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THE EFFECT OF MELATONIN ON THE BULL LIQUID SEMEN AND ENZYMATIC RELEASE IN SEMINAL PLASMA

(With 7 Tables and 4 Figures)

By

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تأثير الميلاتونين على السائل المنوى المخفف فى العجول البقرى وكذلك الإنزيمات فى بلازما السائل المنوى

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تمت دراسة تأثير الميلاتونين على السائل المنوي المخفف للعجول البقري وكذا على الإنزيمات الموجودة في بلازما السائل المنوي أثناء حفظه في درجة حرارة ٤°م . تم تخفيف السائل المنوي باستخدام المخفف الروسي للحصول على تركيز ١٠٠ × ١٠٦ حيوان منوي لكل واحد ملليمتر . ثم أضيف الميلاتونين بالتركيزات الآتية : ١ ، ٥ ، ١٠ ، ١٥ ، ٢٠ ميكروجرام لكل ١٠٠ × ١٠٦ حيوان منوي وأيضا عينة بدون إضافة (العينة الضابطة) . وتم فحص هذه العينات يوميا ولمدة ستة أيام بعد حفظها عند درجة حرارة ٤°م . وأظهرت هذه الدراسة أن الميلاتونين وخاصة التركيزات العالية (١٠ ، ١٥ ، ٢٠ ميكروجرام) لها تأثير معنوي في تحسين الحركة الذاتية للحيوانات المنوية لمدة طويلة وأيضا لها تأثير معنوي على نسبة الحيوانات المنوية الميتة وكذلك إختزال الطاقة معنويا في بلازما السائل المنوي . وأثبتت هذه الدراسة أن إضافة ٢٠ ميكروجرام ميلاتونين للسائل المنوي المخفف يؤدى إلى تحسين جودة

السائل المنوي والمحافظة على جودته لمدة اكثر من ثلاثة أيام أي حتى ٥-٦ أيام في درجة حرارة ٤°م .

SUMMARY

The effects of melatonin upon the bull liquid semen and enzymatic release in seminal plasma during storage at 4°C were studied. The semen was extended by using Russian diluent to obtain 100 X 10^6 sperm per ml. The melatonin was added to extended semen as 0.0, 1.0, 5.0, 10.0, 15.0 and 20.0 µg /100 X 10^6 sperm. The samples in this study were stored at 4°C for six days and examined daily for motility, alive, abnormalities % as well as fructose content, Acid phosphatase, ATPase and hyaluronic acid in seminal plasma. The results in this study demonstrated that melatonin, particularly the high concentrations (10.0, 15.0 and 20.0 µg), improved sperm motility ($P<0.01$) for a long period of storage, reduced dead sperm % ($P <0.01$) as well as conservation of energy in seminal plasma ($P< 0.01$). The ultrastructure investigation of the treated bull semen after 3 and 5 days storage revealed that, the high concentration (especially at 20 µg) melatonin had a good protective effect for sperm. The effect of melatonin on sperm cells may be through activation of microtubular function, protection of DNA from damage by free radical moreover, it was antiaging on sperm cells. These results also arise the possibility of using the melatonin with the extender or diluent (especially at 20µg / 100 X 10^6 sperm per ml) for improving the liquid semen quality and storage for long time at 4°C.

Key words: Melatonin, bull semen, Enzymes, seminal plasma.

INTRODUCTION

Melatonin-N-acetyl-5- methoxytryptamine is a hormonal product of the pineal gland. Its synthesis is higher at night than during the day in all vertebrates including man *(REITER, 1993)*. Many investigators discussed the effect of melatonin on reproductive system *(REITER , 1985 and ANWAR, 1987)*.

The effect of extensive dilution on mammalian sperm was a subject of great interest during the early years of sperm research *(MANN, 1964)*. The dilution of semen in appropriate media not only provides an environment suitable for extended storage of the spermatozoa, but also dilutes the sperm

concentration to a level appropriate for making multiple insemination *(FOOTE, 1980 and GARNER, 1991)*. Preservation of spermatozoa is a process in which not only the viability of the cells but also their fertilizing capacity must be maintained during storage *(SALISBURY, et al., 1978)*. The spermatozoa can be stored in a liquid media which is maintained either at ambient temperature or in a refrigerator at 4 - 5°C. For successful short-term liquid storage of semen, it is necessary either to extend the time that spermatozoa were able to maintain high metabolic rates or to slow the process down *(GARNER, 1991)*.

Various additives have been incorporated into semen extenders to enhance sperm preservation and fertility *(MAULE, 1962)*. For example, catalase, vitamins analogue and various hormonal preparations have been added to semen *(SALISBURY, et al., 1978)*. Several substances can interfere with microtubular function including colchicine and melatonin *(BORNMAN, et al., 1989)*. They reported also that melatonin has similar effects as colchicine on microtubles. Sperm motility is possibly under control of an unknown hormone acting through the receptors or adenylate cyclase or cyclic AMP or protein phosphorylation cascade *(VUURNER, et al., 1992)*.

The aim of this study is to investigate the effect of melatonin on liquid semen through sperm viability and storage for long time which will help in artificial insemination (A.I). In addition the acrosomal integrity will be studied which will be of help in invitro fertilization (IVF).

