Shielding Effect of Melatonin Improves Seminal Quality and Oxidative Stress Indices During Chilled Storage of Ram Semen

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Research Article

Keywords: Magra Ram, Semen liquid storage, Melatonin, oxidative stress, Sperm quality, vaccination

Posted Date: November 10th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-985285/v1>

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Abstract

Supplementation of antioxidant to semen extender maintains seminal quality by reducing oxidative stress during preservation time period. The main aim of this study was to investigate the effect of different concentration of melatonin supplementation on liquid storage of Magra ram semen. This study was performed on adult Magra ram (n=8) and seminal ejaculates (48) were collected and evaluated for various macroscopic and microscopic seminal quality parameters for further processing. After preliminary evaluation, ejaculates of each collection session were mixed and divided into four equal aliquots. All the aliquots were diluted (1:10) with Tris-citric fructose egg yolk extender contained sans melatonin served as control whereas the other three aliquots were supplemented with 0.5, 1 and 2 mM MLT were grouped as MLT0.5, MLT1 and MLT2, respectively. Thereafter, the samples were stored at 4°C for 72 h and various seminal parameters (individual sperm progressive motility, viability, abnormalities, plasma membrane functionality) along with oxidative stress parameters (total antioxidant capacity (TAC), malondialdehyde (MDA) were evaluated at 0, 24, 48 and 72 h of preservation. The results indicated that the mean percent values for progressive sperm motility, viability, plasma membrane functionality and TAC were significantly higher ($p < 0.05$) in treated groups with the highest values in MLT1 group. Significantly lower ($p < 0.05$) percentage of total sperm abnormalities and MDA level were observed in treatment groups compared to control group. The results report that supplementation of 1mM melatonin efficiently maintain the seminal quality and ameliorate oxidative stress during preservation up at 4°C to 72h.

1. Introduction

Artificial insemination (AI) is considered as one of the most pragmatic approach to overcome, hurdles associated with low productive potential and scarcity of elite germplasm of sheep (Redden and Thorne, 2020). The major problem is that, ovine semen collapse fast at ambient temperature, making transportation almost impossible in the remote areas which limit application of AI in field conditions. Hence, it is need of the day to extend semen storage life without reducing fertility potential to facilitate the transport and widespread use of AI technique in areas located far from the semen production centres.

Long term storage of ram semen is still a researchable issue as ram semen stored at ultralow temperature presents more damaged sperms and highly reduced ability to fertilize ova compared to liquid storage. Therefore, it is still advised to use liquid chilled semen within 24 h post collection for the AI to achieve better lambing rates (O'Hara et al., 2010). Storage of the semen at refrigerator temperature for longer times would assist in rectifying in reproductive management of large flocks in field conditions. This scenario imposes researchers to work on storage of semen at 4°C as inexpensive and valid alternative of cryopreserved semen. Compared to other mammalian species, ram semen is more sensitive to cooling process and produce more reactive oxygen species (ROS) and lipid peroxidation (LPO) causing compromised sperm fertility (Coyan et al., 2012; Shafiei et al., 2015). This situation paved the way for inclusion of antioxidant in semen extender to increase the storage life span of sperm by combating the excess ROS and LPO production during liquid semen storage (Santiani et al., 2014a).

Melatonin (MLT, N-acetyl-5-methoxytryptamine), is a potent non-enzymatic antioxidant synthesized and secreted in the pineal gland of all mammalian species (Reiter and Fraschini, 1969; Jang et al., 2010). In recent years, the antioxidant ability of MLT and its metabolic derivatives has been uncovered which act synergistically with other classic antioxidants to protect organisms from oxidative stresses (Reiter et al. 2014; Pang et al., 2016). Recent studies have shown that MLT in semen extender could protect seminal quality by preventing oxidative stress damage and augmenting antioxidant activity (Ashrafi et al., 2013; Khalifa et al., 2017). The information on the effect of addition of MLT on seminal quality and oxidative stress indices during chilled liquid storage of the ram semen is little explored. Considering the importance of sheep husbandry in Indian rural economy, hurdle faced by the farmers and urgent need of elite germplasm for sheep improvement breeding programmes, the present study was undertaken to assess the effect of this antioxidant on Magra ram seminal quality and biomarkers of oxidative stress during chilled storage at 4°C up to 72h.

