EFFECT OF MELATONIN ON BUFFALO BULL SPERM FREEZABILITY, ULTRASTRUCTURE CHANGES AND FERTILIZING POTENTIALS

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ABSTRACT

Cryopreservation induces sublethal damage to the spermatozoa, which leads to reduce their fertile life. The objective of the present study was to investigate the effect of melatonin on freezability, ultrastructure and fertilizing potentials of the buffalo spermatozoa. Buffalo spermatozoa were cryopresreved with Tris egg yolk extender containing 7% glycerol supplemented with (0.100, 0.250, 0.500, 0.750 and 1 mM melatonin) or Tris-based extender only (control). Cryopresreved spermatozoa were assessed for post-thawing sperm motility, viability and acrosomal integrity, ultrastructure changes and fertilizing potentials. The current results clearly indicated that adding 0.1 mM melatonin to Tris extender significantly improved ($P<0.05$) post-thawing sperm motility and viability index following cryopreservation (60.00±2.9% and 157.5±11.8, respectively) compared with the control spermatozoa (45.00±2.90% and 97.00±9.09, respectively). The current results illustrated that addition of 0.1mM melatonin protected the plasma membrane, acrosomal region and mitochondria and maintained the ultrastructure integrity of the cryopresreved spermatozoa compared with the control spermatozoa. The present data indicated that addition of melatonin at a dose of 0.1 and 0.25 mM to the semen extender significant increased ($P< 0.05$) the positive HOST percent (83.66 and 81.33%, respectively) compared with the control semen (43.33 %). Additionally, the current results revealed that addition of 0.1 mM and 0.25 mM melatonin to the semen extender highly significant increased ($P< 0.01$) the pregnancy rate (66.67 and 60.43%, respectively) compared with the control semen (45.65%). Therefore, the present results revealed that addition of melatonin to the freezing extender might improve semen quality and reduce cryodamage of the buffalo bull spermatozoa.

Key words: *Melatonin, semen extender, cryopreservation, HOST, ultrastructure and conception rate.*

INTRODUCTION

Semen cryopreservation offers many advantages to the livestock industry, particularly in conjunction with allowing the widespread dissemination of valuable genetic material by means of artificial insemination (Bucak *et al.,* 2009). The success of an AI program depends on the proper management of semen collection, storage and use (Leboeuf *et al.,* 2000). Although, many protocols have been developed for semen cryopreservation, sperm cryosurvival rate is still not optimum in the buffalo. Cryopreservation induces some irreversible damages in sperm cells (Medeiros *et al.,* 2002). Factors responsible for these damages includes; changes in temperature, ice formation, access of reactive oxygen species and lipid peroxidation, alterations in sperm membrane, toxicity of cryoprotectants and osmotic stress which reduces the post thaw quality of semen (Watson, 2000). To keep the cell alive during cryopreservation process, plasma membrane is a key component that must be maintained (Aboagla and Terada, 2003). The plasma membrane of mammalian spermatozoa contains high concentrations of polyunsaturated fatty acids, which make it susceptible to reactive oxygen species (ROS) induced peroxidative damage with a subsequent loss of sperm functions (Lenzi *et al.,* 2002). There are several substances used to protect sperm plasma membrane during cryopreservation, one of the most important of

them melatonin. Melatonin (N-acetyl-5-Methoxytryptamine) an indole derivative secreted rhythmically from the pineal gland and played a major role in regulating the circadian clock in mammals in general and regulating the reproductive functions in particular (Reiter, 1991). The role of melatonin in modulating the reproduction is still obscure (Berlinguer *et al.,* 2009). More recent studies had demonstrated that, melatonin had an antioxidant and powerful direct scavenger effect that protected the cells from the free radicals (Adriaens *et al.,* 2006 and Kang *et al.,* 2009). Melatonin and its metabolites potently scavenge ROS (Reiter *et al.,* 2005), thus altering redox-sensitive events and preventing oxidative damage and improve sperm motility during sperm liquid storage or in the unfrozen state (Ashrafi *et al.,* 2001 and Kang *et al.,* 2009). Additionally, melatonin might be involved in the protection of different cell types against damage-induced apoptosis (Baydas *et al.,* 2005, Casao *et al.,* 2010 and Espino *et al.,* 2011). Moreover, melatonin exerts several beneficial actions on sperm fertilizing ability such as induction of capacitation (Bornman *et al.,* 1989) and hyperactivation (Fujinoki 2008). However, the effect of melatonin on the integrity and fine plasma membrane structure of buffalo spermatozoa was rarely evaluated. So the current study aimed to estimate the influence of melatonin on the fine ultrastructure changes and the fertilizing potentials of buffalo spermatozoa.

