pooled and diluted (1:10) with glycerolated Tris-citric egg yolk extender, and were split into 4 portions. The first portion served as control (melatonin-free), whereas the other 3 portions were supplemented with 0.1, 0.2 or 0.3 mM melatonin. The post-thaw objective assessment of cryopreserved spermatozoa, in all groups, was conducted by a computer-assisted sperm analysis (CASA) system. The results revealed that melatonin supplementation was positively correlated (P<0.01) with post-thaw total motility, progressive motility, viability, acrosome integrity, sperm cell membrane integrity, VSL, VAP and WOB (r= 0.88, 0.71, 0.78, 0.85, 0.96, 0.95, 0.68, 0.57 and 0.53, respectively. However, it was negatively correlated (P<0.01) with primary and secondary sperm abnormalities, as well as non-progressive motility and immotile spermatozoa (r= -0.73, -0.85, -0.49 and -0.76, respectively. These results elucidate that adding melatonin to ram semen extender substantially enhanced post-thaw sperm physical properties, which implies its powerful potential as an exogenous antioxidant supplement.

Keywords: Melatonin; ram; semen; cryopreservation; oxidative stress

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Introduction

Artificial insemination with cryopreserved semen has become one of the most effective methods in animal biotechnology and breeding techniques. Accordingly, semen cryopreservation became essential for the commercial application of this assisted reproductive technology (Ashrafi et al. 2013). However, during the process of semen cryostorage, particularly freezing and thawing stages, mammalian spermatozoa are exposed to stress and, thus, exhibit sever damage (Watson, 2000).

Basically, cryopreservation has been reported to associate with production of reactive oxygen species (ROS; i.e. superoxide anion radical, hydrogen peroxide), which lead to lipid peroxidation of sperm membranes and consequence accumulation of lipid hydro-peroxides (Bilodeau et al., 2002; Alvarez and Storey 2005). This, in turn, induces sperm premature activation in the female genital tract and altered membrane responses to physiological stimuli of sperm that survive the freezing-thawing process (Viswanath and Shannon 2000) and , consequently, a loss of sperm motility, viability and fertility (Sariözkan et al. 2009). Furthermore, extending and freezing-thawing of semen decrease the antioxidant capacity of semen (Sariözkan et al. 2009).

Several investigations were carried out over the past years to reduce the negative effects of ROS accumulation and lipid peroxidation on spermatozoa exposed to cryopreservation and thawing stress by supplementing the extender with different antioxidants (Donoghue & Donoghue, 1997; Bucak and Tekin, 2007; Câmara et al., 2011; Ashrafi et al. 2013).

Melatonin is an indole derivative that is secreted rhythmically from the pineal gland and is naturally found in mammalian seminal fluid (Bornman et al., 1989; Van Vuuren et al., 1992). In addition to its multiple actions on different physiological processes, melatonin and its metabolites are considered powerful antioxidants due to their ability to scavenge excessive ROS and, thus, protect the spermatozoa (Reiter and Tan, 2003; Ahn and Bae, 2004; Adriaens et al., 2006; Kang et al., 2009). Therefore, melatonin supplementation was reported to counteract the drastic effects of lipid peroxidation on cryopreserved semen parameters in different species (Sariözkan et al. 2009; El-Raey et al., 2014).

The present work was therefore conducted to evaluate the effects of various concentrations of melatonin supplementation in the freezing extender on CASA-derived sperm motility variables, viability, morphology and plasma membrane integrity in post-thawed ram semen.

Materials and methods

Animals and Management

The present investigation was carried out at the Artificial Insemination Lab., Mariout Research Station, Desert Research Center, Egypt. Five adult Barki rams aged 36 - 48 months and an average body weight of 45.0 ± 2.0 kg were used from January - April, 2017. The rams were housed in a fenced open yard throughout the period of the study and were allowed to graze daily from 0800 to 1400 hr. Thereafter, Egyptian clover hay was provided *ad libitum*, and a concentrate mixture was presented to fulfill their protein and energy requirements (NRC, 2007). Fresh water was presented once daily after returning from the pasture. All rams were clinically examined prior to conducting the investigation and were found free of disease or reproductive disorders.

