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Effect of supplementing ram semen extender with melatonin on oxidative stress indices and physical properties of chilled spermatozoa

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ABSTRACT

The current study aimed to evaluate the efficiency of supplementing ram semen extender with melatonin on chilled storage capacity of spermatozoa. Eighty ejaculates were collected from 5 Barki rams, 16 ejaculates each, using an artificial vagina throughout the period from January – February, 2017. The ejaculates of each collection session were pooled and diluted (1:10) with Tris-citric egg yolk extender, and were split into 4 aliquots. 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. Thereafter, the samples were stored at 4 oC for 48 hrs, during which sperm physical and morphological properties were evaluated at 24 hr interval. Simultaneously, oxidative stress indices in terms of total antioxidant capacity (TAC), malondialdehyde concentration (MDA), resazurin reduction test, in addition to enzymatic activities of AST, ALT and ALP were determined. The results revealed that, over the 48 hr preservation period, level of melatonin in the diluent was positively correlated (P<0.01) with sperm motility, viability, percent of normal sperm, intact acrosome, sperm cell membrane integrity and total antioxidant capacity (r= 0.75, 0.96, 0.82, 0.95, 0.96 and 0.74, respectively). Contrarily, a negative correlation (P<0.01) was observed between melatonin level and each of primary and secondary sperm abnormalities, MDA, AST, ALT and ALP over time of storage (r= - 0.71, - 0.85, - 0.46, - 0.71, - 0.95 and - 0.84, respectively). These results elucidate that supplementing the diluent with 0.3 mM melatonin efficiently reduced oxidative stress and improved chilled storage of ram spermatozoa.
Keywords: Melatonin; ram; semen; chilled preservation; oxidative stress

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1. Introduction

Among different domestic animals, sheep possess a remarkable stature for their multi-productive potential of meat, milk, lamb and wool under harsh environmental conditions. The utilization of assisted reproductive technologies (ARTs) substantially contributed in developing genetically-improved sheep breeds with high productivity, resistant against diseases, and having high capacity to cope with environmental conditions (Baldassarre and Karatzas, 2004).

Artificial insemination (AI), either with fresh or cooled diluted semen, was reported to increase the rate of genetic progress in sheep breeding (O'Hara et al., 2010). It is preferable using ram semen within a short time after collection to perform AI rather than using cryopreserved semen, which exhibit poor quality and fertilization capacity due to freezing/thawing induced sperm injuries (Maxwell and Watson, 1996; King et al., 2004). However, liquid chilled storage of semen has been reported to deleteriously affect both sperm motility and morphology and, thus, sperm fertilizing capacity over chilling-preservation time (Alam et al., 2005; Munsi et al., 2007; Kasimanickam et al., 2007). Therefore, maintenance of the fertilization potential of ram sperm during chilled storage has become in focus of recent research (Jha et al., 2013).

Chilled storage of semen has been reported to expose spermatozoa to the drastic effects of oxidative stress and consequential loss of motility and increased sperm damage in different species (Foote et al., 2002; Agarwal et al., 2003). Oxidative stress during cooled preservation of semen has been reported to occur due to spontaneous lipid peroxidation of polyunsaturated fatty acids comprising sperm cell membrane, as well as accumulation of free radicals in the storage medium throughout the period of storage. At optimum physiological concentrations, free radicals play important roles as mediators of normal sperm function (de Lamirande and Gagnon, 1993; Fialkow et al., 1994), in addition to regulating fertilization, acrosome reaction, hyperactivation, motility and capacitation (Omu et al. 1998; WHO, 1999). However, excessive levels of ROS damage spermatozoa's biomolecules; i.e. lipids, proteins and DNA, and promotes alterations in sperm membrane fluidity and, hence, alteration in sperm motility parameters (Sikka, 1996; Khinde et al. 2003).