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, $800x10^6$ 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 (0.1, 0.25, 0.5, 0.75 and 1 mM) vs. Tris-based extender only (control) to obtain 120×10^6 sperm/ml. Melatonin (M5250-250mg, 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 (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 hour 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).

Hypo-osmotic swelling test

Hypo-Osmotic Swelling Test (HOST) of spermatozoa, as an in-vitro fertility test, was conducted to evaluate the semen sample in the procedure as described by Revell and Morde (1994). Proportion of sperm cells exhibiting hypo-osmotic swollen positive response was expressed as percent (Correa and Zavos, 1994).

Ultrastructure analysis of the cryopresreved spermatozoa

The ultrastructure changes occurred for the cryopresreved buffalo spermatozoa were evaluated by transmission electron microscopy (TEM). Straws from each treatment were washed three times by centrifugation at 1000 rpm for 5 min using PBS (Phosphate Buffered Saline). The frozen-thawed semen was prefixed for 2-3 h with PBS containing 2% glutaraldehyde, washed three times by centrifugation at 1000 rpm with PBS (pH 7.4) for 5 min at 4°C and post-fixed in 1% osmium tetroxide for 1-2 h at 4°C (Boonkusol, 2010). Spermatozoa were dehydrated in propylene oxide and embedded in epon resin. Ultrathin sections were cut using the Leica EM UC6 ultramicrotome and stained with uranylacetate

and lead citrate. Randomly fields were examined by a transmission electronic microscope (JEOL-EM-100 S at 80 Kv at VACSERA- Electron Microscopy Unit) and photographed for further analysis.

Evaluation of fertilizing potentials of the semen

A preliminary fertility trial was performed to compare between control semen and melatonin treated semen. Buffalo cows were randomly assigned to one of six treated groups: group 1 (92 buffaloes) was inseminated using control semen; group 2 (93 buffaloes) was inseminated using 0.1 mM melatonin treated semen, group 3 (91 buffaloes) was inseminated using 0.25 mM melatonin treated semen, group 4 (87 buffaloes) was inseminated using 0.5 melatonin treated semen, group 5 (86 buffaloes) was inseminated using 0.75 mM melatonin treated semen and group 6 (87 buffaloes) was inseminated using 1 mM melatonin treated semen. Pregnancy diagnosis was performed 45 days post-insemination by transrectal palpation.

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 Fig (1), The current results clearly indicated that adding 0.1 and 0.25 mM of melatonin to Tris extender significantly improved ($P<0.05$) post-thawing sperm motility (60.00 ± 2.9 and 55.00 ± 5.01, respectively), viability index (157.5 ± 11.8 and 150.83 ± 8.21, respectively) compared with the control spermatozoa (45.00±2.905% and 97.00±9.09, respectively).

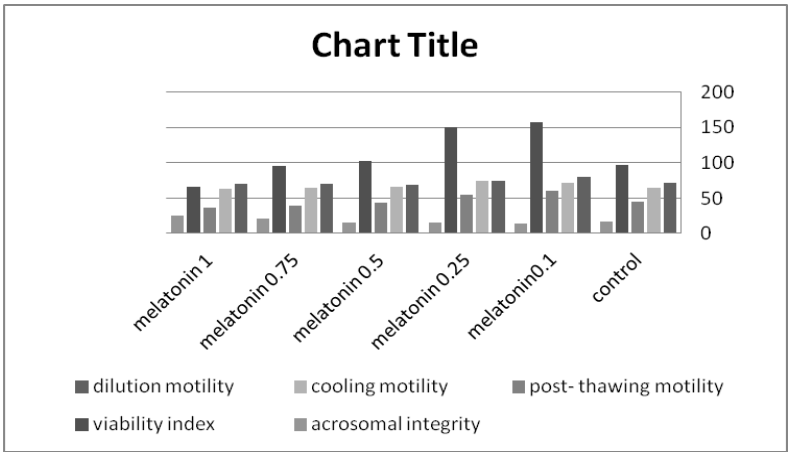

Fig. 1: Effect of melatonin supplementation to buffalo semen extender on the frozen-thawed spermatozoa characteristics.

