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EFFECT OF CALCIUM CHANNEL BLOCKER ON QUALITY OF EGYPTIAN BUFFALO LIQUID SEMEN (ROLE OF CALCIUM ON SPERM APOPTOSIS)

(With 10 Tables, 12 Figures and 2 Plates)

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تأثيرمثبطات مجرى الكالسيوم على خاصية السائل المنوى المخفف لعجول الجاموس المصرى ودور الكالسيوم فى موت الحيوان المنوى

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تمت دراسة تأثير مثبطات مجرى الكالسيوم على السائل المنوى المخفف لعجول الجاموس المصرى وكذا دوره على موت الحيوانات المنوية أثناء حفظه فى درجة حرارة ٤°م ولمدة ستة ايام . فتم تخفيف السائل المنوى للحصول على تركيز ١٠٠ × ١٠٦ حيوان منوى لكل واحد ملليمتر ثم أضيفت مادة مثبطات مجرى الكالسيوم بتركيزات ٥ ، ١٠ ، ١٥ ، ٢٠ ، ٣٠ ميكروجرام لكل ١٠٠ × ١٠٦ حيوان منوى وأيضاً عينة ضابطة. وفحصت جميع العينات يومياً ولمدة ستة أيام بعد حفظها فى درجة حرارة ٤°م. وأظهرت النتائج أن مادة مثبطات مجرى الكالسيوم ذات تأثير واضح ومعنوى فى رفع وتحسين الصفات والخواص للحيوانات المنوية وخاصة التركيزات العالية (٢٠ ، ٣٠ ميكروجرام) وساعدت فى تحسين الحركات الذاتية وتقليل نسب تفتيت القلنصوة للحيوانات المنوية بعد حفظه لمدة طويلة . كما ساعدت على تقليل مستوى LDH وأيضاً Lipid peroxide فى بلازما السائل المنوى وذلك دلالة على مقدرة التركيزات العالية لمثبطات مجرى الكالسيوم فى المحافظة على أغشية الحيوانات المنوية من التحطيم والتى أظهرتها الفحوصات بالمجهر الالكترونى .

SUMMARY

The effect of calcium (Ca^{2+}) blocker upon the buffalo-bull liquid semen was studied through increasing the life-span of sperm during

storage with keeping the penetrating ability and decreasing the acrosomal and membrane integrity. Semen was diluted and mixed with 0.0, 5.0, 10.0, 15.0, 20.0 and 30.0 µg Ca^{2+} blocker/100 x 10^6 sperm. The samples were stored at 4°C for 6 days and examined daily. The obtained results revealed that, sperm motility, alive sperm percentages and sperm penetrating ability were improved and increased significantly with the concentrations of Ca^{2+} blocker especially higher concentrations (20 and 30 µg). In addition, significant decrease in sperm abnormalities and acrosomal integrity percentages were observed after adding Ca^{2+} blocker. LDH and lipid peroxide levels were decreased significantly with the higher concentrations of Ca^{2+} blocker. Moreover, the ultrastructure of treated sperm head showed that, the high concentration of Ca^{2+} blocker had a better protective effect against membrane integrity. The effect of Ca^{2+} blocker on sperm cells may be through blockage of Ca^{2+} uptake with subsequent protection from damage (apoptosis) and indirect through preventing more cell damage during storage which in turn decreased free radical oxygen that had a dangerous effect upon sperm.

Key words: *Egyptian buffalo - Semen quality - Calcium channel blocker*

INTRODUCTION

The artificial insemination (A.I) industry enhances the longevity of semen by cryopreservation, but semen may also be stored in a liquid state using reduced temperature or other means to depress metabolism. However, metabolism is not completely arrested during liquid storage; the main changes which occur include an irreversible reduction in motility, morphological integrity and fertility of spermatozoa (Maxwell and Stojanov, 1996). These changes may be contributed to the accumulation of the toxic metabolic products and, more importantly,from the reactive oxygen (Maxwell and Stojanov, 1996). These changes may be contributed to the accumulation of the toxi metabolic products and, more importantly, from the reactive oxygen (Maxwell and Salamon, 1993). A significant correlation has been observed between the percentage of spermatozoa with intact acrosomes and fertility (Whitfield and Parkinson, 1992), while the

ability of spermatozoa to penetrate cervical mucus (Aitken et al., 1986) has also been employed to predict fertility of semen.

The stresses in cryopreservation causes damage to sperm structure which might be involved in reducing the fertilizing ability of preserved spermatozoa (Shannon and Vishwanoth, 1995 and Zhao and Buhr, 1996). These stresses also affect the ability of spermatozoa to regulate calcium (Robertson and Watson, 1986) and formation of lipid peroxides which are extremely toxic to spematozoa. Lipid peroxides are related to loss of membrane, structural damage to DNA and destroy the structure of the lipid matrix which associated with loss of motility (Jones and Mann, 1977; Wishart, 1984 and White, 1993). Furthermore, the direct action of lipid peroxides can be observed on the enzymes which lead to inactivation of enzymes in spermatozoa, in particular those that are membrane-bound and contain sulfhydryl (SH) groups (Jones and Mann, 1977).

Ultrastructural changes in the spermatozoa plasma membrane resulting from the preservation may disrupt Ca^{2+} dependent phenomena, contributing to the reduced fertilizing capacity (Buhr and Zhao, 1992). Certain aspects of Ca^{2+} flux were correlated with fertility (Bailey and Buhr, 1994) and other aspects of Ca^{2+} flux were correlated wtih motility. The change in membrane permeability during storage may cause an increase in the intracellular Ca^{2+} concentration with changes in the head as well as a significant levels of acrosomal integrity (Fraser and McDermott, 1992; Adeoya-Osiguwa and Fraser, 1993; Fuller and Whittingham, 1996 and Januskauskas et al., 1996).

Apoptosis is a programmed cell death, including cell shrinkage and nuclear fragmentation (Howie et al., 1994). Apoptosis commonly involves the activation of endonucleases which lead to DNA fragmentation (Oberhammer et al., 1993). Ca^{2+} ion is one of the most important factor stimulating endonucleases activities and thereby the apoptosis (Gaido and Cidlowski, 1991). The high concentrations of Ca^{2+} in the external media may decrease the motility of sperm (Tash and Means, 1983), presumably by rasing intracellular Ca^{2+} levels and this may be a factor in sperm apoptosis (DasGupta et al., 1994).

The aim of the present work was to increase the life-span of liquid buffalo semen during preservation. This was carried out by using calcium channel blocker (CCB) to keep the fertilizing capacity of the spermatozoa through increasing their penetrating ability

together with minimizing the decrease in the integrity of sperm cell membranes and acrosome.