2. Materials And Methods

The experimental procedures related to animals were executed following the standard guidelines issued by the Institute Animal Ethics Committee (IAEC). All the chemicals used in this study, were purchased from Sigma Aldrich (St. Louis, MO, USA), unless specified.

2.1 Experimental animals

The present study was conducted at the Artificial Insemination Lab., Indian Council of Agricultural Research-Central Sheep & Wool Research Institute, Arid Region Campus, Bikaner, Rajasthan (India) during July-September of the year 2019. During the research period, the average temperature, percent humidity and thermal humidity index (THI) were 32.44°C, 55.16 and 30.17, respectively. Eight breeding Magra rams, with an average age of 1.5-3.0 years and body weight of 38±5 kg, trained to donate semen by artificial vagina technique, were selected for the study. Throughout the study period, the rams were housed iso-managerial in a shed having fenced open yard and allowed to graze on the improved pasture of Sewan grass (*Lasiurus scindicus*) for at least 7 h per day. Then the animals were fed of a concentrate mixture in evening to fulfil the protein, energy and other nutrients requirements. Fresh water was offered twice in a day (ad libitum) after returning from the pasture. Before commencement of the study, all animals were clinically inspected and were found free of any kind of disease. The general health management practices for deworming, vaccination etc were followed as per health calendar of the research institute.

2.2 Semen extender preparation

The semen extender was prepared on each day of semen collection as per Evans and Maxwell (1987). Stock solution of melatonin was composed by solvating melatonin powder in dimethyl sulfoxide (DMSO) (Succu et al., 2011) and supplemented in the extender as per the following described protocols.

2.3 Semen collection and processing

Each day of semen collection, pre-stimulation in terms of two false mountings was adopted to get optimum quantity and quality of semen production. A total of six ejaculates from each ram were collected, twice a week. Soon after collection, collection cups were docketed and transferred to water bath at 37°C and evaluated for acceptability of semen in terms of concentration $>2.5 \times 10^9$ spermatozoa/ml; mass motility $>3+$; individual motility $>70\%$, and total abnormality $< 10\%$ (Gil et al., 2003). Acceptable ejaculates of each day were mixed to eliminate individual variation and was treated as a single ejaculate. This pooled ejaculate was further divided into four equal parts using split sample technique. These aliquots were step wise extended with pre-warmed (37°C) extender to reach a final concentration of approximately 150×10^6 sperm/ml. The aliquot extended sans melatonin served as control and remaining 3 aliquots were added stock solution of MLT to make final concentration of 0.5, 1 and 2 mM melatonin and grouped as MLT0.5, MLT1 and MLT2, respectively. Diluted semen aliquots were kept in a glass tube stand and chilled from 37°C to 4°C at a rate of 0.2-0.3 °C/min and stored at 4°C up to 72 h. The time when the temperature of aliquots reached at 4°C was considered as 0 h.

2.4 Semen quality evaluation

All the chilled semen aliquots (in duplicate) were thawed at 37°C for 5 min in water bath and evaluated at 24 h intervals during the preservation time up to 72 h for following parameters.

2.4.1 Sperm physical parameters

Sperm physical parameters were assessed in terms of sperm motility, viability and abnormality. Sperm motility was assessed subjectively in five random microscopic fields by placing each sample on a pre-warmed glass slide, covered with pre-warmed cover slip (37°C) under microscope at 40X magnification (Dewinter Binocular Microscope, Italy). The mean of these different microscopic fields was recorded in terms of percentage progressive motile sperms (0-100) (Gil et al., 2003). Sperm viability (live sperm percentage) and abnormalities were examined using differential staining technique. The final composition of the stain was: eosin 1 g, nigrosin 5 g and 2.94% sodium citrate dihydrate solution dissolved in 100 ml of distilled water. Sperm suspension smear was prepared by gentle mixing of small drop (30 µl) of semen with same amount of eosin-nigrosin stain and spreading the mixture with another pre-warmed slide followed by air drying. The sperm viability and abnormality were determined by counting 400 spermatozoa in different microscopic fields at 40X and 100X, respectively. Sperm displaying partial or complete purple staining represented dead sperm while live had complete exclusion of stain. The morphological deformity observed in structures of sperm like head, body or tail was considered as abnormality (Evans and Maxwell, 1987).