MATERIAL and METHODS

Three bulls were used in this study maintained under identical nutritional and managemental conditions at the farm belonging to Faculty of Veterinary Medicine, Assiut University, Egypt. The semen collections were done at the early morning hours using artificial vagina (42 - 43°C) and female in oestrus as used as teaser. Within 2 - 3 minutes, after collections, semen of the three bulls were taken to the laboratory where it was pooled and kept in water bath at 37°C. Evaluation of collected semen was carried out including mass activity, individual motility and sperm cells concentration after dilution to 1:200 using citrate buffer and addition of three drops of formaline and three drops of 0.5% eosin solution for staining.

The semen was extended with a Russian diluent *(MILOVANOV , et al., 1964 and AZAWI, et al., 1990)* to give a final concentration of 100 X 10^6 sperm per ml. Melatonin was added to the extended semen as 0.0 µg (control sample), 1, 5, 10, 15 and 20 µg / 100 X 10^6 sperm. Three samples were prepared from each concentration and control. All samples (treated and control) were stored in refrigerator (4°C) and examined daily for six days for motility %, alive sperm % (using eosin - nigrosin stain) and sperm abnormalities % (using alkaline methyl violet stain).

After daily examination, the samples were centrifuged at 3000 rpm for 20 minutes. The supernatant fluid (seminal plasma and diluent) were collected then kept at -20°C till used for determination of fructose content according to *BERGMEYER (1974)*, non-prostatic acid phosphatase (ACP) according to *MOSS (1984)*, ATP-ase enzyme according to *HOSIE (1965)* and Hyaluronic acid according to *GREILING (1974)*. The sediment (spermatozoa) was prepared for examination by Transmission electron microscope for any changes in the plasma membrane of spermatozoa after being stained by using uranyl acetate and lead citrate. The results were analyzed by Analysis of Variance (ANOVA) with repeated measures on all factors *(PC-STATE, 1985)*.

RESULTS

The obtained results in this study are presented in Tables 1 - 7 and Figures 1 and 2. Sperm motility % (S.M. %) was affected by the addition of melatonin (Table 1 and Fig. 1 a). High concentration of melatonin (10, 15 and 20.0 µg) had a significantly increasing effect ($P< 0.01$) on S.M % when compared with the control samples at the first day of incubation. S.M % increased significantly ($P< 0.01$) with all melatonin concentrations at 2-5 days of storage at 4°C. However, S.M % increased significantly ($P<0.01$) only with 5, 10, 15 and 20 µg melatonin at 6th day of storage. It also observed that, the percent increase in S.M % was noticed with high concentrations of melatonin at the last days of incubation.

Table (2) and Fig. (1 b) show the effect of melatonin on the alive sperm % (A.S %). It was increased significantly ($P< 0.01$) with all concentrations of melatonin during all storage time except with 1.0 and 5.0 µg melatonin at the first day of storage. High concentration of melatonin

(10, 15 and 20.0 µg) had a highest effect on the A.S % especially at last 3 days of storage thereby the increasing percent.

Sperm abnormalities % (S.Ab %) is reduced by the addition of melatonin to the liquid bull semen (Table 3 and Fig. 1 c). All concentrations of melatonin had a reducible effect ($P< 0.01$) upon S.Ab % during the storage time, however, 1.0 µg at first day had a non-significant effect upon S.Ab %. The reduction percent of S.Ab% was increased with the highest concentrations of melatonin and prolonged storage time.

The variation of fructose content in seminal plasma after addition of melatonin was presented in Table (4) and Fig. (2 a). The overall mean of the fructose was significantly increased ($P< 0.01$) with the addition of 20.0 µg melatonin among storage time. However, 15.0 µg melatonin had a significant ($P< 0.01$) effect at 3 - 6 days of storage as well as 4 - 6 days in case of 10.0 µg melatonin. The effect of 1.0 or 5.0 µg melatonin was non-significant except during the last day of storage ($P< 0.01$). The increased % of fructose in seminal plasma is elevated with the increasing of the concentrations of melatonin as compare with the control samples.

Non-prostatic acid phosphatase (ACP) levels varied according to the concentrations of melatonin and storage time (Table 5 and Fig. 2 b). All concentrations of melatonin had a significant decreasing ($P< 0.01$) effect upon ACP among storage time but 1 µg melatonin had a significant effect ($P< 0.05$) at 4^{th} and 5^{th} as well as non-significantly at 6^{th} day of storage. The reduction of the percent of ACP increased with the increasing of melatonin concentration.

The changes in ATP-ase level in treated samples among storage were illustrated in Table (6) and Fig. (2 c). The 15.0 and 20.0 µg melatonin had a significant increasing ($P< 0.01$) effect especially during 2 - 6 days of storage. However, 10.0 µg melatonin had a significant ($P< 0.01$) effect at 2 and 3 days but at 4 - 6 days of storage the increasing effect observed significantly at $P< 0.05$ level. Generally, the increase percent of ATP-ase increased with increasing of melatonin concentration and storage time.

The results presented at Table (7) and Fig. (2 d) show the effect of melatonin on the level of hyaluronic acid (H. acid). All concentrations of melatonin had a significant increasing ($P< 0.01$) effect upon H. acid among

storage time except the first day where the effect was non-significant in case of 1.0 and 5.0 μg of melatonin concentrations.

The ultrastructure investigation of the treated bull semen after 3 and 5 days storage revealed that , the high concentration (20 μg) of melatonin had a good protective effect for sperm (Fig. 3b & Fig.4a) as compared with control (Fig. 3a). At 10 μg melatonin, the changes in the sperm plasma membrane were in the form of swelling and mild disintegration of the plasma membrane (Fig. 3c & Fig.4b).The lower concentration of melatonin (1μg) had a bad action. The changes were in the form of great expansion and severe disintegration of the sperm plasma membrane (Fig.3d & Fig.4c).