MATERIAL and METHODS

Semen collection:

Semen samples were obtained from two healthy buffalo bulls raised in the artifical insemination center, at the local Vet. service, Assiut. The animals were under the same conditions of vaccination, management and nutrition. They appeared in good condition and nearly of similar age (6-8 years). The semen was collected twice weekly for three weeks at early morning using artifical vagina and female buffalo used as a teaser. Immediately after collection, the samples were placed in a water bath and transferred to the laboratory.

Semen evaluation:

Semen quality was evaluated according to Ahmad et al. (1996). Ejaculates having less than 60% motility were discarded and the good quality samples were pooled before dilution for the subseqent examination. Morphology and alive sperm percentages were assessed by using alkaline methyl violet and eosin-nigrosin stains respectively. Acrosomal integrity was determined by using Giemsa staining technique according to Watson (1975). The sperm mucus penetration test was estimated by using cappilary tubes and calibrated microscope slide according to Dev et al. (1996).

Semen extension and treatment:

The pooled semen was extended with a Russian diluent to give a final concentration of 100×10^6 sperm/ml according to Azawi et al. (1990). The calcium channel blocker (Diltiazem hydrochloride, Sigma Chem. Co., USA) was added to the extended semen as 0.0 µg (control sample), 5, 10, 15, 20 and 30 µg/100×10^6 sperm. These serial doses were suggested according to Verheyen (1996). Three samples from diluted pooled semen were prepared for each of the above mentioned, concentration and control. All samples were stored in refrigerator (4°C) and examined daily for 6 days for sperm motility, livability and abnormalities (secondary abnormalities especially free loss head and bent tail) percentages. In addition, assessment of acrosomal integrity and sperm mucus penetration test were performed.

Biochemical analysis:

After examination, the remainder of samples were centrifuged at 3000 rpm for 20 minutes. The supernatant fluid was collected and kept at -20°C till used for determination of fructose content according to Bergmeyer (1974), Ca^{2+} and Mg^{2+} according to Fraser et al. (1987) and Gindler (1971) respectively, lactate dehydrogenase (LDH) by using commercial kit No. 0940, Stanbio Lab. INC., San Antonio, Texas. Lipid peroxide concentration was determined by the malonaldehyde (MDA) level according to Benge and Aust (1978).

Electronic microscopical examination:

After centrifugation, the sediment was prepared, in the unit of Electronic Microscope, Assiut Univ., and divided into two parts. The first part was prepared for examination by transmission electron microscope for any changes in the plasma membrane of spermatozoa after being stained by uranyl acetate and lead citrate. The second part was prepared for X-rays analysis by using scanning technique and Link ISIS programme for intracellular Ca^{2+} and Mg^{2+}.

Statistical analysis:

Data were expressed as the mean ± S.D for all treatments. Analysis of variance (ANOVA) for all treatments was done and differences between treatments were analysed by least significant difference (LSD) using PC-stat computer programme. Results were considered significant at $P< 0.05$ or less.

RESULTS

The obtained results in this study are presented in tables 1-10 and figures 1-12. Sperm motility % (S.M%) was affected by the addition of CCB (Table 1 and Fig. 1). S.M % increased significantly ($P< 0.01$) with all CCB concentrations and all days of storage at 4°C. It was observed that, the increase of S.M % was noticed with high concentration of CCB till the last days of incubation when compared with the control samples at the same day of storage. All concentrations of CCB had a significant increasing ($P< 0.01$) effect on the alive sperm % (A.S %) except 5.0 µg CCB which has a non-significant effect (table 2 and figure 2). High concentration of CCB (20 and 30 µg) had a highest effect on the A.S % among all days of storage. However, 10 and 15 µg of CCB has a highest effect till 4th

and 5th days of storage respectively. Sperm abnormalities % (S.Ab %) reduced by the addition of CCB to the liquid buffalo semen (table 3 and figure 3). All the concentrations of CCB had a significant ($P< 0.01$) reduciable effect, however, 5.0 µg CCB had a non-significant effect at first and second days of storage.

The variations in the effect of CCB upon the acrosomal integrity % were presented at table (4) and figure (4). CCB had a significant decreasing ($P< 0.01$) effect on the acrosomal integrity % with all concentrations among storage time when compared with the control samples at the same days. The changes in increasing ability of sperm penetration distance through cervical mucus were illustrated in table (5) and figure (5). The overall means of sperm penetration distance were significantly increased ($P< 0.01$) with addition of all concentrations of CCB except 5.0 µg CCB at 4th and 5th or 6th days of storage which had a non-significant effect.

Calcium and Magnisum levels in seminal plasma were presented in tables (6 and 7) and figures (6 and 7). Addition of 5 µg and 10 µg CCB not altered significantly the levels of Ca^{2+} and Mg^{2+} in seminal plasma in all days of storage except at last days of storage with 10 µg CCB when compared with the control samples. Also, all concentrations of CCB had a non-significant decreasing effect on the Ca^{2+} levels during first and second days of storage, however, in case of Mg^{2+}, there was a non-significant effect with all concentrations of CCB except 30 µg among first day of storage.

Table (8) and figure (8) showed the fructose levels in seminal plasma after additon of CCB. It was observed that, the overall means of fructose were increased significantly ($P<0.01$) from control in each day to the same concentration and in the same day with all concentrations. The changes in the levels of LDH and MDA were illustrated in tables (9 and10) and figures (9 and 10) respectively. The significant decrease ($P<0.01$) was noticed for each of LDH and MDA levels from control in all concentrations among the first 3 days of storage in case of LDH but among all days of storage in case of MDA. LDH levels were decreased significantly ($P< 0.01$) in all concentrations and among 4th, 5th and 6th days of storage. However, 5.0 µg CCB had a non-significant effect at 4th and 5th days of storage but at 4th day of storage only in case of 10 µg CCB.

The ultrastructure examination of treated and control samples after 2, 4 and 6 days of storage (Plates 1 and 2) revealed that, the protective effect of CCB for sperm aganist plasma membrane integrity increase with high concentration (30 μg) of CCB when compared with control. The lower concentration (5 μg) of CCB had a bad effect which observed in the form of severe disintegration of the sperm plasma membrane. X-rays analysis of control and treated samples (Figures 11 and 12), revealed that, the CCB somewhat protect the intracellular Ca^{2+}% especially with the high concentration of CCB, however, the control sample showed firstly increasing of intracellular Ca^{2+}% which decreased rapidly with the advance of storage time.

DISCUSSION

Many investigators have reported that mammalian spermatozoa deteriorate as a result of extensive dilution in artificial media (Harrison et al., 1978 and Ashworth et al., 1994). The presence of Ca^{2+} in seminal plasma of diluted semen accelerates acrosomal and membranal damage or exocytosis during storage (Fraser and McDermott, 1992). For this reason, the principle aim of the present study was to find a means for preventing the accelerated acrosomal damage and death of spermatozoa (apoptosis) after dilution.