2.4.2 Sperm functional parameter

Hypo osmotic swelling test (HOST) was carried out to assess the sperm functionality by evaluating sperm plasma membrane integrity. The test was assayed by mixing 0.1 ml of semen with 1.0 ml of a 150 mOsm/l hypo osmotic solution (1.351 g fructose + 0.735 g sodium citrate dehydrate/100ml of distilled water) followed by incubation at 37°C for 60 min in water bath. To obtain better visibility, 0.2 ml eosin

solution (0.5 % w/v in sodium citrate 2.9 % + 10 % formalin) was added to the test tube after incubation and 0.2ml mixture placed on slide and mounted with cover slip. A total of 200 spermatozoa were evaluated in five different fields at 40X magnification and spermatozoa with swollen and curled tails were counted as HOS responsive (Mosaferi et al., 2005).

2.4.3 Oxidative stress assay

For determination of oxidative stress, 2 ml of each aliquot at times parallel to semen quality evaluation was centrifuged at 150g for 10 min at 4°C (Eppendorf, 5430R, Hamburg, Germany). The supernatant was aspirated and stored at -20°C until used. Malondialdehyde (MDA; byproduct of LPO) and total antioxidant capacity (TAC) were assayed in duplicate to determine oxidative stress in seminal plasma using ELISA Kits (SinoGeneClon Biotech Co., Ltd. China) according to the manufacturer's instructions.

3. Statistical Analysis

Variables were first checked by Shapiro-Wilk test for adherence to the assumptions of normal distribution for metric variables. One-way ANOVA was used to compare the mean differences among the groups for sperm motility, plasma membrane integrity, sperm viability, sperm abnormality, TAC and MDA levels. To compare each treatment mean, Duncan's multiple range test served as *post-hoc* test and was used for analysis of data. The results are expressed as Mean±SE. The data analysis was done with SPSS software (IBM® SPSS® statistics, Version 25.0).

4. Results

The effect of supplementing MLT in semen extender was found to be significant on seminal parameters and oxidative stress indices up to 72h of storage of liquid semen at 4°C which are tabulated under Table 1 and Fig. 1, 2. The results of the same were analyzed in the following sections:

Table 1

Effect of different concentration of melatonin on semen quality parameters (Mean $\pm$ SE) during cooled storage of Magra ram semen

Parameters	Preservation Time (h)	Treatment (Melatonin)			
		Control	MLT0.5	MLT1	MLT2
Progressive sperm motility	0	70.50 ^a $\pm$ 0.67	72.00 ^{ab} $\pm$ 0.82	74.67 ^c $\pm$ 0.88	73.84 ^{bc} $\pm$ 0.60
	24	57.50 ^a $\pm$ 1.12	61.50 ^b $\pm$ 0.76	69.17 ^d $\pm$ 0.48	64.67 ^c $\pm$ 0.92
	48	47.84 ^a $\pm$ 1.12	51.50 ^{ab} $\pm$ 0.76	59.00 ^c $\pm$ 0.48	53.84 ^b $\pm$ 0.92
	72	36.34 ^a $\pm$ 1.09	39.67 ^b $\pm$ 1.15	48.34 ^d $\pm$ 0.69	43.67 ^c $\pm$ 0.66
HOS reactive spermatozoa	0	63.50 $\pm$ 1.38	64.00 $\pm$ 0.77	65.66 $\pm$ 1.15	63.33 $\pm$ 0.49
	24	49.67 ^a $\pm$ 0.80	52.67 ^b $\pm$ 0.71	59.17 ^c $\pm$ 1.19	54.34 ^b $\pm$ 0.67
	48	40.84 ^a $\pm$ 0.60	43.67 ^b $\pm$ 1.31	49.84 ^c $\pm$ 0.79	45.17 ^b $\pm$ 0.87
	72	31.67 ^a $\pm$ 1.17	33.34 ^{ab} $\pm$ 0.95	39.50 ^c $\pm$ 1.57	35.67 ^b $\pm$ 1.33
Viability of spermatozoa	0	71.16 $\pm$ 0.85	73.50 $\pm$ 0.76	75.00 $\pm$ 0.58	74.16 $\pm$ 0.60
	24	59.84 ^a $\pm$ 1.08	63.50 ^b $\pm$ 0.56	69.34 ^d $\pm$ 0.71	66.67 ^c $\pm$ 1.12
	48	48.84 ^a $\pm$ 0.91	52.00 ^{ab} $\pm$ 0.89	60.50 ^c $\pm$ 2.43	55.50 ^b $\pm$ 0.76
	72	42.50 ^a $\pm$ 1.18	46.34 ^b $\pm$ 1.12	52.67 ^c $\pm$ 1.26	48.84 ^b $\pm$ 0.87
Sperm abnormalities	0	6.67 $\pm$ 0.33	6.00 $\pm$ 0.37	5.67 $\pm$ 0.33	6.33 $\pm$ 0.33
	24	7.50 ^c $\pm$ 0.43	7.17 ^b $\pm$ 0.31	5.83 ^a $\pm$ 0.31	6.67 ^a $\pm$ 0.33
	48	9.67 ^c $\pm$ 0.33	9.00 ^b $\pm$ 0.37	7.67 ^a $\pm$ 0.33	8.33 ^a $\pm$ 0.33
	72	13.00 ^c $\pm$ 0.37	10.83 ^b $\pm$ 0.60	9.17 ^a $\pm$ 0.31	10.00 ^a $\pm$ 0.37
Within rows means with different superscripts differ significantly (P<0.05).					