Semen extender

Unless otherwise stated, all chemicals were obtained from Sigma (Sigma-Aldrich). A Tris-citric egg yolk extender was prepared for dilution of ram semen according to Kulaksiz et al. (2012) with modification. The extender comprised Tris buffer (0.25 Mol, 3.63 %), citric acid (1.99 %), glucose (0.5 %), antibiotics (0.1 % streptomycin sulphate and 100000 IU penicillin), and was further supplemented with egg-yolk (40 %). Immediately after preparation the diluent was centrifuged at 6000 rpm for 15 min, and the clarified supernatant was separated and was supplemented with glycerol (4%). The extender was prepared 24 hr prior to each collection session and was stored at 5 ^{o}C until semen dilution.

Semen collection

A total number of 140 ejaculates were collected from the rams, 28 ejaculates each, twice weekly at 0700 hr throughout the period from January - April, 2017. Collection of semen was performed using an artificial vagina according to El-Bahrawy et al. (2004). The collection tubes were modified with outer plastic water jackets to maintain semen samples at 37 ^{o}C during the collection sessions. Only ejaculates manifested normal physical properties were processed.

Experimental design

Immediately after collection, raw ejaculates were transported to the laboratory, and were further subjected to physical and morphological analysis. Semen was kept in a water bath at 37 ^{o}C throughout the assessment. Thereafter, all good quality specimens were pooled. Mean values of pooled semen physical and morphological properties, throughout the experimental period, are displayed in table (1).

Afterwards, the pooled semen was diluted (1:10) with glycerolated Tris-citric egg yolk extender, and was split into 4 portions. The first portion (melatonin-free) served as control, whereas each of the other 3 portions was supplemented with one of three levels of melatonin (N-Acetyl-5-methoxytryptamine, Sigma-Aldrich, USA; Cat. no. M5250) as follows: low (melatonin $_{LD}$, 0.1 mM), medium (mmelatonin $_{MD}$, 0.2 mM) or high (melatonin $_{HD}$, 0.3 mM). All diluted semen groups were equilibrated at 4 ^{o}C for 5 hr (T_5) and were packed in 0.5 mm French straws using a mini-tübe filling and sealing machine (Model 133, Mini-tübe, Germany). The straws were then placed in a mini-tübe biological freezer and were exposed to nitrogen vapor (-140 °C) for 10 min before being immersed in liquid nitrogen (-196 °C). The frozen straws were stored under liquid nitrogen surface until analyses.

Computer-assisted semen analysis (CASA)

The frozen straws were thawed in a programmable thawing device (mini-tube, Germany) adjusted at 38°C for 40 seconds. Immediately after thawing, each sample was evaluated for sperm physical and dynamic characteristics by a computer-assisted semen analysis (CASA) system (Mira-9000, Mira Lab, Egypt). Basically, the system was designed to follow the world health organization strict criteria of human semen (WHO, 2010). Prior to the evaluation, the system was calibrated for normal ram sperm motility and morphometric properties. A 10 µl drop of semen was placed onto the CASA's slide and covered with a coverslip. Ten random fields were assessed at 500x magnification, and a minimum of 200 sperm were evaluated for motility parameters; i.e. total motility (%), progressive motility (%), non-progressive motility (%), proportion of immotile sperm (%) and viability (%). Sperm dynamics criteria in terms of straight line velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), path velocity (VAP, µm/s), amplitude of lateral head displacement (ALH, µm), wobble movement (WOB, %), linearity (LIN, %) and straightness (STR, %) were also recorded.