In the present study, the obtained results revealed that, the addition of Ca^{2+} blocker decrease the plasma membrane and acrosomal integrity as well as decrease the sperm abnormalities. Moreover, the damaged or dead spermatozoa proved to have a high intracellular calcium [$(Ca^{2+})_i$] level which then rapidly decreased. These results were in agreement with Fuller and Whittingham (1996), Whitfield and Parkinson (1995) and Zhong et al. (1993). They reported that, the changes in the membrane permeability during storage may cause an increase in the $(Ca^{2+})_i$ level due to decrease in the cell ability to maintain its normal low level. The rapidly decrease of $(Ca^{2+})_i$ level can be attributed to severe damage of plasma cell membrane during storage (DasGupta et al., 1994). This can be inhibited by Ca^{2+} blocker substance as well as increase the alive sperm percentage with decreasing acrosomal integrity (Zhong et al., 1993). The increasing of $(Ca^{2+})_i$ with CCB treatment, especially with 30μg can be attributed to the presence of other sources Ca^{2+} inside the cell

as cytosol, sarcoplasmic reticulum and mitochondria (Verheyen, 1996).

The controlling of Ca^{2+} flux in the head of diluted spermatozoa was more critical to fertilization than Ca^{2+} control in the tail (Zhao and Buhr, 1996). Moreover, Ca^{2+} flux was correlated with motility and fertility (Bailey and Buhr, 1994 and Bailey et al., 1994). The increase Ca^{2+} influx is considered as apoptotic stimuli which induce translocation of cytochrome C into the cytoplasm and subsequent activation of endonuclease enzyme. These processes are necessary for DNA cleave between nucleosomes with apoptotic effects (Green, 1997, Kumar and Lavin, 1996, Oberhammer et al., 1993 and Peistch et al., 1993) . The above mentioned reasons might be interpreted to the obtained results which observed that, the highest Ca^{2+} blocker concentration had a significant decreasing effect on acrosomal and cell membrane integrity as well as increasing effect on the sperm survival percentage.

In this work, a low concentrations of LDH were negatively correlated with the high concentration of Ca^{2+} blocker during storage. This is in agreement with Dhami and Kadagali (1990) and Upreti et al. (1995 & 1996). They reported that LDH is predominantly localized in cytosol of spermatozoal membrane and its leakage has been correlated with cell membrane damage as well as there are a negative correlation between the leakage of LDH and sperm motility and sperm survival percentages after dilution. This was evident in the present study that, with a high concentration of Ca^{2+} blocker, the percentages of sperm motility and survivability were increased and decreased of LDH leakage.

Lipid peroxide levels (MDA), in this study, decreased in treated samples when compared with control as well as the higher concentration of Ca^{2+} blocker had a significant decreasing effect on MDA and increasing effect on sperm motility and alive sperm percentages than the lower. These results are in agreement with Slaweta et al. (1988) and White (1993). They concluded that, the lipid peroxidation produced a harmful agents responsible for damage of cell and DNA. Moreover, peroxidation brings to irreversible loss of sperm motility due to loss of cytosolic enzymes and essential substrates such as adenine and pyridine nucleotides. In addition, it

also influences sperm fertility where decreased the penetrating ability (Aitken and Clarkson, 1987).

In conclusion, it might be considered that, calcium channel blocker might act for prolonging the sperm survival time of buffalo semen and delayed its apoptosis during storage. This is occurred through direct mechanism via blocked of Ca^{2+} uptake and subsequent protect its damage. The indirect mechanism was observed through prevention of more sperm damage which in turn decreased the free radical oxygen that leads to decrease the lipid peroxidation.

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EXPLANATION OF PLATES

Plate I:

Fig. 1: Sagital section through the head of buffalo-bull spermatozoa (at time zero) with intact and normal plasma membrane.

Fig. 2: Sagital section through the head of untreated buffalo-bull spermatozoa after 2 days of storage and showing swelling and broken of the plasma membrane.

Fig. 3 to 5: Sagital section through the head of treated buffalo-bull spermatozoa after 2 days of storage.

Fig. 3: Shows swelling and rupture of plasma membrane (arrow head) (treated with 5 µg CCB).

Fig. 4: Showing corrugate plasma membrane but intact (treated with 15µg CCB).

Fig. 5: Showing nearly normal plasma membrane (treated with 30 µg CCB).

Plate II:

Fig. 1: Sagital section through the head of untreated buffalo-bull spermatozoa after 4 days of storage. Note that severe swelling and broken plasma membrane (arrow).

Fig. 2 to 4: Sagital section through the head of treated buffalo-bull spermatozoa after 4 days of storage.

Fig. 2: Showing moderate swelling of plasma membrane (arrow head) (treated with 5 µg CCB).

Fig. 3: Showing slight swelling of plasma membrane (arrow head) (treatd wtih 15 µg CCB).

Fig. 4: Showing normal intact plasma membrane (treated with 30 µg CCB).

Fig. 5: Sagital section through the head of untreated buffalo-bull spermatozoa after 6 days of storage. Note that complete loss of the inner plasma membrane and sever swelling of the outer plasma membrane (*) with disintegration of acrosomal membrane (arrow head).

Fig. 6 to 8: Sagital section through the head of treated buffalo-bull spermatozoa after 6 days of storage.

Fig. 6: Showing slight swelling of the outer (arrow head) plasma membrane (treated with 5 µg CCB).

Fig. 7: Showing intact but corrugate plasma membrane (treated with 15µg CCB).

Fig. 8: Showing intact plasma membrane like normal (treated with 30µg CCB).

Table (1): Effect of CCB on sperm viability* % stored at 4°C for 6 days.

Storage time (days)	control (n = 18)	Treated samples concentration of CCB				
		5.0 µg (n =18)	10.0 µg (n =18)	15.0 µg (n =18)	20.0 µg (n =18)	30.0 µg (n =18)
	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D
1	52.67±2.52	61.33 ± 1.53	62.67 ± 0.58	66.33 ± 2.31	67.67 ± 1.53	70.00 ± 1.00
2	47.67±2.52	57.33 ± 3.79	62.33 ± 2.08	62.89 ± 3.61	63.00 ± 4.58	68.99 ± 1.01
3	40.33±5.68	52.00 ± 7.01	54.33 ± 1.53	59.00 ± 4.36	60.00 ± 4.35	64.00 ± 1.05
4	38.00±2.65	48.67 ± 5.53	49.67 ± 2.52	52.00 ± 1.00	56.00 ± 8.15	57.67 ± 3.03
5	28.67±5.51	40.67 ± 2.08	43.33 ± 6.11	49.00 ± 1.00	51.33 ± 2.08	53.33 ± 4.51
6	14.67±4.51	26.33 ± 3.06	29.33 ± 3.21	33.67 ± 3.22	35.00± 2.65	45.98± 3.61

All parameters are significant at the level of 0.01.

* before storage = 71.66 ± 2.88 %

Table (2): Effect of CCB upon alive sperm* % stored at 4°C for 6 days.

