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IMPACT OF ZINC AND TOCOPHEROL ON FUNCTIONAL COMPETENCE OF CRYOPRESERVED BUFFALO PERMATOZOA

(With 4 Tables and 2 Figures)

By

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تاثير إضافة الزنك و فيتامين هـ على خصائص السائل المنوي المجمد للجاموس

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استخدم في هذه الدراسة عدد خمس طلائق جاموسي ، حيث قسمت إلى أربعة مجموعات٠ اشتملت المجموعة الأولى على طلوقة واحد استخدم كضابط ، اشتملت المجموعة الثانية على طلوقة واحد اعطى ٦.٩جم أكسيد الزنك كل أسبوع لمدة ثلاثة اشهر ٠اما المجموعة الثالثة اشتملت على طلوقة واحد اعطى ١مجم فيتامين هـ لكل كجم من وزن الحيوان كل يوم لمدة ثلاثة اشهر ، أما المجموعة الرابعة اشتملت على طلوقتين حيث تم إعطائهم ٦.٩جم أكسيد الزنك كل أسبوع بالإضافة ١مجم فيتامين هـ لكل كجم من وزن الحيوان كل يوم لمدة ثلاثة اشهر ٠ تم تجميع عينات السائل المنوي قبل و بعد المعاملات السابقة من كل مجموعة ثم تم تخفيف السائل المنوي في مخفف الترس المضاف إليه ٢٥, مجم فيتامين هـ ، ١ مللي مول كلوريد الزنك ، ١.٥مجم فيتامين هـ + ١ مللي مول كلوريد الزنك وبعد ذلك تم التبريد والتجميد باستخدام النظام الفرنسي ٠ تم تقييم عينات السائل المنوي بعد الاساله من حيث النسبة المئوية للحيامن بعد الاسالة ، حيوية السائل المنوي ، النسبة المئوية لتشوهات القلنسوة وكذلك مسافة اختراق الحيوانات المنوية للمخاط بالإضافة لتقييم معدل فقد الأنزيمات الموجودة داخل خلية الحيوانات المنوية٠ أسفرت النتائج على أن النسبة المئوية الحيوانات المنوية بعد التخفيف لم تتأثر إلا بعد إضافة الزنك سواء على العليقة أو على المخفف٠اما الحركة الأمامية للحيامن بعد الإسالة فقد ازدادت زيادة معنوية مع كل المعالجات بالمقارنة بالمجموعة الضابطة٠ وبالنسبة لحيوية السائل المنوي بعد الإسالة فقد ازدادت زيادة معنوية مع كل المعالجات ماعدا عند إضافة الزنك على العليقة حيث لم تتأثر معنويا ٠اما إضافة الزنك فقط أو فيتامين هـ فقط آو كليهما معا إلى مخفف الترس قبل تجميد السائل المنوي

المجمع من طلائق تم تغذيتها بعلائق مزودة بالزنك أو فيتامين هـ نتج عنها نقص معنوي في النسبة المئوية لتشوهات القلنسوة . كما أن مسافة اختراق الحيوانات المنوية للمخاط تأثرت تأثرا عاليا مما يشير إلى أهمية إضافة فيتامين هـ على المخففات المستخدمة في تخفيف السائل المنوي الجاموسى كما أسفرت النتائج عن الدور الفعال لإضافة الزنك فقط أو فيتامين هـ فقط أو كليهما معا على الغشاء الخلوي للحيوانات المنوية بعد التجميد والاسالة وذلك من معدل فقد الأنزيمات الموجودة داخل خلية الحيوانات المنوية والتى سجلت اقل معدل. في ضوء ما تقدم من نتائج يمكن استنتاج أن إضافة الزنك فقط أو فيتامين هـ فقط أو كليهما معا سواء على العليقة أو على المخففات المستخدمة في حفظ السائل المنوي الجاموسى كان لها الدور الفعال فى حماية الغشاء الخلوي للحيوانات المنوية أثناء عملية التجميد والإسالة.

SUMMARY

Five buffalo-bulls aged 3-4 years were used in the present work. They were classified into four groups. Group I involved one buffalo- bull, which served as a control. Group II included one buffalo - bull which was orally supplemented with 6.9 grams zinc oxide every week for three months. Group III involved one buffalo-bull, which was orally supplemented with 1mg dl-α-Tocopherol acetate/day/kg live body weight for three months. Group IV consisted of two buffalo- bulls which were orally supplemented with zinc oxide and dl-α-Tocopherol acetate. Semen was collected (before and after dietary supplementation) from each buffalo-bull in a regular service. Ejaculates were extended in tris- based extender without or with 0.25 mg dl-α-Tocopherol acetate, 1 mM zinc chloride and 1.5 mg/ ml dl-α-Tocopherol acetate + 1 mM zinc chloride as additives. Cooling and freezing of extended semen was done using IMV system. Frozen-thawed straws were evaluated for percentage of sperm motility, Viability Index, acrosomal integrity percent and the distance that buffalo spermatozoa passes through the cow cervical mucus .In addition, detection of the structural integrity of sperm plasma membrane was done by estimating the loss of the intracellular aspartate aminotransferase after freezing and thawing. The obtained results revealed that the percentage of individual motility after dilution did not significantly increase except after in vivo and in vitro supplementation of zinc, the post thawing motility was highly significant ($p<0.01$) increase in all treatments over that was found in the control group except after in vitro addition of zinc or tocopherol the figures obtained were significantly ($p<0.05$) higher from the control. Also, viability index was significantly increased in all