Electron microscopic images of sagital sections of the frozen thawed buffalo sperm cells in the control group showed, swollen plasma membrane segmentation of the outer acrosomal membrane and swollen acrosome (fig, 2 and 3). Mean while, the frozen thawed buffalo semen treated with 0.25 mM melatonin illustrated a well defined and intact plasma membrane and intact outer and inner acrosomal membranes (fig, 4 and 5). Moreover, the control semen showed severe degeneration marked vacuolation in the mitochondria with complete absence of the transverse cristae (fig, 6). While, semen treated with 0.25 mM melatonin illustrated homogenous mitochondria content and high-quality mitochondrial dense electron spaces with appeared transverse cristae (fig, 7 and 8).

Hypo-Osmotic Swelling Test (HOST):

Data regarding the hypo-osmotic swelling test are illustrated in fig. 9. A significant difference ($P<0.05$) was found to exist between the melatonin treated semen at a concentration of 0.1 and 0.25 mM (83.66 and 81.33%, respectively) compared with the control group (43.33%).

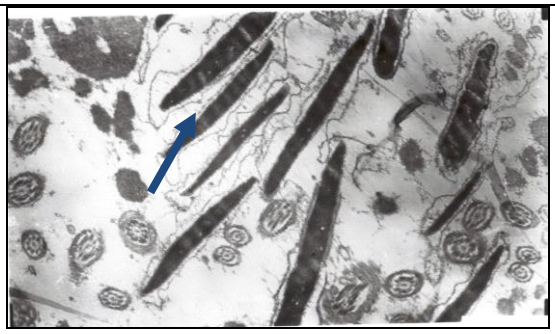

Fig 2

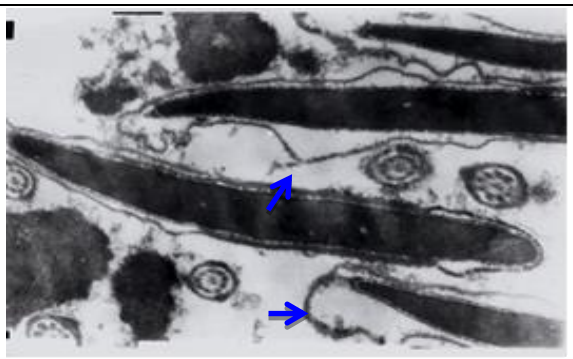

Fig 3

Fig. 2 & 3: Electron micrograph for a sagital section in the sperm head from a frozen-thawed semen sample of control group illustrating swollen, degenerated and vacuolated plasma membrane, segmentation of the outer acrosomal membrane (OAM) and swollen acrosome (X 14000).