Storage time (days)	control (n =18)	Treated samples concentration of CCB				
		5.0 µg (n =18)	10.0 µg (n =18)	15.0 µg (n =18)	20.0 µg (n =18)	30.0 µg (n =18)
	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D
1	70.95±1.73	72.65±0.86$^{N.S}$	76.72 ± 1.37	78.30 ± 0.65	79.56 ± 0.68	80.82 ± 0.81
2	67.15±3.21	69.31±0.42$^{N.S}$	73.47 ± 1.76	76.96 ± 1.58	78.89 ± 0.28	79.93 ± 1.19
3	61.73±0.88	63.71±1.21$^{N.S}$	68.78 ± 2.79	74.58 ± 2.31	76.41 ± 0.67	78.85 ± 0.25
4	53.40±0.95	55.25±1.77$^{N.S}$	65.94 ± 3.13	68.23 ± 2.82	73.19 ± 3.44	75.75 ± 1.97
5	44.28±2.01	48.98±3.17$^{N.S}$	58.84 ± 7.01	65.98 ± 6.52	69.68 ± 3.84	72.43 ± 2.61
6	33.07±1.78	34.83±3.67$^{N.S}$	49.85 ± 8.55	58.44 ± 2.88	60.14 ± 4.05	63.74 ± 3.35

N.S = Non-significant

All parameters are significant at the level of 0.01.

* before storage = 84.43 ± 1.04

Table (3): Effect of CCB on sperm abnormalities* % stored at 4°C for 6 days.

Storage time (days)	control (n =18)	Treated samples concentration of CCB				
		5.0 µg (n =18)	10.0 µg (n =18)	15.0 µg (n =18)	20.0 µg (n =18)	30.0 µg (n =18)
	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D
1	19.85±0.44	18.95±0.01$^{N.S}$	18.76 ± 0.19	18.69 ± 0.20	18.53 ± 0.48	18.41 ± 0.10
2	21.99±0.26	20.77±0.22$^{N.S}$	19.15 ± 0.32	18.94 ± 0.05	18.87 ± 0.10	18.52 ± 0.18
3	24.95±0.36	22.65 ± 0.79	20.87 ± 0.71	19.66 ± 0.47	18.94 ± 0.32	18.72 ± 0.15
4	27.89±0.16	24.50 ± 0.50	22.03 ± 0.83	20.74 ± 0.79	19.71 ± 0.55	18.88 ± 0.38
5	29.62±0.64	25.94 ± 0.20	23.04 ± 0.27	21.69 ± 0.92	20.68 ± 0.98	19.65 ± 0.77
6	32.88±0.58	26.71 ± 0.31	24.16 ± 0.61	22.62 ± 0.45	21.86 ± 0.39	20.74 ± 0.54

N.S = Non-significant

All parameters are significant at the level of 0.01.

* before storage = 17.86 ± 0.84 %

Table (4): Effect of CCB upon sperm acrosomal integrity* % stored at 4°C for 6 days.

Storage time (days)	control (n =18)	Treated samples concentration of CCB				
		5.0 µg (n =18)	10.0 µg (n =18)	15.0 µg (n =18)	20.0 µg (n =18)	30.0 µg (n =18)
	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D
1	12.78±2.41	08.62 ± 1.04	08.08 ± 0.41	07.08 ± 0.23	06.71 ± 0.68	05.98 ± 0.97
2	16.76± 2.25	11.68 ± 1.33	10.16 ± 1.43	07.52 ± 0.53	06.52 ± 0.53	06.19 ± 0.33
3	22.98±2.25	14.28 ± 1.19	11.61 ± 1.02	08.20 ± 0.34	07.45 ± 0.96	07.01 ± 0.48
4	25.85±1.90	16.07 ± 1.75	13.62 ± 1.62	09.32 ± 1.16	08.98 ± 0.50	08.33 ± 0.57
5	28.24±2.76	23.25 ± 5.48	19.63 ± 2.12	12.96 ± 1.01	11.51 ± 1.94	10.62 ± 0.62
6	30.18±3.11	26.93 ± 0.95	22.16 ± 1.91	16.26 ± 1.25	14.89± 0.21	12.35± 0.67

All parameters are significant at the level of 0.01.

* before storage = 05.88 ± 0.33 %

Table (5): Effect of CCB on sperm penetration distance* (mm/ 30 min.) stored at 4°C for 6 days.

Storage time (days)	control (n =18)	Treated samples concentration of CCB				
		5.0 µg (n =18)	10.0 µg (n =18)	15.0 µg (n =18)	20.0 µg (n =18)	30.0 µg (n =18)
	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D
1	24.23±0.49	25.79 ± 0.41	26.25 ± 0.49	27.09 ± 1.02	27.23 ± 0.22	27.93 ± 1.25
2	22.12± 0.48	24.29 ± 0.56	25.81 ± 0.72	25.92 ± 1.01	26.17 ± 2.12	26.79 ± 0.48
3	21.13±0.18	23.09 ± 0.77	25.11 ± 0.67	25.54 ± 0.74	25.74 ± 0.79	25.97 ± 0.63
4	20.25±1.28	21.51±0.51$^{N.S}$	23.34 ± 1.04	24.88 ± 0.73	24.52 ± 0.80	25.59 ± 0.60
5	18.02±0.31	19.95±1.21$^{N.S}$	21.37 ± 1.95	23.22 ± 1.68	23.59 ± 0.74	24.87 ± 2.34
6	17.57±2.09	18.93±1.65$^{N.S}$	20.91 ± 3.23	21.89 ± 2.14	22.41± 1.25	23.85± 1.88

N.S = Non-significant All parameters are significant at the level of 0.01.

* before storage = 29.0 ± 0.45 mm/30 min.

Table (6): Effect of CCB on seminal plasma Ca^{2+} levels* (mg/100ml) stored at 4°C for 6 days.

Storage time (days)	control (n =18)	Treated samples concentration of CCB				
		5.0 µg (n =18)	10.0 µg (n =18)	15.0 µg (n =18)	20.0 µg (n =18)	30.0 µg (n =18)
	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D
1	39.85±0.90	39.75±0.26$^{N.S}$	39.63±0.42$^{N.S}$	39.18±0.24$^{N.S}$	38.59±0.35$^{N.S}$	38.25±0.95$^{N.S}$
2	39.95± 2.35	39.83±0.47$^{N.S}$	39.75±0.59$^{N.S}$	39.35±0.24$^{N.S}$	38.78±0.50$^{N.S}$	38.45±0.39$^{N.S}$
3	45.27±2.00	42.72±1.52$^{N.S}$	41.88±2.72$^{N.S}$	40.19 ± 0.78	39.45 ± 0.64	39.25 ± 0.27
4	48.28±1.97	43.87±2.01$^{N.S}$	43.30±2.62$^{N.S}$	41.07 ± 1.31	40.52 ± 0.38	40.12 ± 0.54
5	50.30±2.07	47.40±4.98$^{N.S}$	45.57±2.17$^{N.S}$	41.32 ± 2.45	42.60 ± 0.85	41.98 ± 1.98
6	55.92±6.08	50.73±2.39$^{N.S}$	47.28 ± 2.42	42.93 ± 2.54	41.18± 1.01	40.78± 1.44

N.S = Non-significant. All parameters are significant at the level of 0.01.