4.1 Effect of supplementing MLT on sperm motility, viability and abnormality

Sperm motility, viability and abnormality are considered as major vital parameters for defining quality semen. Melatonin supplemented groups exhibited significantly ($p<0.05$) higher sperm motility as compared to control group at each time point of the study (Table 1). The study revealed that supplementation of MLT in extender could maintain individual sperm motility up to 72h post storage period and showed most profound effect at the level of 1mM MLT concentration ($p<0.05$, Table 1). The

sperm viability and abnormality was comparable ($p>0.05$) among all the groups at 0 h but it exerted significant ($p<0.05$) positive effect on both the attributes 24h onwards. The most significant effect of MLT was observed at 1mM MLT concentration (significantly higher sperm viability and lower sperm abnormality percentage) ($p<0.05$, Table 1).

4.2 Functional attributes of spermatozoa

Effect of supplementing MLT on functional attributes was assessed by observing plasma membrane integrity of spermatozoa. The plasma membrane integrity was assayed by HOST. The percentage of osmolarity responsive spermatozoa did not differ significantly ($p>0.05$) among all the groups at 0h of incubation period, however, it was significantly improved by inclusion of MLT in extender 24h onwards compared to control group with the highest values in MLT1 group ($p<0.05$, Table 1).

4.3 Oxidative stress indices

The storage potential of MLT supplemented semen extender was assessed as the level of TAC and MDA in the seminal plasma up to 72h of storage period. The TAC level was significantly ($p<0.05$) higher in MLT treated group compared to control groups during storage period (Fig. 1). MDA values were significantly lower ($p<0.05$) in MLT treated group compared to control groups during the incubation period (Fig. 2). Among the treatment groups significantly ($p<0.05$) higher TAC and lower MDA value in 1mM MLT treated group indicated the potent antioxidant potential of MLT at 1mM concentration in semen extender.

5. Discussion

Refrigerated stored ram semen is practical alternative to ultralow frozen semen, as AI in field conditions with refrigerated semen is inexpensive, non-invasive and easy to carry out with satisfactory semen quality. Now a days, antioxidants gained great attention of semen biologists for the improvement of semen quality during preservation. In recently past, the substantial antioxidant capacity of melatonin has been recognized (Hardeland et al., 2009). Therefore, we evaluated the antioxidant effect of different concentrations of MLT on seminal attributes, commonly observed to represent seminal quality and oxidative stress indices during the chilled storage of Magra ram semen. Sperm motility is an important parameter to assess seminal quality during storage period which decreases with the advancement of the incubation period at refrigerated temperature. The highest motility of the sperm was observed in the semen supplemented with 1mM MLT concentration. Further, the proportion of live sperm with high sperm membrane integrity and least sperm abnormalities was better sustained in 1mM MLT treated group compared to other MLT treated as well as control group, which may be one of the reasons for optimum motility in this group (du Plessis et al., 2010). These seminal quality findings are consistent with previous findings in rams (Khalifa, 2017; Dai et al., 2018) and buck (El-Battawy and El-Nattat, 2013; Daramola et al., 2016) where addition of MLT in semen extender improved seminal characteristics compared to control.