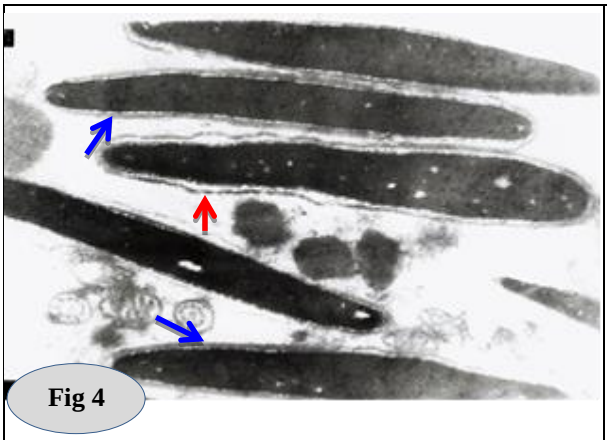

Fig 4

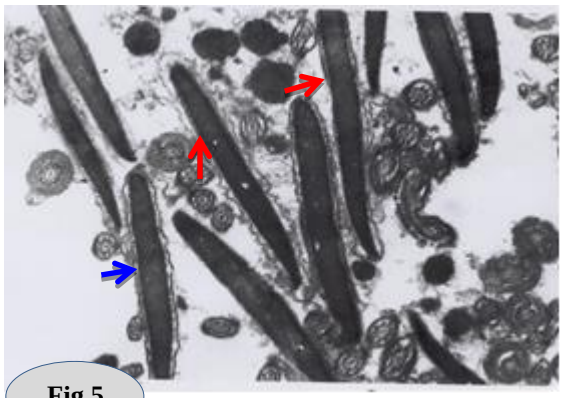

Fig 5

Fig. 4&5: Electron micrograph for a sagital section in the sperm head from frozen-thawed semen sample treated with 0.1 mM melatonin illustrating intact plasma membrane (PM) and the nucleus content (N) is homogenous in the electron density. Also, outer and inner acrosomal membranes are intact and the subacrosomal space is evident (× 2000). Also electron micrograph for a sagittal section in the sperm from a frozen-thawed semen sample illustrating intact outer acrosomal membrane (OAM) inner acrosomal membrane (IAM) in the electron density (×10000).

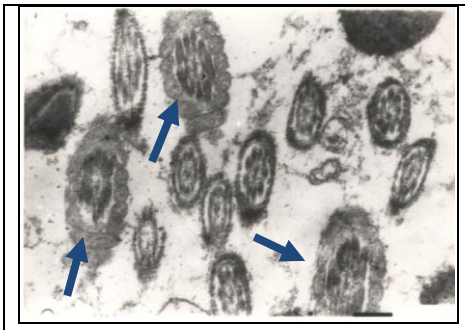

Fig 6

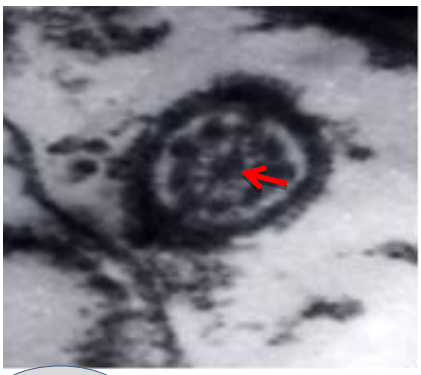

Fig 7

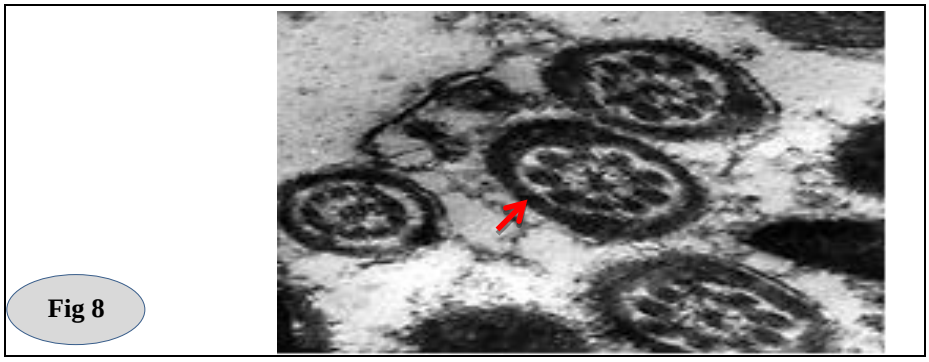

Fig. 6: Electron micrograph of a cross section in the neck region (note the presence of mitochondria in different orientation) of sperm from a frozen-thawed semen sample of control group showing severe degeneration (marked vacuolation) in the mitochondria that contained electron-translucent spaces with complete absence of the transverse cristae and some mitochondria are completely disappeared (× 20000).

Fig: 7&8: Electron micrograph of a cross section in the mid-piece region and the tail from a frozen-thawed semen sample treated with 0.1mM melatonin illustrating good mitochondrial dense electron spaces with appeared transverse cristae (×25000)

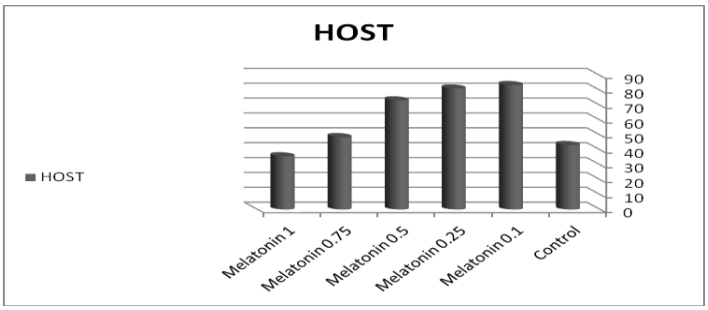

Fig. 9: Effect of melatonin addition to the freezing extender on the HOST%.