treatments except in vivo supplementation of zinc which did not evoke any significant effect if compared to that observed before treatments. The effect of zinc and/or tocopherol in tris based diluent before freezing of semen from dietary zinc and tocopherol supplemented buffalo bulls resulted in highly significant reduction of the percentage of defected acrosomes. The sperm penetration distance was proved a highly significant which focusing the importance of adding Tocopherol to semen diluents. Also, The present study confirmed the protective action of zinc and /or vit. E on the plasma cell membrane of buffalo spermatozoa after freezing and thawing as indicated from the minimum rate of extracellular enzyme (Aspartate aminotransferase and acid phosphatase) released. It was concluded that in vitro and /or vivo supplementation of Vit.E and /or zinc proved to protect the plasma membrane of buffalo spermatozoa during freezing and thawing.

Key words: *Competence, Cryopreserved, Buffalo, Spermatozoa*

INTRODUCTION

Buffalo spermatozoa contain comparatively more unsaturated fatty acids than in other species (Guraya and Sidhu, 1975; Jain and Anand, 1976a, b and Singh et al., 1989) which make them more susceptible to lipid peroxidation (Singh et al., 1989). Peroxidation reaction was proved to induce an extensive damage to sperm plasma membrane, particularly in the region of the acrosomes (Jones and Mann, 1977) accompanied by a fast and irreversible loss of motility, a deep change in sperm metabolism and a high rate of release of intracellular components (Mann and Lutwak-Mann, 1981) Moreover, literature survey emphasized the fundamental role of zinc and tocopherol in the antioxidant defense system of living cells and prevention of peroxidative damage (Eliasson and Lindholmer, 1976; Abu-Erreish, et al.., 1978; Fujihara and Howarth, 1978; Mann and Lutwak-Mann, 1981, Wishart, 1984; Keen and Graham, 1989;McDowell, 1989 and Beconi et al.,1993). As it was shown that zinc and tocopherol (vitamin E) have to be supplied via the feed as they can not be produced by the animal itself (Ullrey, 1980). These considerations have led us to investigate the influence of dietary as well as in-vitro Zinc and/or tocopherol supplementation on post thowing motility, viability, and sperm plasma membrane and acrosomal integrity of cryopreserved buffalo spermatozoa.

MATERIALS and METHODS

Animals:

Five buffalo-bulls aged 3-4 years, were used in the present work. All animals were kept at Animal Reproduction Research Institute, Al-Ahram, Giza Governorate. Throughout the experimental period which lasted six months, the animals were fed on two basal rations, basal ration 1 which was fed for one month and basal ration 2 fed for the rest of the six months of the experimental period. The zinc and vitamin E. contents of basal ration are listed in Table (1). Water was offered to these animals ad libitum all the day long using manual water trough system containing ungalvanized pipes.

Dietary Supplementation:

The five buffalo-bulls under investigation were randomly classified into four groups. Group I involved one buffalo-bull which served as a control. Group II included one buffalo-bull which was orally supplemented with 6.9 grams zinc oxide (El-Gomhouria Co., Egypt) every week for three months. Group III involved one buffalo-bull which was orally supplemented with 1 mg dl-α-Tocopherol acetate/ day/kg live body weight (Hydrovit E, Rhône-Poulenc, France) for three months. Group IV consisted of two buffalo-bulls which were orally supplemented with zinc oxide and dl-α-Tocopherol acetate. The above mentioned doses for dl-α-Tocopherol acetate and zinc oxide were recommended by Putnam and Comben (1987) and Mohamed et al. (1994), respectively.

Semen collection, extender, in-vitro supplementation and general semen processing: Semen was collected (before and after dietary supplementation) from each buffalo-bull in a regular service. Ejaculates with good wave motion and initial sperm motility not less than 65% were used for processing. Two consecutive ejaculates were pooled and split-extended to a rate of 1:5 in one-step dilution at 30°C using tris-based extender (Abdel-Malak, 1994).