DISCUSSION

This study is a trial to investigate the effect of different concentrations of melatonin (1, 5, 10, 15 and 20 μg / 100 X 10^6 sperm) on bull liquid semen, incubated at 4°C for 6 days. It was observed that addition of melatonin produced significant increase in sperm motility, alive sperm percentage, fructose content, hyaluronic acid and ATP-ase. In addition, melatonin produced a significant decrease in sperm abnormalities and non-prostatic acid phosphatase. The effects of melatonin addition were clear and observed markedly in high concentrations and at the last three days of incubation.Unfortunately, there is no available literatures in this field.

The effect of melatonin on sperm motility and survival may be attributed to one and/or all of the following possibilities:

Firstly, ATP is the main energy source used by the sperm flagellum to initiate and propagate forward motility *(BURGER, et al., 1991)*. Sperm motility was thus dependent on two factors: the ability to produce sufficient ATP and the ability to utilize this energy effectively *(TOIT et al., 1993)*. They reported also, that glycolysis or more specifically fructolysis was the main energy pathway for the generation of ATP. In our results ATP-ase level was increased as well as alive sperm % by the addition of melatonin. *TOIT et al. (1993)* were inagreement with our results. They reported that a reduction of ATP is correlated the reduction of ATP-ase in seminal plasma. *HEPPEL and HILMOE (1953)* reported that ATPase catalyses the reaction $ATP+H_2O \rightarrow AMP$ + pyrophosphate and energy.

MANN and LUTWAK-MANN (1981) found that the movement of sperm was entirely linked to the structure of the sperm flagellum. They added that tubulin and dynein are the principal structure protein in the axonemal fibrils of the tail sperm. Also, they have a specific ATP-ase activity and essential for hydrolysis of ATP and sperm movement.

Secondly, Melatonin could be a potent cyclic AMP (c, AMP) stimulator *(YUNG , et al .,1995)*. Also, *GURAYA (1987)* observed that melatonin increased c, AMP production in seminal plasma. Cyclic AMP and its enzyme adenyl cyclase were important for sperm motility and viability *(LINEMANN, 1978 and BHATTACHARYYA and PAKRASHI ,1993)*. *LINDEMANN (1978)* reported also, that any substance; such as caffeine when added to semen would stimulate markedly sperm motility through activation of c, AMP production. It was evident that, c, AMP stimulates sperm motility by direct action on the axoneme of the tail *(LINDEMANN, 1978)* or by indirectly acting on the cell membrane as a secondary messenger *(GARBERS and KOPF, 1980)*. Moreover, the sperm cell has three sites of melatonin receptors, in the head, middle piece and microtubular complex in the tail flagellum *(VUURNER, et al., 1992)*.

The third, possible mechanism through calcium ions which play an important role in microtubular fibrils contractility. *YANAGIMACHI (1988)* found that Ca^{+2} ions were essential to mammalian sperm motility. Moreover, *DELGADILLO et al. (1994)* reported that melatonin stimulate cellular influx of Ca^{+2} ions which help and enhance sperm cells motility. The influx of calcium into sperm cells stimulated its motility, therefore, the cytophysiological mechanism of the hormone antimotility action is probably mediated by stimulation of influx of calcium from sperm cells *(RATNSOORIYA and PREMAKUMARA, 1993)*.

The fourth possible mechanism of action may be through protection of sperm cell DNA and plasma membrane. *MOSS and HENDERSON (1993)* reported that the level of acid phosphatase could be used as an indicator for cellular death or damage. In our results, we found a reduction of non-prostatic acid phosphatase after melatonin additions. This observation may be attributed to the protective mechanism of melatonin (especially with 20 μg) to the sperm against any changes in the plasma membrane (Fig. 3 & 4). In addition, *POEGGELER et al. (1993)* reported that melatonin was a potent antioxidant. It was a very potent and efficient

endogenous radical scavenger. It reacted with the highly toxic hydroxyl radical and provides protection against oxidative damage to biomolecules.It was observed that the number of abnormal and dead sperms after ejaculation were reduced after additional of melatonin. This may be coincided with *POEGGELER et al. (1993)* who reported that melatonin had antiaging effect on cells.

Finally it may be concluded that melatonin can be used as an agent for prolonged sperm cell survival as well as indication of sperm motility. Also, it help in conservation of energy in seminal plasma. The ideal concentration of melatonin is 20 μg which may be used with the diluent semen for long time of storage rather than three days. Also, the addition of melatonin may be had a value when added to human semen. Moreover, the effect of melatonin on sperm cells and its receptors need more investigations.