Ruminant semen normally contains antioxidants that can offset lipid peroxidation (Griveau et al., 1995; Kantola et al., 1998). However, this endogenous antioxidative capacity of semen may be insufficient to prevent lipid peroxidation during prolonged chilled storage (Brezezinska-Slevbodzinska et al., 1995; Aurich et al., 1997). Besides, dilution of semen lowers antioxidant capacity of seminal plasma even further (Cerolini et al., 2000). Therefore, the concept of improving fertilization potential of spermatozoa exposed to such oxidative stress, using antioxidants supplementation, has gained considerable attention in assisted reproductive technology practices (Paulenz et al. 2002; Michael et al., 2007; Maia et al. 2009, 2010].

Previous reports indicated that supplementing semen extenders with different antioxidants efficiently counteracted the drastic impact of generated oxidants, which were produced during liquid storage of sperm in small ruminants (Bucak et al., 2008; Bucak et al., 2010; Câmara et al., 2011; Zeitoun and Al-Damegh, 2015). In the present investigation, we evaluated the capacity of supplementing ram semen diluent with melatonin on neutralizing ROS-induced oxidative stress and, hence, enhancing sperm physical properties during chilled storage of spermatozoa.

2. Materials and methods

2.1. 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 - February, 2017. Throughout the period of the study, the rams were housed in a fenced open yard and were allowed to graze daily from 0800 to 1400 hr. Thereafter, a concentrate mixture was presented to fulfill their protein and energy requirements (NRC, 2007) while Egyptian clover, *Trifolium alexandrinum*, hay was provided *ad libitum*. Fresh water was presented once daily after returning from the pasture. Prior to executing the experiment, all rams were clinically examined and were found free of disease or reproductive disorders. All experimental pro-

cedures were conducted in conformity with the EU Directive for protection of experimental animals (2010/63/EU).

2.2. 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 by slightly modifying the extender reported by Kulaksiz et al. (2012). 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. The extender was prepared 24 hr prior to each collection session and was stored at 4 ^{o}C until use.

2.3. Semen collection

A total number of 80 ejaculates were collected from the rams, 16 ejaculates each, throughout the period of study (January - February, 2017). Collection of semen was performed twice weekly at 0700 hr using an artificial vagina according to the method of El-Bahrawy et al. (2004). Collection tubes, with modified plastic water jackets, were used to maintain semen samples at 37 ^{o}C during the collection sessions. Ejaculates manifested contamination with urine, strange substances, strange color or odors were discarded.

2.4. Experimental design

Immediately after collection, raw semen samples were transported to the laboratory, and were further subjected to physical and morphological analysis. Semen was kept in a water bath adjusted at 37 ^{o}C throughout the assessment. Upon examination, only normospermic ejaculates were processed, whereas oligospermic or teratospermic specimens were ditched. 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).

The pooled semen was diluted (1:10) with Tris-citric egg yolk extender, and was split into 4 aliquots using a split-sample technique. The first aliquot served as control (melatonin-free), whereas each of the other 3 aliquots 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 (melatonin $_{MD}$, 0.2 mM) or high (melatonin $_{HD}$, 0.3 mM). Afterwards, all diluted semen groups were stored at 4 ^{o}C for 48 hr, during which sperm physical and morphological characteristics were assessed immediately after dilution (T_0) and at 24 hr interval (T_{24} and T_{48}).

2.5. Semen assessment

Sperm progressive motility was estimated in all diluted samples using a phase-contrast microscope (Leica) at 400 X magnification, where an average of 5 random fields was obtained to the nearest 5%. Sperm vitality (live and dead sperm, %) were examined using the differential staining technique, where a mixture of 10 μl of semen and 5 μl of freshly-prepared eosin-nigrosin stain was smeared on a warm stage, and were examined under high power magnification (1000×). Sperm abnormalities and acrosome integrity were evaluated using Romanowski's triple-stain technique (DIFF-QUICK III, Vertex, Egypt). Smears preparation and staining procedure were conducted following instructions provided by the manufacturer, and the stained smears were evaluated using a phase-contrast microscope at 1000x magnification. Sperm plasma membrane integrity was determined by the hypo-osmotic swelling test (HOST) as described by Mosaferi et al. (2005), where at least 200 sperm were evaluated at 400 X magnification.