Gwayi et al. (2002) and Ashrafi et al. (2013) reported dose dependent negative effects of MLT on sperm motility in rat and bull which may be due to different processing procedure and sperm membrane

composition in species (Zhang et al., 2015). Shoaie and Zamiri (2008) and Ashrafi et al. (2011) showed that the higher level of antioxidants caused high fluidity of plasma membrane above the desired point, making sperm more prone to acrosomal damages. In addition, the excess quantity of antioxidant may be detrimental to spermatozoa due to physiological changes in the semen extender. Destruction of integrity causes a rise in the membrane permeability and a decrease in the ability of sperm to control the intracellular concentrations of ions that in turn are involved in sperm motion (Baumber et al., 2000), which further may negatively affect other seminal parameters. Our study also revealed that when the threshold level of MLT was increased beyond 1mM, it significantly reduced the spermatozoa viability and functionality. These observations were in agreement with findings in bull where freezing of sperm at a higher concentration of MLT decreased the viability and quality of spermatozoa (Ashrafi et al., 2013; Asma-ul-Husna et al., 2017).

TAC and MDA were analyzed as indicator of oxidative stress level. Compared to control group, TAC activity owned significant improvement whereas MDA was significantly lower in MLT supplemented group indicating positive effect of MLT on seminal quality during incubation period. These results were in agreement with previous reports in ram (Dai et al., 2018; Mohamadi et al., 2018); buck (Dessouki and Sakr, 2019) and bull (Ashrafi et al., 2013; El-Raey et al., 2015). Moreover, the increased concentration of TAC along with decreased levels of MDA in MLT supplemented group confirm and explain the antioxidant potential of MLT in protecting seminal quality throughout the chilled storage. The excess generation of ROS utilizes antioxidants, thus leading to reduction in TAC and increase in MDA level with advancement of the incubation period. The higher TAC and lower MDA level in treatment group may also be attributed to stimulatory effects of MLT on the activity of enzymes involved in intrinsic antioxidant defence and reduced production of free radicals, thereby maintain quality of semen (Tsantarliotou et al., 2008; IZADPANAH et al., 2015).

Ram sperm membrane is rich in polyunsaturated fatty acids which render it more prone to lipid peroxidation and generation of ROS leading to reduced sperm motility, viability and plasma membrane integrity during the liquid storage (Falchi et al., 2018). In addition to this, intrinsic scavenger mechanism of ram semen is not enough to neutralize the ROS due to dilution of semen, small size of sperm and intrinsic scavenger mechanism decreased with advancement of incubation period. This further accelerates impairment in sperm functions (Bilodeau et al., 2002; Alvarez and Storey, 2005; Camara et al., 2011). Sperm mitochondria encase the axosomes, connect with dense fibres in the middle piece and produce adenosine triphosphate (ATP) which may be damaged by a high level of ROS (Aitken and Clarkson, 1987) resulting in poor sperm motility and other seminal quality parameters. It has been proved that seminal fluid contains melatonin and its receptors are present on the sperm membrane (Van Vuuren et al., 1992; Reppert et al., 1995). Therefore, addition of MLT is beneficial in semen extender and helps in maintaining sperm motility, viability, plasma membrane integrity and reduced sperm abnormality, oxidative stress by virtue of its antioxidant potential (Perumal et al., 2013).

In conclusion, findings of the present study revealed that supplementation of melatonin in semen extender improve semen quality parameters and counteract oxidative stress as evident from oxidative

stress indices during liquid storage at 4°C. In addition, 1mM melatonin was found to be optimum concentration for preservation of liquid semen. However, further study are warranted to prove its efficacy *in vivo* by conducting fertility trails and also to determine its role in protecting the seminal quality in cryopreserved semen.

Declarations

Acknowledgements: Authors express gratitude to Dean, CVAS, Bikaner; Dean PGS, RAJUVAS; Head, VGO, CVAS, Bikaner for providing financial and technical support. Authors are also thankful to the Director, ICAR-CSWRI, Project Coordinator, NWPSI and PI on Magra; Head, ICAR-CSWRI, ARC, Bikaner for providing animals and lab facilities during the research period.

Availability of data and material: The relevant data has been included in the manuscript. The supplementary data may be provided, if it is necessary to support the research findings.

Code availability: Not applicable.

Author contribution: All the authors have contributed significantly in planning, execution and drafting of manuscript. All the authors have given their consent to submit the manuscript in the Journal of Tropical Animal Health and Production.

Funding: The source of funding has been duly acknowledged.

Ethics approval: The study does not include any in-vivo animal experimentation. The semen samples were collected from the animals following standard method of semen collection from Magra ram using artificial vagina method.