Data regarding the effect of fortification of semen extender with different melatonin concentrations on the pregnancy rate is demonstrated in Fig. 10. The results showed that addition of 0.1 mM and 0.25 mM melatonin to the semen extender highly significant (P< 0.01) augmented the pregnancy rate (66.67 and 60.43%, respectively) compared with the control semen (45.65%). Moreover, addition of high concentration (1mM) of melatonin to the semen extender drastically reduced the pregnancy rate (28.74%).

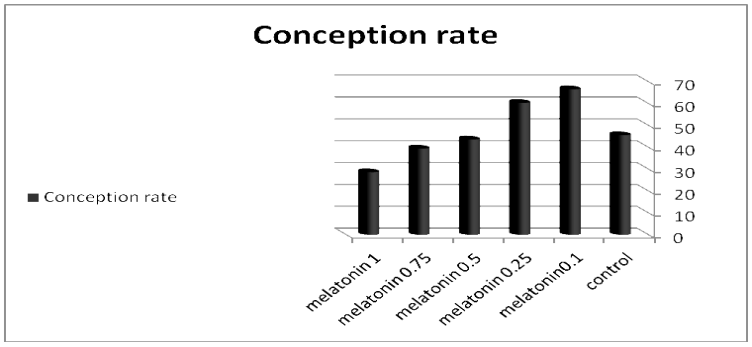

Fig. 10: Effect of melatonin addition to the freezing extender on the conception rate.

DISCUSSION

The current results revealed that melatonin supplementation to Tris extender prior to cryopreservation resulted in fantastic and protecting functions on the post-thaw semen quality, conception rate and preserved the integrity of the fine structure and the mitochondrial arrangement of the spermatozoa at a dose dependent manner. The high concentrations of melatonin inhibited the quality of sperm motility. These data are in accordance with results of (Fujinoki, 2008, Du Plessis *et al.*, 2010 and

Succu *et al.*, 2011), who reported that the addition of melatonin to the semen freezing extender protected spermatozoa during cryopreservation and had beneficial effects on the post-thaw semen motility, viability and membrane integrity and significantly decreased the rate of lipid peroxidation of the cryopresreved spermatozoa in a dose-dependent manner.

However, the exact mechanism of sperm protection by melatonin has not been fully discovered and remains unclear. Variety of hypotheses and peculations has been proposed by various authors to explain the protective mechanism of melatonin. The current results may provide a novel mechanism of melatonin on the cryopresreved buffalo spermatozoa quality and fertilizing potentials. The positive effect of melatonin might be built on its ability to preserve the integrity of plasma membrane fine structure and mitochondrial arrangement of the spermatozoa, creating it less vulnerable to cryo-damage.

The results of the current study may be indicated by the results of (Tan *et al.*, 2002, Du Plessis *et al.*, 2010 and Espino *et al.*, 2011) who found that melatonin might be due to potent scavenging effect of melatonin for reactive oxygen species (ROS). It had been established that ROS are highly reactive with complex cellular molecules such as proteins, lipids and DNA resulting in damaging of the cell membranes, triggering of apoptosis in spermatozoa, oocytes and embryos (Tatemoto *et al.*, 2004 and Agarwal *et al.*, 2006), inducing DNA fragmentation (Guérin *et al.*, 2001), serious dysfunction of many enzymes (Halliwell and Gutteridge, 1989), mitochondrial abnormality and dysfunction (Lopes *et al.*, 1998), damaging of RNA and protein synthesis (Comporti, 1989); which were generally thought to be harmful for embryonic development (Guérin *et al.*, 2001). Therefore, melatonin is effective in lowering molecular damage under conditions of elevated oxidative stress due to potent scavenging effect of melatonin for ROS (Reiter *et al.*, 2005).