Sperm viability, abnormalities and acrosome integrity were evaluated after staining fixed semen smears with Romanowski's triple-stain technique (DIFF-QUICK III, Vertex, Egypt). Smears preparation and staining procedure were conducted according to the manufacturer's instructions, and the stained smears were evaluated by a phase-contrast microscope at 500x magnification. Sperm plasma membrane integrity was assessed by the hypo-osmotic swelling (HOS) test as described by Mosaferi *et al*. (2005). At least 200 sperm were evaluated using a phase contrast microscope under high-power magnification (400x).

Statistical procedure

The Shapiro-Wilk test was used to check the normal distribution of data, and when the distribution was not normal data were logged. Mean values of pooled (raw) sperm physical and morphological characteristics and their 95% confidence intervals, throughout the period of the study, were analyzed by Student's T-test. Repeated measures analysis of variance (ANOVA) was used to determine the fixed effects of treatment, time (T_0, T_5 and $T_{post\text{-}thaw}$) and treatment by time interaction. The correlations between melatonin level and post-thaw sperm characteristics were obtained by Spearman's correlation coefficient. The data were analyzed by IBM-SPSS statistics (IBM-SPSS, 2013), and the statistical significance threshold was set at 5%. The results are expressed as means ± standard error of mean (SEM).

Results

The results of CASA-derived spermiograms showed that melatonin supplementation, regardless of the level in the extender, improved ($P<0.05$) physical properties of ram spermatozoa following freezing/thawing (Table 1). However, the highest ($P<0.05$) post-thaw sperm motility (71.7 ±1.9 %) and viability (86.2 ±0.9 %) percentages were recorded in the high melatonin level group ($Melatonin_{HD}$) compared to those of control (54.0 ±1.2 and 51.4 ±1.4 %, respectively) (Table 1). A similar trend was observed in the percent of post-thaw normal sperm. Contrariwise, the Melatonin $_{HD}$ group recorded the lowest ($P<0.05$) percentages of post-thaw primary and secondary sperm abnormalities (2.6 ±0.6 and 13.0 ±1.1 %) compared to the melatonin-free group with corresponding values 6.8 ±0.5 and 47.0 ±0.9 %, respectively (Table 1).

The results also showed that the highest ($P<0.05$) percentages of post-thaw intact acrosome and sperm cell membrane integrity were observed in the same melatonin-supplemented group (Melatonin $_{HD}$) with values 91.0 ±0.4 and 85.2 ±0.2 % compared to those of control (42.8 ±2.3 and 39.4 ±0.9 %), respectively (Table 1).

In the meantime, melatonin supplementation affected ($P<0.05$) sperm motion criteria except for both curve linear velocity (VCL) and amplitude of lateral head displacement (ALH) (Fig. 1). The highest ($P<0.05$) mean values of sperm progressive motility, sperm linearity (LIN), wobble movement (WOB), and straight-line velocity (STR) were recorded in the melatonin-supplemented specimens, regardless of level in the diluent, compared to those of control (Fig. 1). However, the lowest ($P<0.05$) mean values of post-thaw non-progressive motility and immotile spermatozoa were observed in Melatonin $_{HD}$ group compared to all other groups (Fig. 1).

Collectively, melatonin supplementation was positively correlated ($P<0.01$) with post-thaw total motility, progressive motility, viability, acrosome integrity, sperm cell membrane integrity, VSL, VAP and WOB (r= 0.88, 0.71, 0.78, 0.85, 0.96, 0.95, 0.68, 0.57 and 0.53, respectively. However, it was negatively correlated ($P<0.01$) with primary and secondary

Table 1. Physical properties of pooled ram semen (raw) throughout the period of the study (mean ±SEM).

Parameter		Parameter	
Volume (ml)	0.96±0.06	Progressive motility (%)	90.0 ±2.7
pH	7.2 ±0.1	Live sperm (%)	90.4 ±2.2
Sperm concentration (X 10^6 /ml)	2374.4 ±26.2	Normal sperm (%)	89.2 ±1.5
Mass motility score (5-0) *	4.26±0.16	Intact acrosome (%)	88.6 ±2.8

* Mass motility score: 5= highly motile 0= immotile

Table 2. Effect of different levels of melatonin supplementation on physical properties of cryopreserved ram sperm (mean ±SEM).