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Table (1) : The effect of melatonin upon sperm motility % stored at 4°C for 6 days.

storage time (days)	control	Treated samples				
		concentration of melatonin				
		1.0 µg	5.0 µg	10.0 µg	15.0 µg	20.0 µg
	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D
1	79.67 ± 0.58	80.33 ± 0.58 n.s (0.83 %)	80.67 ± 1.16 n.s (1.23 %)	84.33 ± 1.16 (5.66 %)	85.33 ± 0.58 (5.66 %)	85.67 ± 1.16 (7.33 %)
2	65.00 ± 1.00	75.00 ± 1.16 (15.38 %)	75.00 ± 2.08 (15.38 %)	79.67 ± 2.08 (23.1 %)	79.67 ± 2.08 (23.1 %)	80.33 ± 2.08 (23.58 %)
3	52.67 ± 4.73	70.00 ± 1.00 (32.29 %)	70.00 ± 1.00 (24.68 %)	75.00 ± 1.00 (42.39 %)	75.00 ± 1.00 (51.89 %)	80.00 ± 1.00 (51.89 %)
4	30.00 ± 1.00	60.00 ± 1.00 (100 %)	60.00 ± 1.00 (83.33 %)	75.00 ± 1.00 (150 %)	75.00 ± 1.00 (150 %)	80.00 ± 1.00 (166.67 %)
5	05.00 ± 1.00	10.00 ± 1.00 (100 %)	15.33 ± 2.52 (206.6 %)	20.00 ± 1.00 (300 %)	50.00 ± 2.00 (900 %)	60.00 ± 1.00 (1100 %)
6	01.00 ± 1.00	01.00 ± 1.00 (0.00%)	05.00 ± 1.00 (400 %)	10.00 ± 1.00 (900 %)	30.00 ± 2.00 (2900 %)	39.67 ± 1.53 (3867 %)

n.s = non-significant.

All parameters are significant at the level of 0.01.

Figures in parentheses indicate per cent increase in motility in treated samples over control levels.

Table (2) : The effect of melatonin upon alive sperm % stored at 4°C for 6 days.

Storage time (days)	control	Treated samples				
		concentration of melatonin				
		1.0 µg	5.0 µg	10.0 µg	15.0 µg	20.0 µg
	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D
1	81.79 ± 1.85	81.80 ± 0.89 n.s (0.01 %)	82.72 ± 0.94 n.s (1.14 %)	85.98 ± 0.49 (5.12 %)	86.98 ± 0.81 (6.35 %)	87.17 ± 0.75 (6.57 %)
2	68.29 ± 0.47	78.22 ± 0.21 (14.54 %)	79.19 ± 0.17 (15.96 %)	80.77 ±0.39 (18.28 %)	81.56 ± 0.51 (19.43 %)	81.97 ± 0.01 (20.00 %)
3	58.49 ± 0.05	72.56 ± 0.51 (24.06 %)	77.84 ± 1.61 (39.92 %)	78.15 ± 0.74 (33.61 %)	79.99 ± 0.99 (36.76 %)	81.28 ± 0.49 (38.96 %)
4	32.45 ± 0.51	58.14 ± 1.03 (79.17 %)	66.59 ± 1.47 (105.21 %)	75.72 ± 1.03 (133.30 %)	78.16 ± 0.37 (140.86 %)	79.39 ± 0.45 (144.70 %)
5	25.88 ± 0.96	33.15 ± 0.36 (28.09 %)	34.53 ± 0.50 (33.42 %)	49.42 ± 0.37 (90.95 %)	67.64 ± 0.35 (161.36 %)	70.75 ± 0.65 (173.40 %)
6	22.49 ± 0.5	28.58 ± 0.36 (27.10 %)	30.15 ± 0.27 (34.00 %)	44.74 ± 1.58 (98.93 %)	50.79 ± 1.58 (125.80 %)	60.39 ± 1.62 (168.50 %)

n.s = non-significant.
All parameters are significant at the level of 0.01.
Figures in parentheses indicate per cent increase in alive sperm in treated samples over control levels.

Table (3) : The effect of melatonin upon sperm abnormalities % stored at 4°C for 6 days.

Storage time (days)	control	Treated samples				
		concentration of melatonin				
		1.0 µg	5.0 µg	10.0 µg	15.0 µg	20.0 µg
	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D
1	12.03 ± 0.89	12.01 ± 0.24 n.s (0.17 %)	09.47 ± 0.45 (21.28 %)	09.15 ± 0.22 (23.94 %)	09.04 ± 0.12 (24.85 %)	08.24 ± 0.25 (31.50 %)
2	13.14 ± 0.91	11.31 ± 0.97 (13.93 %)	10.53 ± 0.41 (19.86 %)	09.60 ± 0.55 (26.94 %)	09.18 ± 0.15 (30.14 %)	08.46 ± 0.78 (35.61 %)
3	16.25 ± 0.56	11.37 ± 1.90 (31.29 %)	11.25 ± 0.45 (32.02 %)	10.76 ± 1.08 (34.98 %)	10.16 ± 1.59 (38.61 %)	09.95 ± 0.13 (39.88 %)
4	19.65 ± 0.61	14.29 ± 0.39 (27.28 %)	13.22 ± 0.47 (32.72 %)	11.69 ± 0.92 (40.51 %)	10.85 ± 0.22 (44.78 %)	10.43 ± 0.71 (46.92 %)
5	20.62 ± 0.54	14.64 ± 0.35 (29.00 %)	13.65 ± 0.56 (33.80 %)	11.96 ± 0.18 (41.99 %)	11.31 ± 0.59 (45.15 %)	10.64 ± 0.47 (48.39 %)
6	25.79 ± 0.68	18.09 ± 0.35 (29.86 %)	14.14 ± 0.43 (45.17 %)	13.54 ± 0.43 (47.49 %)	12.35 ± 0.33 (52.11 %)	11.17 ± 1.19 (56.67 %)

n.s = non-significant. All parameters are significant at the level of 0.01.
Figures in parentheses indicate per cent decrease in abnormalities in treated samples over control levels.