Additionally, the beneficial action of melatonin on the cryopresreved buffalo spermatozoa and its fertilizing potentials may be due to preserve the mitochondrial dense structure and arrangement of the spermatozoa. Therefore, it would ultimately enhance the fertilizing potentials of the cryopresreved spermatozoa. Moreover, Martin *et al.* (2000) found that intra mitochondrial melatonin levels 100-fold higher than those of plasma indicating that melatonin had a potent physiological role in mitochondrial homeostasis (Martin *et al.*, 2000). It had been reported that healthy condition of mitochondria was important for ATP production (Mtango *et al.*, 2008), and regulation of cell survival (Dumollard *et al.*, 2007). Whereas, aged mitochondria displayed decreased membrane potential and increased fragility (Dirks *et al.*, 2006).

Furthermore, melatonin acts as an anti-apoptotic hormone through down-regulation of mitochondrial cytochrome C release and inhibition of activation of casp-9 and casp-3 (Molpeceres *et al.*, 2007).

Therefore, results emerged from this study clearly demonstrated that supplementation of semen extender with melatonin exerted valuable effects on the quality and the fertilizing potentials of the cryopresreved buffalo spermatozoa. These constructive effects appeared due to its role in preserving the integrity of the plasma membrane fine structures, the mitochondrial dense structure and activity of the cryopresreved spermatozoa.

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دراسة تأثير الميلاتونين علي قدرة حيامن الجاموس للتجميد ، التغيرات في التركيب الدقيق وقدرتها الاخصابية

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أجري هذا البحث لدراسة تأثير اضافة الميلاتونين الي ممدد السائل المنوى علي قابلية حيامن الجاموس للتجميد وكذا قدرتها الاخصابية في الحقل. تم تجميع عينات السائل المنوي من ستة طلائق وبعد تقييم السائل المنوي معمليا تم تمديده في ممدد التريس المضاف اليه تركيزات مختلفة من الميلاتونين (٠.١٠٠، ٠.٢٥٠، ٠.٥٠٠، ٠.٧٥٠ و١ ملليمول علي التوالي) أو مخفف التريس فقط (المجموعة الضابطة). وبعد تبريد وتجميد الحيامن بالنظام الفرنسي تم تقيمه من حيث نسبه الحركه الأماميه والحيويه وتشوهات القلنسوه وكذا اثر التجميد علي سلامة التركيب الدقيق للحيامن وسلامة الغشاء البلازمي باجراء اختبار الهوست منخفض الضغط الاسموزي كذلك قدرته الاخصابيه في الحقل. ولقد أوضحت نتائج الدراسة الحالية أن تجميد السائل المنوى الجاموسي في ممدد مضاف اليه الميلاتونين بتركيز ٠.١٠٠ ملليمول ميلاتونين نتج عنة زيادة معنوية كبيرة في نسبة الحركة الأمامية بعدالإسالة ومعدل الحيوية (٦٠.٠٠% و١٥٧.٥٠ علي التوالي) مقارنة بالمجموعة الضابطة (٤٥.٠٠% و ٩٧.٠٠ علي التوالي) كما حافظ علي سلامة التركيب الدقيق للحيامن بالمقارنة بالمجموعة الضابطة. كذلك نتج عنة زيادة معنوية كبيرة في سلامة الغشاء البلازمي بعد اختبارالهوست منخفض الضغط الاسموزي (٨٣.٦٦%) مقارنة بالمجموعة الضابطة (٤٣.٣ %). كما اوضحت النتائج ارتفاع نسبة الاخصاب في الحقل باستخدام السائل المنوي المجمد باضافة الميلاتونين بتركيز ٠.١٠٠ و٠.٢٥٠ ملليمول (٦٦.٦٧ و ٦٠.٤٣% علي التوالي) مقارنة بالمجموعة الضابطة (٤٥.٦ %). ومن خلال نتائج الدراسة الحالية يمكن أن نستنتج أن إضافة باضافة الميلاتونين بتركيز 0.1 ملليمول الي ممدد التريس يلعب دورا هاما وكبيرا في تحسن وظائف السائل المنوي الجاموسي المجمد ويبدو ذلك من خلال قدرتها علي المحافظة علي سلامة التراكيب الدقيقة للحيامن وزيادة نسبة الاخصاب في الحقل.