Consent to participate: Not applicable.

Consent for publication: The proper procedure for publication has been followed.

Conflict of interest: The authors declare no competing interests.

References

1. Acuna, C.D., Escames, G., Carazo, A., Leon, J., Khaldy, H., & Reiter, R.J., 2002. Melatonin, mitochondrial homeostasis and mitochondrial-related diseases. *Current Topics in Medicinal Chemistry*, 2, 133–151.
2. Aitken, R.J., & Clarkson, J.S., 1987. Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. *Reproduction*, 81(2), 459-469.
3. Aitken, R.J., 1995. Mechanisms and prevention of lipid peroxidation in human spermatozoa. *Human Sperm Acrosome Reaction*, 236, 339–353.

4. Alvarez, J.G., & Storey, B.T., 2005. Differential incorporation of fatty acids into and peroxidative loss of fatty acids from phospholipids of human spermatozoa. *Molecular Reproduction & Development*, 42, 334-346.
5. Appiah, M.O., He, B., Lu, W., Wang, J., 2019. Antioxidative effect of melatonin on cryopreserved chicken semen. *Cryobiology*, 89, 90-95.
6. Ashrafi, I., Kohram, H., Naijian, H., Bahreini, M., & Poorhamdollah, M. 2011., Protective effect of melatonin on sperm motility parameters on liquid storage of ram semen at 5°C. *African Journal of Biotechnology*, 10, 6670-74.
7. Ashrafi, I., Kohrama, H., Ardabili, F.F., 2013. Antioxidative effects of melatonin on kinetics, microscopic and oxidative parameters of cryopreserved bull spermatozoa. *Animal Reproduction Science*, 139, 25-30.
8. Asma-ul-Husna, Ansari, M.S., Rakha, B.A., Ejaz, R., Ullah, N., Akhter, S., 2017. Melatonin supplementation in extender enhances the post thaw quality of buffalo bull spermatozoa. *Pakistan Journal of Zoology*, 49, 163-167.
9. Baumber, J., Ball, B.A., Gravance, C.G., Victor, M., Mina, C.G., Davies-Morel., 2000. The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential, and membrane lipid peroxidation. *Journal of Andrology*, 21, 895-902.
10. Bilodeau, J.F., Chatterjee, S., & Sirard, M.A., 2000. Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. *Molecular Reproduction and Development*, 55, 282–288
11. Bornman, J.F., 1989. New trends in photobiology: Target sites of UV-B radiation in photosynthesis of higher plants. *Journal of Photochemistry and Photobiology B: Biology*, 4(2), 145-158.
12. Camara, D.R., Silva, S.V., Almeida, F.C., Nunes, J.N., & Guerra, M.M.P., 2011. Effects of antioxidants and duration of pre-freezing equilibration on frozen-thawed ram semen, *Theriogenology*, 76, 342-350
13. Cayan, K., Bucak, M.N., Başpınar, N., Taşpınar, M., & Aydos, S., 2012. Ergothioneine attenuates the DNA damage of post-thaw Merino ram sperm. *Small Ruminant Research*, 106, 165–167.
14. Dai, G.C., Meng, Y., Zhang, L.K., Du, Y.Q., Wen Feng, T.Y., & Hu, J.H., 2018. Effect of addition of melatonin on liquid storage of ram semen at 4°C. *Journal of Andrology*, 51, 132-136.
15. Daramola, J.O., Adekunle, E.O., Oke, O.E., Sorongbe, T.A., Iyanda, O.A., & Onanuga, O.D., 2016. Cryosurvival of goat spermatozoa in tris-egg yolk extender supplemented with melatonin. *Bulletin of Animal Health and Production in Africa*, 64, 281-288.
16. Dessouki, S.H.M., & Sakr, O.G., 2019. Effect of melatonin supplementation to freezing extender on post-thaw sperm kinetics, antioxidant capacity and fertility traits in buck rabbits. *Egyptian Poultry Science Journal*, 39, 443-458.
17. Dowling, D.K., & Simmons, L.W., 2008. Reactive oxygen species as universal constraints in life-history evolution, *Proceedings of the royal society B*, 276, 1737-45
18. Du Plessis, S.S., Hagenaar, K., Lampiao, F., 2010. The in vitro effects of melatonin on human sperm function and its scavenging activities on NO and ROS. *Andrologia*, 42, 112–116,