2.6. Determination of seminal plasma oxidative stress indices and enzymatic activities

A portion of each semen group (2 ml) was obtained and centrifuged (2500 rpm for 10 min) at times parallel to those of sperm physical and morphological assessment (T_0, T_{24} and T_{48}). The supernatant was aspirated and stored at -20 ^{o}C until oxidative stress indices and enzymatic activities were analyzed.

Total antioxidant capacity (TAC) and malondialdehyde acetate (MDA) concentration were analyzed using colorimetric kits (Biodiagnostic, Egypt). Likewise, the reduction of the resazurin dye test was performed using colorimetric kits (Biodiagnostic, Egypt). The changes in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) concentrations were analyzed by colorimetric kits (Spectrum, Egypt), whereas alkaline phosphatase (ALP) activity was determined calorimetrically using kits obtained from

Biodiagnostic, Egypt. All procedures were conducted according to the manufacturers' instructions.

2.7. Statistical analysis

The normal distribution of data was checked using the Shapiro-Wilk test, and when the distribution was not normal data were arcsined to improve the approximation of normality. Mean values of pooled (raw) sperm physical and morphological characteristics, throughout the period of the study, were obtained by simple student's t-test. The changes in the same sperm criteria, as well as oxidative stress indices and enzymatic activities, were determined by repeated measures analysis of variance (ANOVA) to determine the fixed effects of treatment, time (T_0, T_{24} and T_{48}) and treatment by time interaction. The differences between means were detected using Tukey's post-hoc test. The correlations between time of preservation or melatonin level and both sperm characteristics and oxidative stress indices were obtained by Pearson's correlation coefficient. The statistical significance threshold was set at 5% and data were analyzed using IBM-SPSS statistics program (IBM-SPSS 2013). The data are expressed as means ± standard error (SEM).