Table (4) : The effect of melatonin upon fructose content (mg/100 ml) in seminal plasma stored at 4°C for 6 days.

Storag time (days)	control	Treated samples				
		concentration of melatonin				
		1.0 µg	5.0 µg	10.0 µg	15.0 µg	20.0 µg
	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D
1	447.33 ± 2.08	449.00 ± 14.0$^{n.s}$ (0.45 %)	455.67 ± 5.13$^{n.s}$ (2.09 %)	456.33 ± 5.50$^{n.s}$ (1.86 %)	457.67 ± 14.16$^{n.s}$ (2.31 %)	466.33±5.51 (4.25 %)
2	443.67 ±10.97	446.00 ± 5.51$^{n.s}$ (0.59 %)	451.00 ± 9.54$^{n.s}$ (1.65 %)	456.00 ± 6.00$^{n.s}$ (2.78 %)	456.33 ± 6.81$^{n.s}$ (2.83 %)	463.00±6.08 (4.36 %)
3	432.33 ± 4.93	437.00 ± 5.29$^{n.s}$ (0.25 %)	438.00 ± 2.65$^{n.s}$ (1.31 %)	444.33 ± 19.14$^{n.s}$ (2.78 %)	450.00 ± 8.96 (4.16 %)	460.00±2.65 (6.40 %)
4	417.63 ± 6.63	427.67 ± 3.79$^{n.s}$ (2.40 %)	432.67 ± 5.51$^{n.s}$ (3.60 %)	444.00 ± 5.13 (6.31 %)	450.00 ± 6.08 (7.75 %)	459,67±8.51 (10.07 %)
5	406.90 ± 4.54	410.67 ± 9.71$^{n.s}$ (0.93 %)	415.33 ± 16.44$^{n.s}$ (2.07 %)	435.67 ± 3.22 (7.07 %)	445.00 ± 4.09 (9.36 %)	450.33±1.53 (10.67 %)
6	354.33 ±12.50	378.00 ± 2.65 (6.68 %)	393.33 ± 5.77 (11.01 %)	415.67 ± 4.93 (17.31 %)	432.67 ± 5.51 (22.11 %)	439.00±1.00 (23.90 %)

n.s = non-significant. All parameters are significant at the level of 0.01.
Figures in parentheses indicate per cent increase in fructose content in treated samples over control levels.

Table (5): The effect of melatonin upon acid phosphatase (U/L) in seminal plasma stored at 4°C for 6 days.

Storage time (days)	control	Treated samples				
		concentration of melatonin				
		1.0 µg	5.0 µg	10.0 µg	15.0 µg	20.0 µg
	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D
1	31.78 ± 1.68	25.48 ± 0.50 (19.82 %)	24.66 ± 0.59 (22.40 %)	19.68 ± 0.59 (38.80 %)	18.35 ± 0.67 (42.26 %)	17.29 ± 0.52 (45.59 %)
2	38.79 ± 1.06	31.07 ± 0.89 (19.90 %)	29.17 ± 0.77 (24.80 %)	26.39 ± 0.54 (31.97 %)	21.60 ± 1.56 (44.32 %)	20.27 ± 0.05 (47.74 %)
3	39.39 ± 0.53	33.07 ± 0.38 (16.00 %)	31.75 ± 0.75 (19.39 %)	30.73 ± 0.45 (21.99 %)	25.31 ± 0.19 (36.75 %)	24.13 ± 4.67 (38.74 %)
4	41.87 ± 1.21	39.46 ± 0.51* (5.76 %)	35.98 ± 1.00 (14.07 %)	33.01 ± 1.00 (21.16 %)	30.73 ± 1.09 (26.61 %)	28.46 ± 0.51 (32.03 %)
5	45.68 ± 0.83	43.65 ± 0.57* (7.20 %)	42.39 ± 0.61 (17.20 %)	39.05 ± 0.29 (14.51 %)	37.52 ± 0.50 (17.86 %)	35.88 ± 0.95 (21.45 %)
6	49.15 ± 0.78	47.31 ± 1.61 n.s (7.28 %)	45.57 ± 0.52 (7.28 %)	39.77 ± 0.69 (19.08 %)	39.04 ± 0.13 (20.57 %)	36.58 ± 0.56 (25.57 %)

n.s = non-significant. * = P <0.05 All parameters are significant at the level of 0.01. Figures in parentheses indicate per cent decrease in ACP levels in treated samples over control levels.

Table (6):The effect of melatonin upon ATPase (μmol/ml/h) in seminal plasma stored at 4°C for 6 days.