19. El-Battawy, K.A., El-Nattat, W.S., 2013. Goat spermatozoa survival rate and freezability in different extenders with/without melatonin supplementation National Research Center Egypt. Cited on www.old.eaap.org/Previous-Annual-Meetings/2004Bled/S3.8-El-Battawy.pdf
20. El-Raey, M., Badr, M., Assi, M., & Rawash, Z., 2015. Effect of melatonin on buffalo bull sperm freezability, ultrastructure changes and fertilizing potentials. *Assiut Veterinary Medical Journal*, 61, 201-208.
21. Evans, G., & Maxwell, W.M.C., 1987. Salamon's artificial insemination of sheep and goats. Butterworths, Sydney, pp.194.
22. Falchi, L., Galleri, G., Zedda, M.T., Pau, S., Bogliolo, L., Ariu, F.b., Ledda, S., 2018. Liquid storage of Ram semen for 96 h: Effects on kinematic parameters, membranes and DNA integrity, and ROS production. *Livestock Science*, 207, 1–6.
23. Gil, J., Lundeheim, N., Soderquist, L., & Rodriuez-Martinez, H., 2003. Influence of extender, temperature, and addition of glycerol on post thaw sperm parameters in ram semen. *Theriogenology*, 59, 1241-55.
24. Gwayi N, Bernard R.T.F., 2002. The effects of melatonin on sperm motility in vitro in Wistar rats. *Andrology*. 34, 391–396.
25. Hardeland, R., Tan, D.X., Reiter, R.J., 2009. Kynuramines, metabolites of melatonin and other indoles: The resurrection of an almost forgotten class of biogenic amines. *Journal Pineal Research*, 47(2), 109–126.
26. Izadpanah, G., Shahneh, A.Z., Zhandi, M., Yousefian, I., & Emamverdi, M. 2015. Melatonin has a beneficial effect on stallion sperm quality in cool condition. *Journal of Equine Veterinary. Science*, 35, 555-59.
27. Jang, H.Y., Kim, Y.H., Kim, B.W., Park, I.C., Cheong, H.T., Kim, J.T., Yang. B.K., 2010. Ameliorative effects of melatonin against hydrogen peroxide induced oxidative stress on boar sperm characteristics and subsequent *in vitro* embryo development. *Reproduction in domestic Animal*, 45(6), 943–950.
28. Kang, J.T., Koo, O.J., Kwon, D.K., Park, H.J., Jang, G., Kang, S.K., & Lee, B.C., 2009. Effects of melatonin on in vitro maturation of porcine oocyte and expression of melatonin receptor RNA in cumulus and granulosa cells. *Journal of Pineal Research*, 46, 22-28.
29. Khalifa, M.A., 2017. Effect of supplementing ram semen extender with melatonin on oxidative stress indices and physical properties of chilled spermatozoa. *International Journal of Animal Research*, 1-14.
30. Lector, C., 1996. Oxidative stress and role of antioxidants in normal and abnormal sperm function. *Fronti. Biological Science*, 1, 78–86.
31. Leon, J., Acuna-Castroviejo, D., Escames, G., Tan, D.X., & Reiter, R.J. 2005. Melatonin mitigates mitochondrial malfunction. *Journal of Pineal Research*, 38, 1–9.
32. Lopez-Saez, A., Ortiz, N., Gallego, L., Garde, J.J., 2000. Liquid storage (5⁰C) of ram semen in different diluents. *Journal of Reproductive Systems*, 44, 155-164.