3. Results

The results showed that time of preservation (T_0-T_{48}) affected (P<0.05) the percent of sperm motility in all semen groups. However, at 48 hr of chilled preservation at 4°C, specimens supplemented either with high or medium melatonin level recorded sperm motility percent higher (P<0.05) than that of control with values 79.0 ±3.7, 72.0 ±4.8 and 49.0 ±4.5 %, respectively (Table 1). Similarly, mean values of sperm viability, normal sperm percent, primary and secondary abnormalities in control and low melatonin level groups were adversely affected (P<0.05) by time of preservation, whereas the aliquots supplemented either with medium or high melatonin level were not affected (Table 1). The high melatonin level group recorded higher (P<0.05) live sperm percent (86.2 ±0.2 %) at T_{48} compared to that of control (61.4 ±0.6 %). However, the group supplemented with high melatonin level efficiently (P<0.05) maintained both intact acrosome percent and sperm cell membrane integrity over the 48 hr preservation period compared to those of control (Table 1). The highest (P<0.05) percent
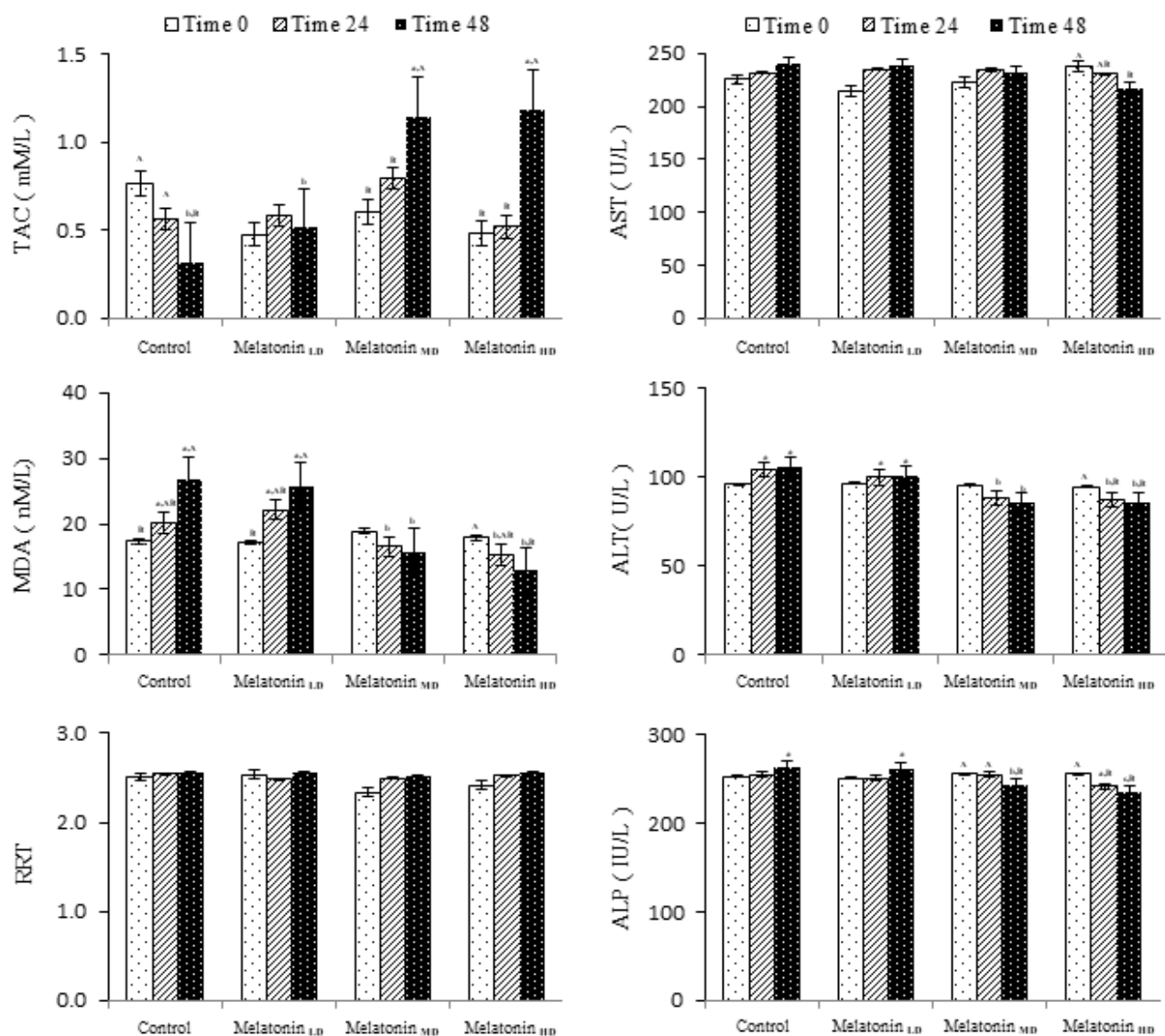
of intact acrosome and sperm cell membrane integrity, at 48 hr of cooled storage, were recorded in the high melatonin level group (82.6 ±0.6 and 78.6 ±0.6 %) compared to those of control with corresponding values 33.2 ±0.7 % and 27.0 ±2.0 % for both criteria, respectively (Table 1).

On the other side, mean values of total antioxidant capacity (TAC, mM/L) in the medium were affected (P<0.05) by time of preservation in all groups (Fig. 1). In this respect, the control group recorded the highest (P<0.05) values at T_0 whereas the lowest were observed at T_{48}. Contrariwise, specimens supplemented with either medium or high melatonin levels exhibited a revers trend. The highest (P<0.05) mean values of TAC at 48 hr of chilled preservation were recorded in high melatonin level group (1.2 ±0.3 mM/L) compared to that of control (0.3 ±0.04 mM/L) (Fig. 1). On the other hand, mean values of malondialdehyde (MDA, nM/L) exhibited a trend opposite to that observed for TAC, in all groups, over the time of preservation. At T_{48}, the high melatonin level group recorded the lowest (P<0.05) MDA concentration (12.9 ±2.3 nM/L), while the highest (P<0.05) concentration was observed in the melatonin-free group (26.7 ±4.8 nM/L) (Fig. 1). No significant difference was observed in the Resazurin reduction test, in all groups, over the time of storage. However, enzymatic activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were drastically affected (P<0.05) by time of preservation (Fig. 1). The highest (P<0.05) mean values of the aforementioned criteria were recorded in the control group, whereas the lowest were noted in both medium and high melatonin level specimens at 48 hr of chilled storage.