* before storage = 38.89 ± 0.96 mg/100 ml

Table (7): Effect of CCB on Mg^{2+} levels* (mg/100ml) in seminal plasma stored at 4°C for 6 days.

Storage time (days)	control (n =18)	Treated samples concentration of CCB				
		5.0 µg (n =18)	10.0 µg (n =18)	15.0 µg (n =18)	20.0 µg (n =18)	30.0 µg (n =18)
	mean±S.D	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D
1	3.77±0.01	3.79±0.49 N.S	4.07±0.12 N.S	4.10±0.11 N.S	4.17±0.15 N.S	4.35 ± 0.13
2	3.89±0.15	3.99±0.19 N.S	4.08±0.57 N.S	4.29 ± 0.33	4.31 ± 0.01	4.55 ± 0.21
3	4.17±0.17	4.21±0.18 N.S	4.37±0.33 N.S	4.57 ± 0.12	4.71 ± 0.01	4.82 ± 0.13
4	5.29±0.11	4.35±0.17 N.S	4.54±0.33 N.S	4.73 ± 0.38	4.85 ± 0.32	4.98 ± 0.34
5	5.89±0.21	5.54±0.44 N.S	5.44±0.2 N.S	5.38 ± 0.09	5.28 ± 0.19	5.15 ± 0.21
6	6.59±0.39	5.99±0.83 N.S	5.79 ± 0.47	5.69 ± 0.75	5.46± 0.09	5.24± 0.34

N.S = Non-significant . All parameters are significant at the level of 0.01.

* before storage = 4.43 ± 0.09 mg/100 ml

Table (8): Effect of CCB upon fructose content* (mg/100 ml) in seminal plasma stored at 4°C for 6 days.

Storage time (days)	control (n =18)	Treated samples concentration of CCB				
		5.0 µg (n =18)	10.0 µg (n =18)	15.0 µg (n =18)	20.0 µg (n =18)	30.0 µg (n =18)
	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D
1	580.82±29.8	606.95 ± 13.9	611.37 ± 3.7	614.09 ± 8.8	617.38 ± 2.2	619.93 ± 0.9
2	556.85± 5.9	601.18 ± 3.3	607.74 ± 1.2	611.93 ± 2.8	614.05 ± 3.1	616.49± 0.8
3	512.38±1.5	591.25 ± 0.6	594.56 ± 0.6	597.87 ± 1.7	605.66 ± 0.6	609.34 ± 0.6
4	496.15±5.4	580.33 ± 0.6	584.94 ± 1.3	591.77 ± 1.1	598.05 ± 0.8	604.59 ± 0.6
5	450.65±0.7	520.51 ± 0.9	575.47 ± 1.2	587.13 ± 0.9	595.31 ± 0.5	597.06 ± 0.9
6	445.28±1.3	510.67 ± 0.7	562.81 ± 2.4	577.64 ± 2.5	583.34 ± 2.6	591.44 ± 1.3

All parameters are significant at the level of 0.01. * before storage = 622.37 ± 1.55 mg/100 ml

Table (9): Effect of CCB on LDH concentration* (U/L) in seminal plasma stored at 4°C for 6 days.

Storage)	control (n =18)	Treated samples concentration of CCB				
		5.0 µg (n =18)	10.0 µg (n =18)	15.0 µg (n =18)	20.0 µg (n =18)	30.0 µg (n =18)
	mean±S.D	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D
1	34.09±1.4	31.45 ± 1.44	30.24 ± 0.89	29.55 ± 0.56	29.19 ± 0.78	28.79 ± 1.16
2	57.58±3.5	37.89 ± 1.02	35.30 ± 1.05	33.55 ± 1.22	32.38 ± 1.03	31.22 ± 0.34
3	74.67±4.5	43.53 ± 1.59	41.97 ± 2.77	39.59 ± 0.64	37.04 ± 2.32	36.65 ± 0.94
4	54.18±1.6	53.57 ± 0.95$^{N.S}$	49.22±2.92$^{N.S}$	47.87 ± 1.64	45.55 ± 0.83	41.88 ± 0.74
5	49.32±2.7	43.04 ± 5.69$^{N.S}$	41.04 ± 1.62	39.69 ± 2.08	38.08 ± 1.95	35.19 ± 1.29
6	44.58±3.1	42.63 ± 1.07	39.08 ± 0.61	38.38 ± 2.34	36.26 ± 1.95	34.76 ± 3.22

N.S = Non-significant

All parameters are significant at the level of 0.01.

* before storage = 26.56 ± 0.64 %

Table (10): Effect of CCB on malonaldehyde (MDA) level* (nmol /L) in seminal plasma stored at 4°C for 6 days.

Storage time(days	control (n =18)	Treated samples concentration of CCB				
		5.0 µg (n =18)	10.0 µg (n =18)	15.0 µg (n =18)	20.0 µg (n =18)	30.0 µg (n =18)
	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D	mean ± S.D
1	0.86 ± 0.08	0.80 ± 0.04	0.69 ± 0.06	0.64 ± 0.10	0.60 ± 0.05	0.56 ± 0.06
2	0.94± 0.04	0.90 ± 0.06	0.78 ± 0.03	0.68 ± 0.02	0.62 ± 0.07	0.59 ± 0.06
3	1.13 ±0.12	1.07 ± 0.09	0.92 ± 0.08	0.75 ± 0.06	0.69 ± 0.07	0.63 ± 0.08
4	1.38 ± 0.11	1.22 ± 0.10$^{N.S}$	1.01 ± 0.09$^{N.S}$	0.94 ± 0.05	0.79 ± 0.06	0.72 ± 0.03
5	1.64 ± 0.12	1.41 ± 0.16$^{N.S}$	1.21 ± 0.12$^{N.S}$	0.98 ± 0.15	0.89 ± 0.06	0.81 ± 0.09
6	1.97 ± 0.11	1.73 ± 0.07$^{N.S}$	1.38 ± 0.17	1.13 ± 0.12	1.07 ± 0.11	0.99 ± 0.08

N.S = Non-significant

All parameters are significant at the level of 0.01.