Parameter	Processing time (hr)	Treatment			
		Control	Melatonin $_{LD}$	Melatonin $_{MD}$	Melatonin $_{HD}$
Total Motility (%)	T_0	85.0 ±1.6 ^{A}	86.0 ±1.6 ^{A}	91.0 ±2.9 ^{A}	95.0 ±1.2 ^{A}
	T_5	65.0 ±1.1 ^{b,B}	78.0 ±1.8 ^{ab,A}	82.0 ±1.8 ^{a,A}	90.0 ±1.2 ^{a,AB}
	Post-thaw	54.0 ±1.2 ^{c,C}	63.4 ±1.7 ^{b,B}	65.5 ±0.9 ^{ab,B}	71.7 ±1.9 ^{a,B}
Live sperm (%)	T_0	82.8 ±1.6 ^{b,A}	84.2 ±2.3 ^{b,A}	89.6 ±2.6 ^{ab,A}	95.6 ±0.4 ^{a,A}
	T_5	83.0 ±1.2 ^{b,A}	81.4 ±1.8 ^{b,A}	85.8 ±1.0 ^{ab,B}	89.8 ±0.2 ^{a,B}
	Post-thaw	51.4 ±1.4 ^{c,B}	54.0 ±1.2 ^{bc,B}	80.2 ±1.7 ^{ab,C}	86.2 ±0.9 ^{a,B}
Normal sperm (%)	T_0	85.8 ±1.2 ^{A}	86.0 ±1.1 ^{A}	89.2 ±0.4 ^{A}	90.6 ±0.8 ^{A}
	T_5	81.2 ±1.5 ^{b,A}	82.4 ±1.7 ^{b,A}	85.2 ±0.9 ^{a,B}	85.8 ±0.6 ^{a,B}
	Post-thaw	47.0 ±2.5 ^{b,B}	43.4 ±1.4 ^{b,B}	79.4 ±0.8 ^{a,C}	84.4 ±0.6 ^{a,B}
Primary abnormalities (%)	T_0	2.0 ±0.4 ^{B}	2.1 ± 0.5 ^{B}	2.0 ±0.4	2.2 ±0.5
	T_5	2.6 ±0.8 ^{B}	2.6 ±0.9 ^{B}	2.4 ±0.7	2.0 ±0.4
	Post-thaw	6.8 ±0.5 ^{a,A}	6.0 ±0.6 ^{ab,A}	3.2 ±0.6 ^{bc}	2.6 ±0.6 ^{c}
Secondary abnormalities (%)	T_0	11.8 ±0.9 ^{B}	12.4 ±1.8 ^{B}	8.0 ±0.6 ^{C}	7.4 ±1.1 ^{B}
	T_5	16.2 ±0.7 ^{a,B}	15.0 ±1.7 ^{a,B}	12.4 ±0.5 ^{a,B}	11.0 ±1.2 ^{b,A}
	Post-thaw	47.0 ±0.9 ^{a,A}	49.8 ±1.9 ^{a,A}	17.4 ±0.5 ^{b,A}	13.0 ±1.1 ^{c,A}
Intact acrosome (%)	T_0	88.0 ±1.7 ^{A}	86.4 ±1.8 ^{A}	91.0 ±0.5 ^{A}	92.0 ±0.3
	T_5	79.4 ±1.2 ^{c,B}	81.6 ±1.6 ^{bc,A}	86.6 ±0.7 ^{ab,AB}	91.8 ±0.3 ^{a}
	Post-thaw	42.8 ±2.3 ^{c,C}	60.8 ±1.2 ^{b,B}	83.8 ±0.7 ^{a,B}	91.0 ±0.4 ^{a}
Intact cell membrane (%)	T_0	87.8 ±0.7 ^{A}	88.0 ±0.8 ^{A}	90.2 ±1.2 ^{A}	92.6 ±0.9 ^{A}
	T_5	79.6 ±0.2 ^{b,B}	83.8 ±0.5 ^{a,A}	84.0 ±1.0 ^{a,AB}	84.6 ±0.9 ^{a,AB}
	Post-thaw	39.4 ±0.9 ^{c,C}	56.2 ±0.9 ^{b,B}	78.0 ±1.0 ^{a,B}	85.2 ±0.6 ^{a,B}