Storae time (days)	control	Treated samples concentration of melatonin				
		1.0 μg	5.0 μg	10.0 μg	15.0 μg	20.0 μg
	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D
1	8.40 ± 0.44	8.42 ± 0.10$^{n.s}$ (0.24 %)	8.47 ± 0.29$^{n.s}$ (0.83 %)	8.57 ± 0.15$^{n.s}$ (2.00 %)	8.57 ± 0.15$^{n.s}$ (2.00 %)	8.58 ± 0.01$^{n.s}$ (2.10 %)
2	7.43 ± 0.38	7.60 ± 0.44$^{n.s}$ (2.20 %)	7.80 ± 0.17$^{n.s}$ (4.10 %)	8.17 ± 0.12 (9.96 %)	8.19 ± 0.01 (10.23 %)	8.23 ± 0.01 (10.77 %)
3	7.40 ± 0.29	7.59 ± 0.10$^{n.s}$ (2.57 %)	7.69 ± 0.01$^{n.s}$ (3.92 %)	7.83 ± 0.06 (5.81 %)	8.10 ± 0.01 (9.46 %)	8.20 ± 0.10 (10.81 %)
4	7.33 ± 0.17	7.53 ± 0.01$^{n.s}$ (2.66 %)	7.60 ± 0.01$^{n.s}$ (3.68 %)	7.65 ± 0.12* (4.37 %)	7.83 ± 0.01 (6.82 %)	7.93 ± 0.01 (8.19 %)
5	7.19 ± 0.10	7.23 ± 0.12$^{n.s}$ (0.56 %)	7.43 ± 0.01$^{n.s}$ (3.34 %)	7.53 ± 0.01* (4.73 %)	7.73 ± 0.01 (7.51 %)	7.83 ± 0.01 (8.90 %)
6	6.97 ± 0.06	6.99 ± 0.42$^{n.s}$ (0.29 %)	7.23 ± 0.01$^{n.s}$ (3.73 %)	7.29 ± 0.01* (4.59 %)	7.50 ± 0.01 (7.60 %)	7.67 ± 0.21 (10.04 %)

n.s = non-significant. * = P <0.05

All parameters are significant at the level of 0.01.

Figures in parentheses indicate per cent increase in ATPase levels in treated samples over control levels.

Table (7) :The effect of melatonin upon Hyaluronic acid (μg/L) in seminal plasma stored at 4°C for 6 days.

Storage time (days)	control	Treated samples concentration of melatonin				
		1.0 μg	5.0 μg	10.0 μg	15.0 μg	20.0 μg
	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D
1	40.33 ± 1.53	40.37 ± 0.11[n.s] (0.09 %)	40.83 ± 0.01[n.s] (1.23 %)	41.70 ± 0.01 (3.40 %)	43.10 ± 0.10 (6.87 %)	44.30 ± 0.10 (9.84 %)
2	33.10 ± 0.01	34.83 ± 0.38 (5.23 %)	36.47 ± 0.31 (10.18 %)	38.30 ± 0.26 (15.70 %)	41.27 ± 0.01 (24.68 %)	42.09 ± 0.45 (27.20 %)
3	30.49 ± 0.46	33.23 ± 0.41 (8.99 %0	34.64 ± 0.53 (11.98 %)	34.89 ± 0.46 (14.43 %)	38.29 ± 0.39 (25.28 %)	40.50 ± 0.50 (32.80 %)
4	21.09 ± 0.65	29.89 ± 0.56 (41.70 %)	32.56 ± 0.51 (54.39 %)	33.46 ± 0.43 (58.65 %)	35.40 ± 0.35 (67.85 %)	36.51 ± 0.46 (73.11 %)
5	20.21 ± 0.74	22.40 ± 0.53 (11.28 %)	23.29 ± 0.31 (15.24 %)	26.18 ± 0.37 (29.50 %)	32.48 ± 0.43 (60.70 %)	33.74 ± 0.38 (66.95 %)
6	19.86 ± 0.24	21.43 ± 0.50 (7.91 %)	21.57 ± 0.49 (7.91 %)	24.39 ± 0.47 (22.80 %)	28.52 ± 0.40 (43.61 %)	31.09 ± 0.18 (56.55 %)

n.s = non-significant. All parameters are significant at the level of 0.01.
Figures in parentheses indicate per cent increase in Hyaluronic acid levels in treated samples over control levels.

Figure (1) : Effect of different concentrations of Melatonin upon :