* before storage = 0.46 ± 0.04 nmol /L

Figure (1) : Effect of different concentrations of CCB upon sperm motility %

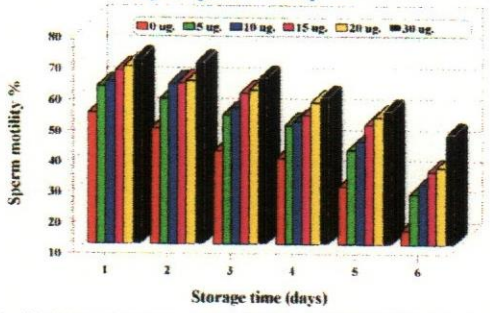


Figure (2) : Effect of different concentrations of CCB upon alive sperm %

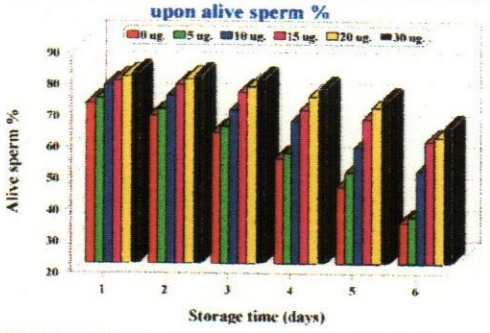


Figure (3) : Effect of different concentrations of CCB upon sperm abnormalities %

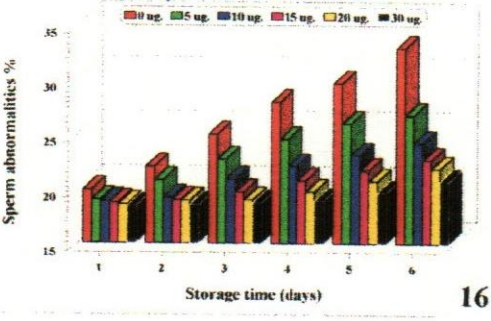


Figure (4) : Effect of different concentrations of CCB upon acrosomal integrity %

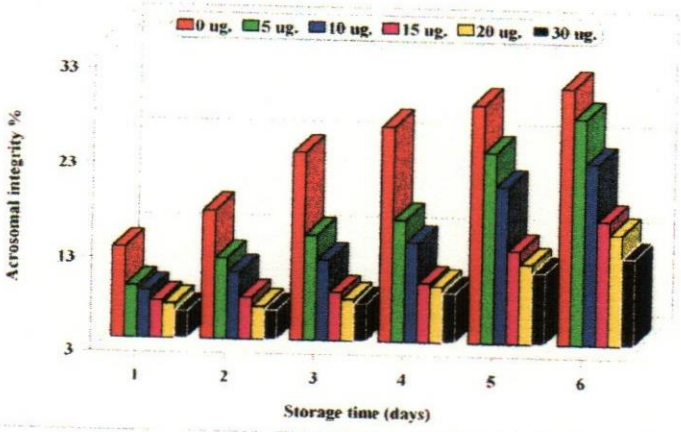
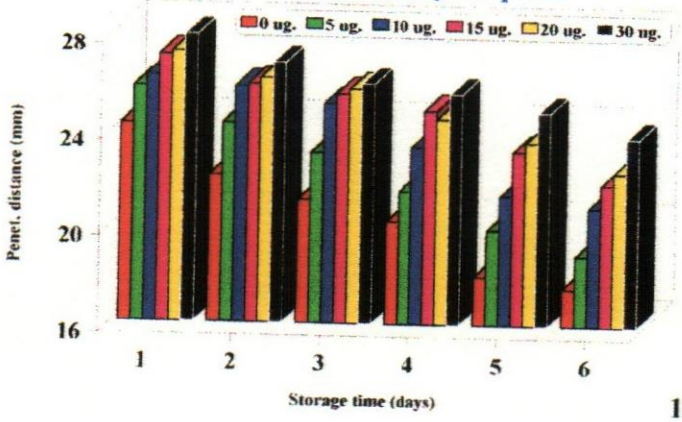


Figure (5) : Effect of different concentrations of CCB upon penetration ability of sperm



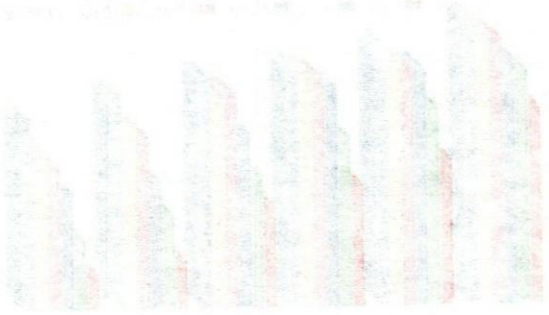


Figure (6) : Effect of different concentrations of CCB upon Ca. levels (mg/ 100 ml).

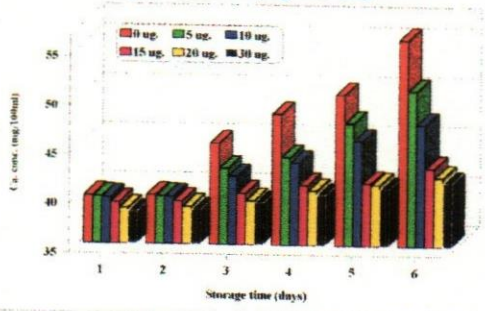


Figure (7) : Effect of different concentrations of CCB upon Mg. levels.

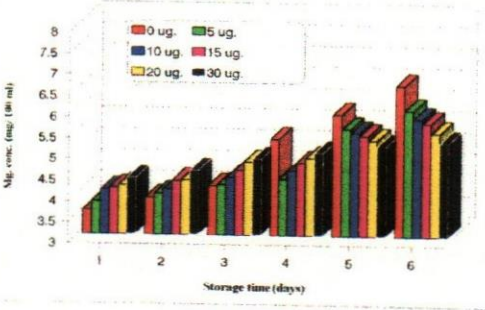


Figure (8) : Effect of different concentrations of CCB upon fructose concentration (mg/ 100 ml).

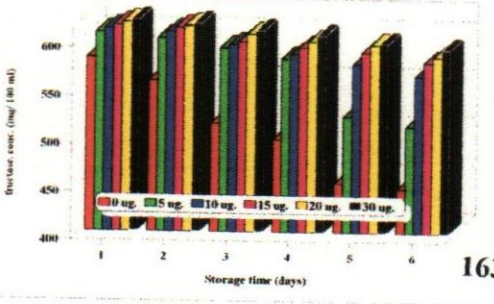


Figure (9) : Effect of different concentrations of CCB upon LDH (U/L) levels.