$^{a-c}$ letters among groups in the same row differ significantly ($P < 0.05$)
$^{A-C}$ letters in the same column within each parameter differ significantly ($P < 0.05$)
T_0 = Immediately after dilution;
T_5 = After 5 hr calibration period at 5 ºC ;
Post-thaw = Immediately after thawing cryopreserved straws.

sperm abnormalities as well as non-progressive motility and immotile spermatozoa (r= -0.73, -0.85, -0.49 and -0.76, respectively.

Discussion

The present results clarified that melatonin supplementation in the diluent improved cryopreservation capacity of ram sperm in a dose depending trend, where 0.3 mM melatonin level efficiently maintained post-thaw sperm motility criteria. This was clearly evident since sperm physical properties in terms of post-thaw motility, viability, normal, intact acrosome and sperm cell integrity percentages in this group of treated semen were 1.3, 1.7, 1.8, 2.1 and 2.2 times higher than those of control, respectively. Additionally, the percentages of post-thaw primary and secondary sperm abnormalities were 2.6 and 3.6 higher in the control group than those of Melatonin $_{HD}$ group, respectively. These results are in conformity with those reported previously in different species (Sariözkan et al. 2009; El-Raey et al., 2014).

Semen processing, particularly cryopreservation, expose the spermatozoa to cold shock during freeze-thaw process, which injure the mitochondria (Pena et al., 2009) as