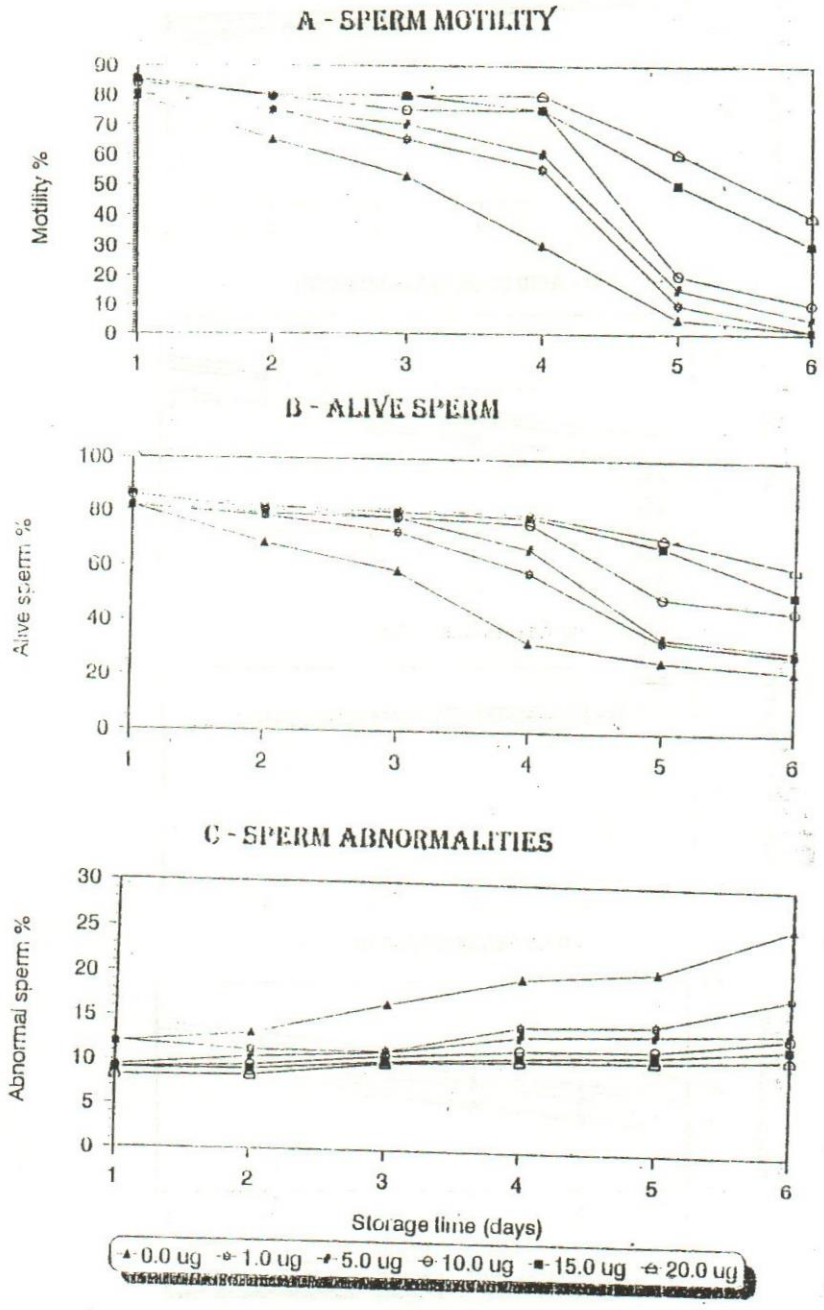
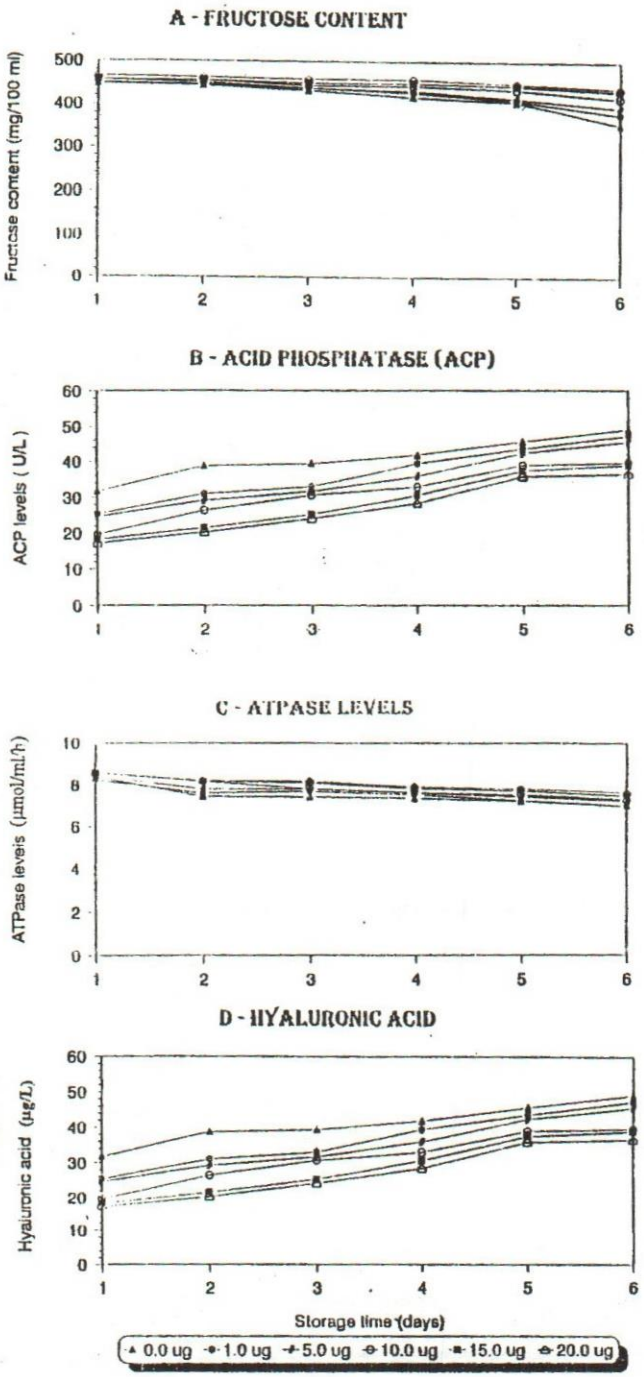
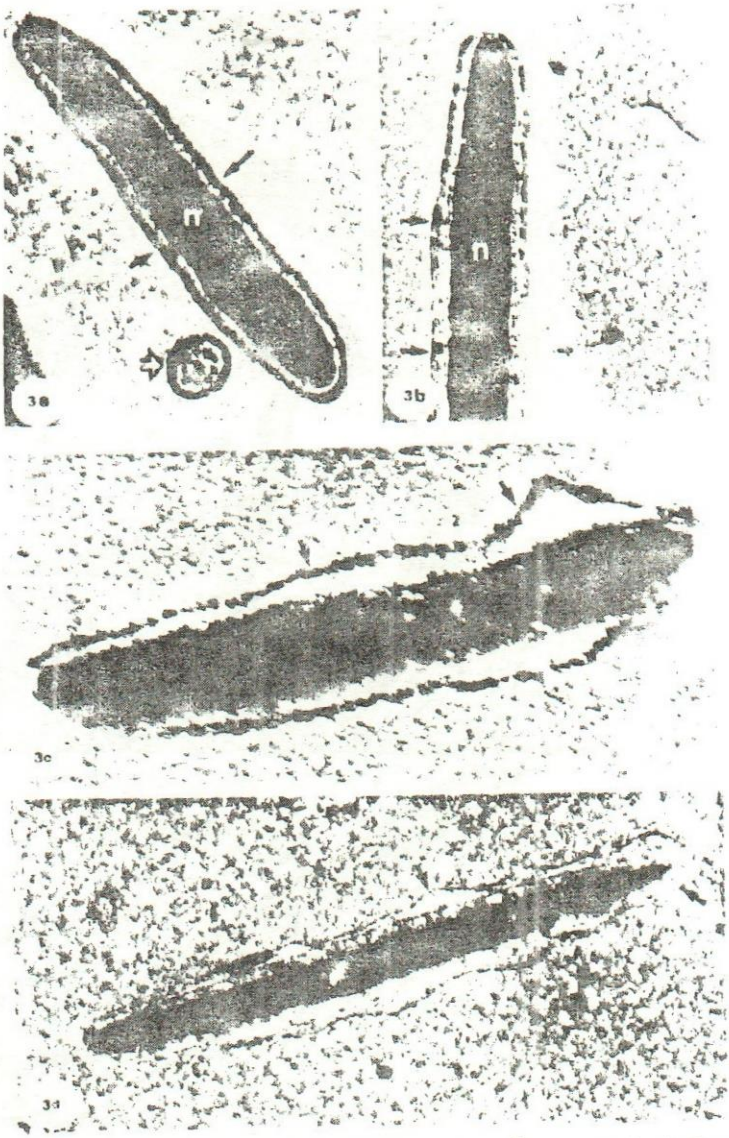