Collectively, over the 48 hr preservation period, level of melatonin in the diluent was positively correlated (P<0.01) with sperm motility, viability, percent of normal sperm, intact acrosome, sperm cell membrane integrity and total antioxidant capacity (r= 0.75, 0.96, 0.82, 0.95, 0.96 and 0.74, respectively). Contrarily, melatonin level was negatively correlated (P<0.01) with primary and secondary sperm abnormalities, MDA, AST, ALT and ALP over time of storage (r= - 0.71, - 0.85, - 0.46, - 0.71, - 0.95 and - 0.84, respectively).

4. Discussion

Semen processing, particularly chilled and cryo-storage of spermatozoa, expose spermato-

a-c letters among groups differ significantly (P < 0.05)
A,B letters within each treatment differ significantly (P < 0.05)

TAC= Total antioxidant capacity; MDA= Malondialdehyde; RRT= Resazurin reduction test; AST= Aspartate

Aminotransferase; ALT= Alanine Aminotransferase; ALP= Alkaline phosphatase.

Fig. 1: Effect of different levels of melatonin supplementation on ram seminal plasma oxidative stress indices and enzymatic activities during 48 hr of chilled preservation at 4°C.

Table 1. Mean values of 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 ram semen during 48 hr of chilled preservation at 4 °C (mean ±SEM).

Parameter	Preservation time (hr)	Control	Treatment		
			Melatonin LD	Melatonin MD	Melatonin HD
Progressive Motility (%)	0	84.0 ±3.3 b,A	85.0 ±1.6 b,A	88.0 ±1.2 ab,A	94.0 ±1.0 a,A
	24	70.0 ±1.6 b,A	76.0 ±2.9 ab,A	78.0 ±1.2 a, AB	81.0 ±2.4 a,AB
	48	49.0 ±4.5 b,B	55.0 ±6.7 b,B	72.0 ±4.8 a,B	79.0 ±3.7 a,B
Live sperm (%)	0	91.4 ±0.7 A	91.4 ±0.7 A	93.2 ±1.2	95.6 ±0.4
	24	72.6 ±1.1 b,B	74.4 ±2.3 b,B	85.4 ±0.4 a	86.8 ±0.4 a
	48	61.4 ±0.6 c,C	72.0 ±0.8 b,B	84.6 ±0.2 a	86.2 ±0.2 a
Normal sperm (%)	0	84.8 ±0.5 A	85.8 ±1.2 A	88.4 ±0.2	87.6 ±0.8
	24	70.8 ±0.4 c,B	72.8 ±0.9 c,B	81.8 ±0.9 b	85.8 ±0.4 a
	48	66.8 ±0.7 b,C	67.0 ±1.0 b,C	80.0 ±3.1 a	81.2 ±0.7 a
Primary abnormalities (%)	0	2.2 ± 0.4 B	2.0 ±0.4 B	2.4 ±0.2	2.3 ±0.5
	24	2.4 ±0.4 B	2.8 ±0.7 B	2.6 ±0.2	2.4 ±0.2
	48	7.0 ±0.8 a,A	7.4 ±0.2 a,A	4.0 ±0.2 b	3.0 ±0.2 b
Secondary abnormalities (%)	0	13.0 ±0.5 B	11.8 ±0.9 B	9.2 ± 1.0 B	11.0 ±1.0
	24	26.8 ±0.5 a,A	24.8 ±0.3 a,A	17.0 ±0.4 b,A	10.0 ±0.5 c
	48	26.2 ±0.8 a,A	23.6 ±0.9 a,A	16.0 ±0.8 b,A	9.0 ±0.8 c
Intact acrosome (%)	0	86.8 ±1.3 b,A	90.4 ±0.7 a,A	91.0 ±0.5 a,A	92.0 ±1.3 a
	24	67.0 ±1.2 b,B	65.4 ±0.9 b,B	82.0 ±2.0 a,B	86.2 ±0.9 a
	48	33.2 ±0.7 c, C	43.2 ±3.1 b,C	75.4 ±2.2 a,C	82.6 ±0.6 a
Intact cell membrane (%)	0	88.6 ±0.4 b,A	87.8 ±0.7 ab,A	90.2 ±2.2 ab,A	92.6 ±0.9 a
	24	57.4 ±1.8 d,B	63.6 ±1.1 c,B	72.4 ±1.1 b,B	87.4 ±0.9 a
	48	27.0 ±2.0 d,C	30.8 ±0.5 c,C	60.4 ±1.1 b,C	78.6 ±0.6 a