33. Maia, M.S., Bicudo, S.D., Sicherle, CC., Rodello, L., & Gallego, I.C.S., 2010. Lipid peroxidation and generation of hydrogen peroxide in frozen-thawed ram semen cryopreserved in extenders with anti-oxidants. *Animal Reproduction Science*, 122, 118-123.
34. Mohamadi, S., Mamouei, M., Vakily, S.T., Fayazi, J., 2018. Antioxidant effects of melatonin on Arabic ram semen parameters after freeze-thawing process. *Iran Journal of Animal Science Research*, 10, 287-96.
35. Mosafari, S., Niasari-Naslaji, A., Abarghani, A., Gharahdaghi, A.A., Gerami, A., 2005. Biophysical and biochemical characteristics of Bactrian camel semen collected by artificial vagina. *Theriogenology*, 63, 92-101.
36. O'Hara, L., Hanrahan, J.P., Richardson, L., Donovan, A., Fair, S., Evans, A.C., Lonergan, P., 2010. Effect of storage duration, storage temperature, and diluent on the viability and fertility of fresh ram sperm. *Theriogenology*, 73, 541-549.
37. Othman, A.I., Edrees, G.M., El-Missiry, M.A., Ali, D.A., Aboel-Nour, M., & Dabdoub, B.R., 2016. Melatonin controlled apoptosis and protected the testes and sperm quality against bisphenol A-induced oxidative toxicity. *Toxicology and Industrial Health*, 32, 1537–1549.
38. Pampori, Z.A., Sheikh, A.A., Aarif, O.V, Hasin, D., & Bhat, I.A., 2018. Physiology of reproductive seasonality in sheep. *Biological Rhythmic Research*, 1-13.
39. Pang, Y.W., Sun, Y.Q., Jiang, X.L., Huang, Z.Q., Zhao, S.J., Du, W.H., & Zhu, H.B., 2016. Protective effects of melatonin on bovine sperm characteristics and subsequent *in vitro* embryo development. *Molecular Reproduction Development*, 83, 993–1002.
40. Perumal, P., Vupru, K., Khate, K., 2013. Effect of addition of melatonin on the liquid storage (50C) of Mithun (*Bos frontalis*) semen. *International Journal of Zoology*, 1-10.
41. Redden, R., & Thorne, J. W., 2020. Reproductive management of sheep and goats. In *Animal Agriculture* (pp. 211-230). Academic Press.
42. Reiter, R.J., Fraschini, F., 1969. Endocrine aspects of the mammalian pineal gland: a review. *Neuroendocrinology*, 5, 219–255.
43. Reiter, R.J., Tan, D.X., Korkmaz, A., & Rosales-Corral, S.A., 2014. Melatonin and stable circadian rhythms optimize maternal, placental and fetal physiology. *Human Reproduction*, 20, 293–207.
44. Reppert, S. M., Weaver, D. R., Cassone, V. M., Godson, C., & Kolakowski Jr, L. F., 1995. Melatonin receptors are for the birds: molecular analysis of two receptor subtypes differentially expressed in chick brain. *Neuron*, 15(5), 1003-15.
45. Saleh, R.A., Agarwal, A., 2002. Oxidative stress and male infertility from research bench to clinical practice. *Journal of Andrology*, 23, 737–52.
46. Santiani, A., Evangelista, S., Sepúlveda, N., Risopatrón, J., Villegas, J., & Sánchez, R., 2014a. Addition of superoxide dismutase mimics during cooling process prevents oxidative stress and improves semen quality parameters in frozen/thawed ram spermatozoa. *Theriogenology*, 82, 884–89.
47. Shafiei, M., Forouzanfar, M., Hosseini, S.M., & Esfahani, M.H.N., 2015. The effect of superoxide dismutase mimetic and catalase on the quality of post thawed goat semen. *Theriogenology*, 83,

1321–27.

48. Shoaie, A., Zamiri, M.J., 2008..Effect of butylated hydroxytoluene on bull spermatozoa frozen in egg yolk-citrate extender. *Animal Reproduction Science*, 104, 414–418.
49. Succu, S., Berlinguer, F., Pasciu, V., Satta, V., Leoni, G.G., Naitana, S., 2011. Melatonin protects ram spermatozoa from cryopreservation injuries in a dose-dependent manner. *Journal of Pineal Research*, 50, 310-318.
50. Tsantarliotou, M., Kokolis, P.N.A., Smokovitis, A., 2008. Melatonin administration increased plasminogen activator activity in ram spermatozoa. *Theriogenology*, 69, 458–465.
51. Van Vuuren, R.J., Pitout, M.J., Van Aswegen, C.H., Theron, J.J., 1992. Putative melatonin receptor in human spermatozoa. *Clinical Biochemistry*, 25, 125-127.
52. Zhang, X.G., Yan, G.J., Hong, J.Y., Su, Z.Z., Yang, G.S., Li, Q.W., & Hu, J.H., 2015. Effects of bovine serum albumin on boar sperm quality during liquid storage at 17°. *Reproduction in domestic animal*, 1-7, doi: 10.1111/rda.12481.

Figures

Figure 1

Effect of different concentration of Melatonin on Total Antioxidant Capacity (TAC) during cooled storage of ram semen

Figure 2

Effect of different concentration of Melatonin on malondialdehyde (MDA) during cooled storage of ram semen