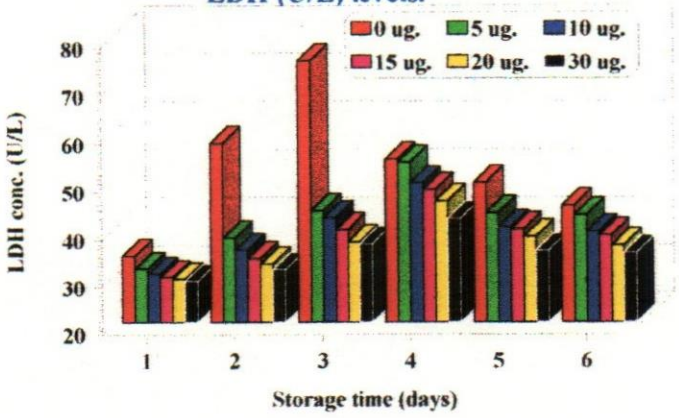
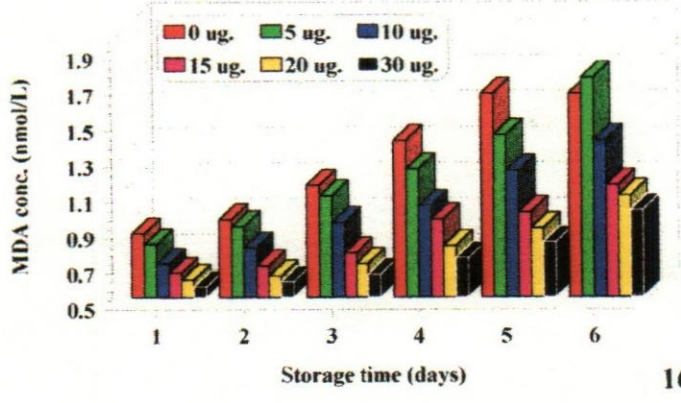


Figure (10) : Effect of different concentrations of CCB on MDA (nmol/L) levels.



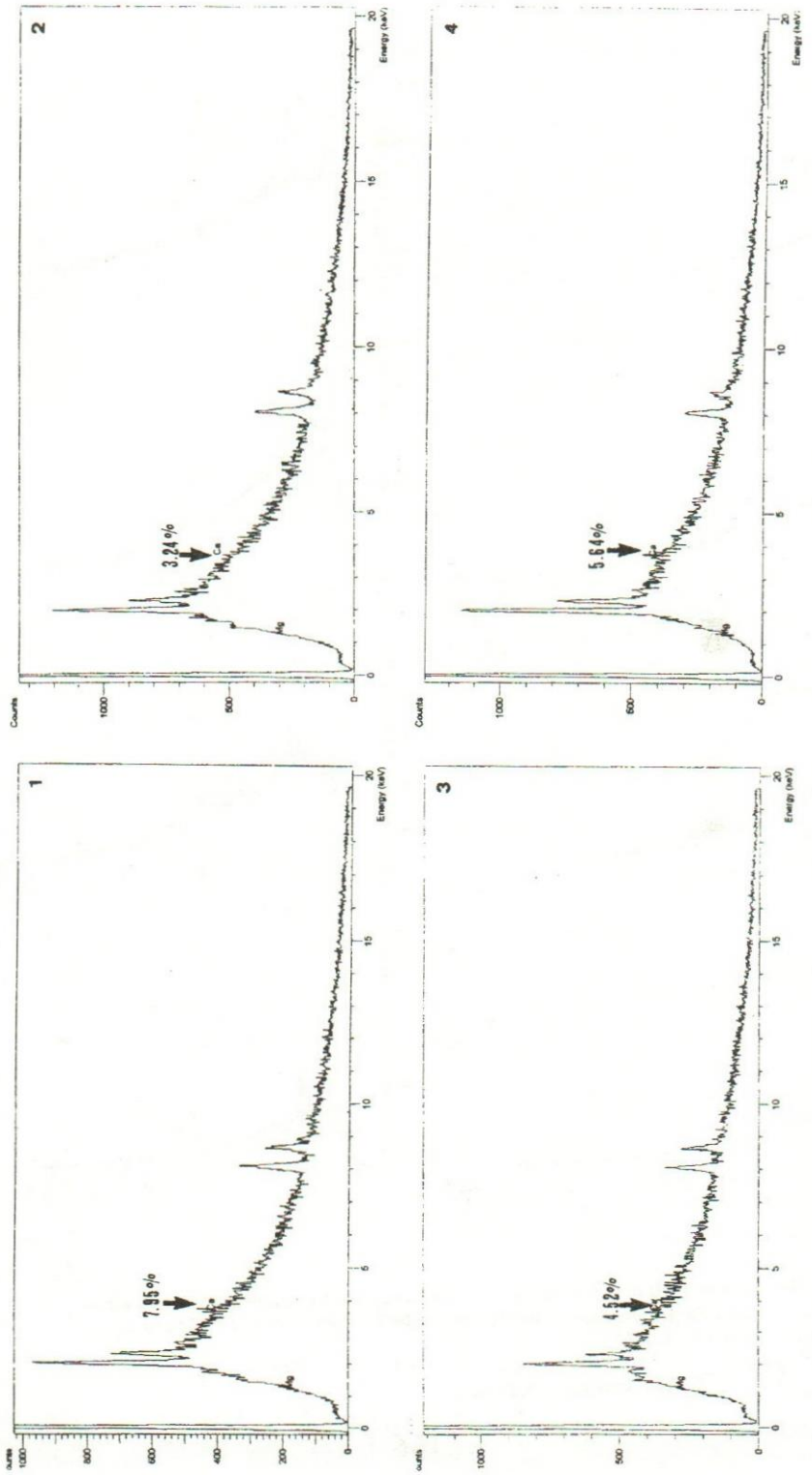


Figure (11) : The effect of CCB on intracellular Ca^{2+} percentages* (by using scanning electron microscope and Linke ISIS programme) in liquid buffalo-bull semen stored at 4°C for 2 days.
1 = control sample 2 = 5 µg CCB 3 = 15 µg CCB
4 = 30 µg CCB * at time zero = 5.23 %