a-d 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)

zoa to sever oxidative stress due to generating excessive amounts of reactive oxygen species (ROS) via peroxides and free radicals which accumulate from sperm respiration, metabolic activity and peroxidation of phospholipid sperm cell membrane (Sanocka and Kurpisz, 2004; Alvarez and Storey, 2005). These ROS promotes alterations in sperm membrane integrity and, hence, alteration in sperm motility parameters; i.e. deprivation of motility, impairment of both the acrosomal region (Bilodeau et al., 2002) and sperm cell membrane (Câmara et al., 2011).

In the present results, it is elucidated that melatonin supplementation in the diluent enhanced the chilled preservation capacity of ram sperm in a dose depending trend, where 0.3 mM melatonin level efficiently maintained sperm motility criteria over 48 hr of cooled storage. This was clearly evident since sperm physical properties in terms of motility, viability, normal, intact acrosome and sperm cell integrity percentages in this group of treated semen were 1.6, 1.4, 1.2, 2.5 and 3.0 times higher than those of control at 48 hr of chilled preservation, respectively. Moreover, the increased concentration of total antioxidant capacity along with decreased levels of lipid peroxidation (presented by the level of MDA), as well as enzymatic activities of ALT, AST and ALP in the 0.3 mM melatonin-supplemented specimens confirm and explain the sustained physical properties of spermatozoa throughout the 48 hr period of chilled storage.

Counteracting the drastic effects of lipid peroxidation on cryopreserved semen parameters by means of melatonin supplementation has been previously reported in buffaloes (El-Raey et al., 2014). Melatonin, an indole derivative secreted rhythmically from the pineal gland, plays a major role in regulating the reproductive functions in mammals (Reiter et al. 1998). Naturally, seminal fluid contains melatonin and the spermatozoa reportedly possess membrane melatonin receptors (Bornman et al., 1989; Van Vuuren et al., 1992) namely MT1, MT2 and MT3 (Reppert et al., 1994; 1995). 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 raised by their metabolism (Reiter and Tan, 2003; Ahn and Bae, 2004; Adriaens et al., 2006; Kang et al., 2009). Moreover, melatonin via modulating the gluta-

thione activity has the potency to improve mitochondrial health state and functions and, thus, improve IVF outcomes (El-Raey et al., 2014).

Malondialdehyde (MDA) is a byproduct of lipid peroxidation, and is commonly analyzed to measure peroxidative damage in spermatozoa (Makker et al., 2009). Further, the enzyme levels of seminal plasma are very important for both sperm metabolism and function (Brooks, 1990). Therefore, they are considered as markers for semen quality since they indicate sperm damage (Pesch et al., 2006). The transaminase activities (AST-ALT) in semen are good indicators of semen quality because they measure sperm membrane stability (Corteel, 1980). Thus, increasing the percentage of abnormal spermatozoa in ejaculate causes high concentration of transaminase enzyme in the extra cellular fluid due to sperm membrane damage and ease of leakage of enzymes from spermatozoa (Gundogan et al., 2010). Likewise, a high positive correlation has been reported between ALP activity released by spermatozoa and semen quality when sperm cells were subjected to sever stress (Ciereszko et al., 1992). These results are is consistent with our findings over the 48 hr of cooled preservation.

5. Conclusion

The present results showed that supplementing ram semen extender with 0.3mM melatonin significantly protected ram sperm from cold storage-induced negative effects on sperm functionality. This was evident from the maintained sperm motility, viability and cell membrane integrity over the chilled storage period compared to the control. Further studies are needed to address the impact of melatonin supplementation on the cryopreservation potential of ram sperm.

6. Acknowledgment

7. Competing interests

The author declares that she has no competing or conflict of interest with respect to the research, authorship, and/or publication of this article.

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