Figure (2) : Effect of different concentrations of Melatonin upon :

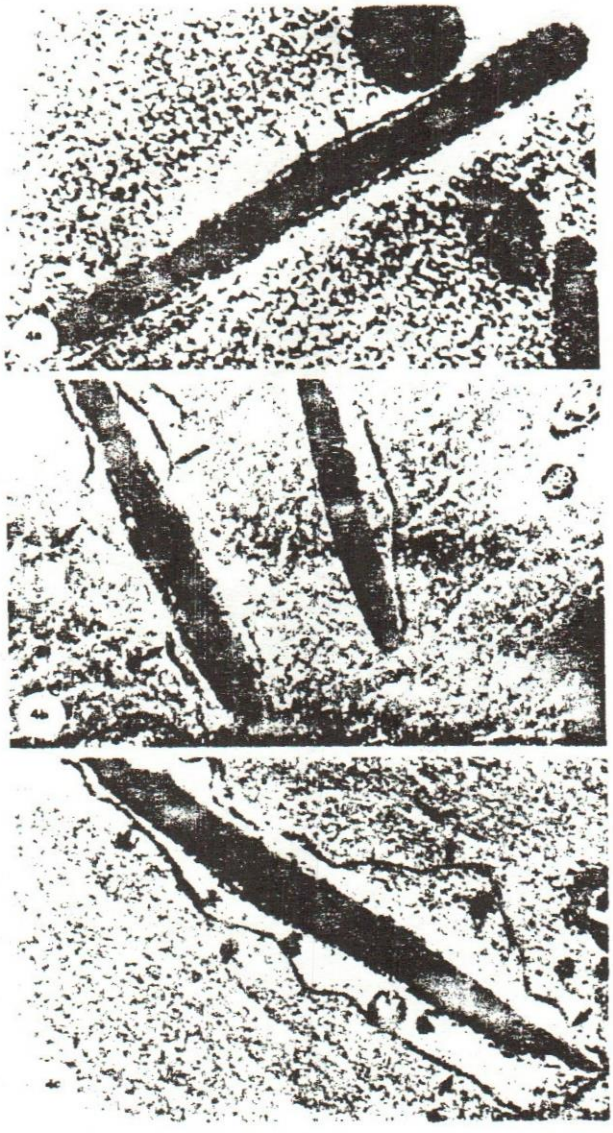


3) : Electron micrograph of bull semen under the effect of different concentrations of melatonin stored at 4°C for 3 days.



a) Showing the normal ultrastructure of the plasma membrane (↑) cross section in the tail piece (⇒).
b) Nearly intact sperm plasma membrane (↑) when treated with 20 µg melatonin (n= nucleus).
c) Swelling of the sperm plasma membrane (↑) when treated with 10 µg melatonin.
d) Severe swelling and disintegration of the sperm plasma membrane at treated with 1 µg melatonin.
Magnification : a) X112000 , b) X78400 , c) X112000 , d) X112000.

Figure (4) : Electron micrograph of bull semen under the effect of different concentrations of melatonin stored at 4°C for 5 days.



a) nearly intact sperm plasma membrane (↑) when treated with 20 µg melatonin.
b) Swelling and mild disintegration of the sperm plasma membrane (↑) when treated with 10 µg melatonin.
c) great swelling and sever disintegration of the sperm plasma membrane (↑) when treated with 1µg melatonin
Magnification : a) X 78400, b) X 56000 , c) X 78400.