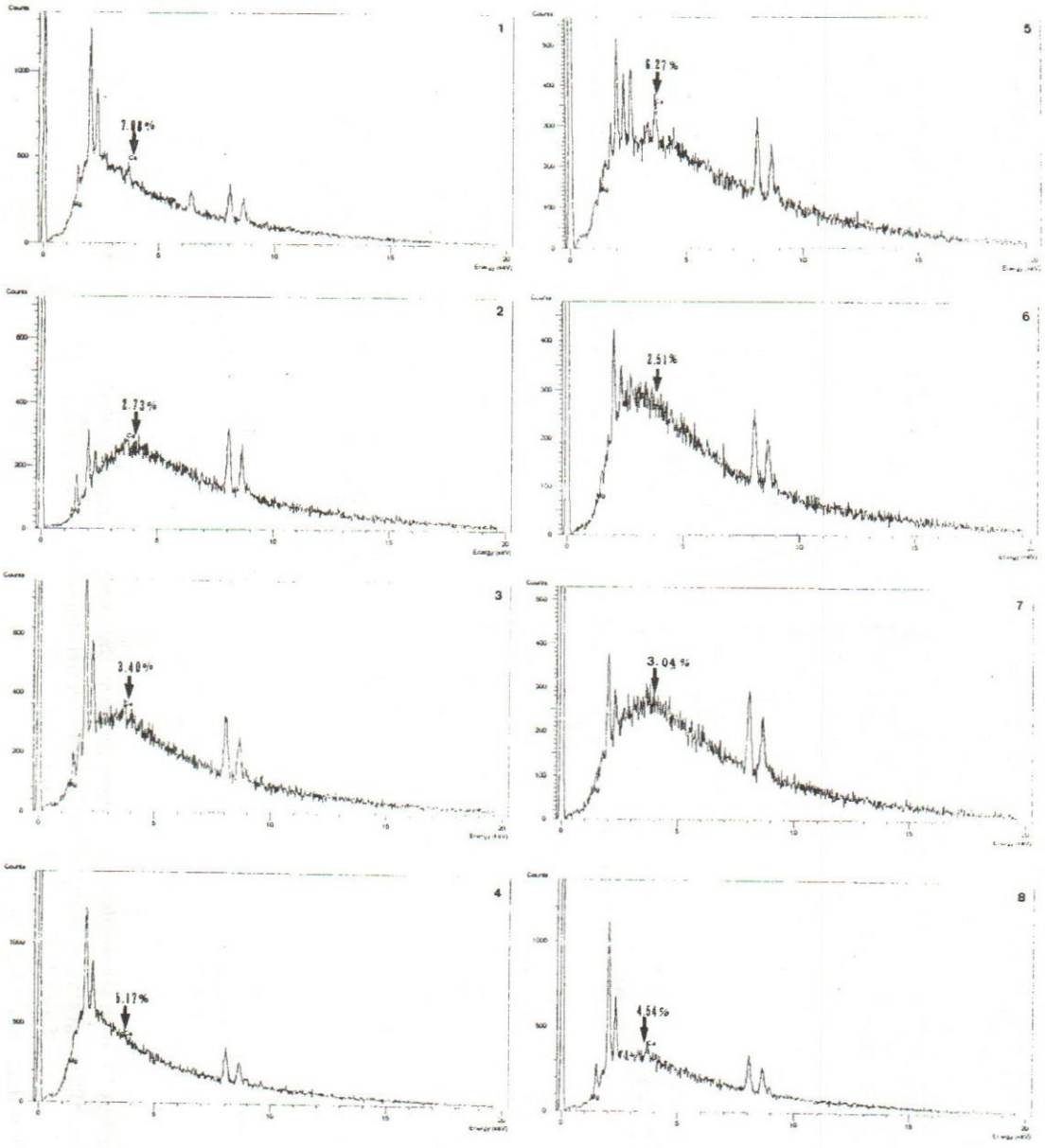


Figure (12) : The effect of CCB on intracellular Ca^{2+} percentages* (by using scanning electron microscope and Linke ISIS programme) in liquid buffalo-bull semen stored at 4°C for 4 days (No. 1,2,3,4) and 6 days (No. 5,6,7,8).

1& 5 = control samples 2& 6 = 5 μg CCB 3&7 = 15 μg CCB

4& 8 = 30 μg CCB *before storage = 5.23 %

Plate I

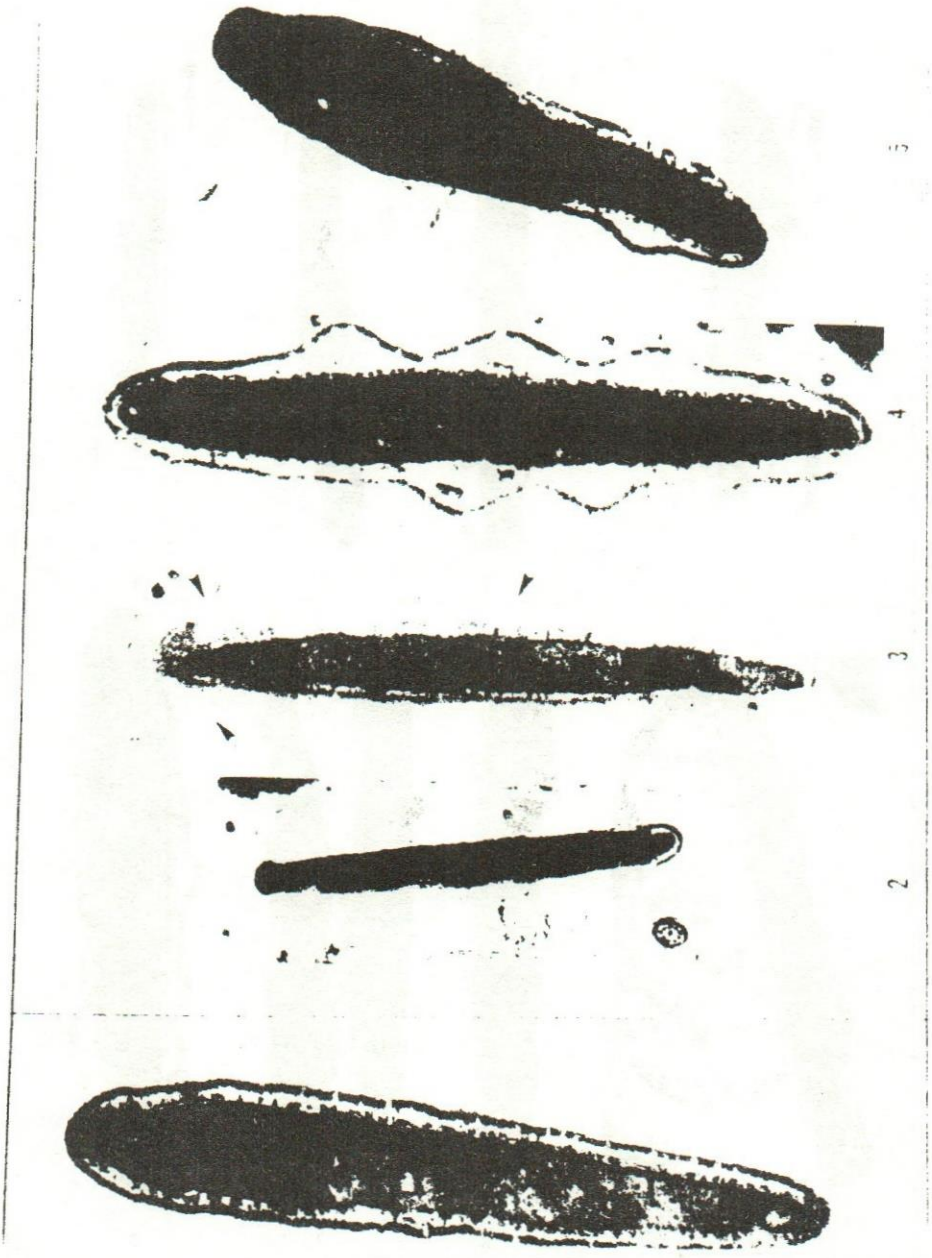


Plate II