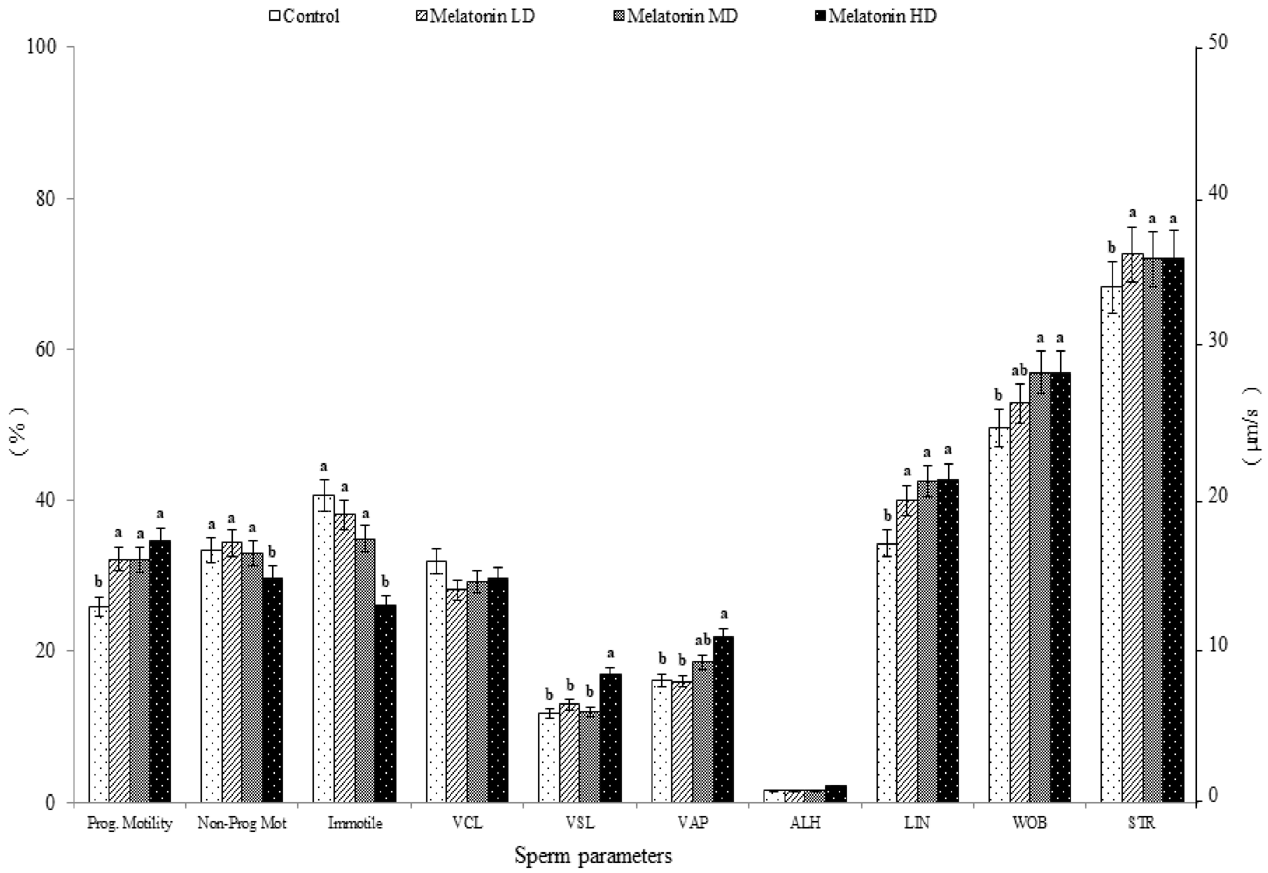

Fig. 1: Effect of different levels of melatonin supplementation on post-thaw kinematic properties of cryopreserved ram spermatozoa (mean ±SEM). Prog. Motility = Progressive motility (%) , VCL = Curvilinear velocity (µm/s), VSL = Straight-line velocity (µm/s), VAP = Average path velocity (µm/s), WOB = Wobble coefficient (%) , LIN = Linearity (%), STR = Straightness (%). [a,b] letters among groups differ significantly (P < 0.05)

well as plasma and acrosome membranes of spermatozoa (Meyers, 2005). This is mainly a consequence to exposing spermatozoa to sever oxidative stress due to generating excessive amounts of reactive oxygen species (ROS) via peroxides and free radicals. These elements and metabolites accumulate due to sperm respiration, metabolic activity and peroxidation of phospholipid sperm cell membrane (Sanocka and Kurpisz, 2004; Alvarez and Storey, 2005). The generated ROS have been reported to promote alterations in sperm membrane integrity and, hence, deprivation of sperm motility, impairment of both the acrosomal region (Bilodeau et al., 2002) and sperm cell membrane (Câmara et al., 2011).

As previously described, melatonin has a powerful antioxidant capacity due to its ability as a scavenger of ROS (Reiter and Tan, 2003; Ahn and Bae, 2004; Adriaens et al., 2006; Kang et al., 2009). Furthermore, via modulating the glutathione activity, melatonin has been reported to improve sperm mitochondrial health state and functions and, thus, improve IVF outcomes (El-Raey et al., 2014).

Conclusion

In conclusion, supplementation the diluent with 0.3 mM melatonin in the freezing medium has beneficial effects on post-thaw motility, viability, normal morphology and plasma membrane integrity of ram spermatozoa. Further studies on the determination of oxidative stress, antioxidative capacity and fertility of cryopreserved ram semen after supplementing the diluent with either enzymatic or nonenzymatic antioxidants to achieve better results in terms of motility, membrane integrity and fertility potential.

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Conflict of interest

All author declares that she has no conflict of interest.

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