A pilot test was conducted to choose the different concentrations of vit. E and zinc that should be added to the buffalo semen diluent, as there is no enough literatures that describe the optimal concentrations of them that should be added to buffalo semen diluents. A total of 88 ejaculates from 5 buffalo bulls were analyzed to find out the normal seminal plasma concentrations of vitamins. E and zinc according to methods of Quaife and Dju, 1949 and Smith, et al., 1979). The frequency

distribution of vitamins. E and zinc were listed in Table (2) where the highest percentages of zinc and vitamins. E concentrations were (2.2- 6.0 ppm and 30.1- 50.0 ppm respectively).The corresponding concentrations to zinc and vit. E that should be added to semen diluent are 0.25 mg/ml dl- -Tocopherol acetate, 1 mM zinc chloride and 1.5 mg/ml dl- -Tocopherol acetate + 1 mM zinc chloride. Cooling and freezing of extended semen was done using IMV system according to Mohammed et al. (1998).

Evaluation of Spermatozoal Function:

Frozen-thawed straws were evaluated for percentage of sperm motility (Salisbury et al., 1978), Viability Index (Milovanov, 1962), acrosomal integrity percent (Wells and Awa, 1970) and the distance that buffalo spermatozoa passes through the cow cervical mucus (El-Sheltawi, 1993).

In addition, detection of the structural integrity of sperm plasma membrane was done by estimating the loss of the intracellular aspartate aminotransferase after freezing and thawing. Enzyme leakage was calculated on the basis of percent increase in post-thaw over prefreez, i.e., rate of increase in enzyme activity in the extracellular medium =

$$\frac{\text{postthaw activity - prefreeze activity}}{\text{prefreeze activity}} \times 100$$

Treatment groups: (1-16)

1- Control. 2- Zinc in vitro. 3- Tocopherol in vitro.
4- (Zinc + Tocopherol) in vitro. 5- Zinc in vivo.
6- Tocopherol in vivo. 7- (Zinc + Tocopherol) in vivo.
8- Zinc in vitro + Zinc in vivo 9- Zinc in vitro + Tocopherol in vivo
10- Zinc in vitro + - (Zinc + Tocopherol) in vivo
11- Tocopherol in vitro + Zinc in vivo.
12- Tocopherol in vitro + Tocopherol in vivo.
13- Tocopherol in vitro + (Zinc + Tocopherol) in vivo.
14- (Zinc + Tocopherol) in vitro + Zinc in vivo.
15- (Zinc + Tocopherol) in vitro + Tocopherol in vivo.
16- (Zinc + Tocopherol) in vitro + (Zinc + Tocopherol) in vivo.

Statistical Analysis:

All data obtained on sperm motility, viability index, abnormal acrosome %, enzyme leakage and sperm penetration distance were analyzed statistically with the GLM procedure of SAS (1987) computer program using Bonferoni t test.

RESULTS

The effect of zinc and/or tochopherol in vivo and in vitro supplementations were listed in Table (3) where the percentage of individual motility after dilution did not significantly increase except after in vivo and in vitro supplementation of zinc (81.25 ± 2.27, 81.25 ± 2.06, 88.50 ± 2.50 and 81.5 ± 1.87 respectively). At the same time, the post thawing motility was highly significant ($p<0.01$) increase in all treatments over that was found in the control group except after in vitro addition of zinc or tocopherol the figures obtained were significantly ($p<0.05$) higher from the control (40.61 ± 2.38 and 39.70 ± 1.79 respectively). Also, viability index was significantly increased ($p<0.05$) in treatments 2, 3, 4, 6, and 15 while it was highly significant ($p<0.01$) in the other treatments except treatment 5, which did not revealed any significant difference if compared to that observed before treatment (134.38 ± 7.07).

The effect of zinc and/or tocopherol in vitro and in vivo supplementation revealed a highly significant ($p<0.01$) reduction in the percentage of defected acrosomes (8-16 treatments). While, the sperm penetration distance was proved to be highly significant in treatments 4,13 and14 only which focusing the importance of adding tocopherol to semen diluent. Table (4) monitor the effect of zinc and /or tocopherol in vitro and in vivo supplementation on the extracellular release of aspartate amino transferase and acid phosphatase enzymes, the results indicated that extra cellular release of aspartate-amino transferase was highly significant reduced ($p<0.01$) in all treatments except after in vitro addition of zinc (39.82 ± 3.54). The maximal reduction (Fig. 1), which was, obtained in treatment 13 (14.31± 0.7) and the minimal one in treatment 3 (25.45 ± 3.04). Also, the extracellular release of acid phosphatase enzyme was highly significant ($p<0.01$) reduced in treatment 2, 5, 6, 7, 9, 11, 12, 13, 14, 15 and 16 and significantly reduced ($p<0.05$) in treatment 4 only (67.15 ± 1.67) while it was comparable to the control and treatment 3, 8, and 10 (68.30 ± 2.46, 67.38 ±3.19 and 69.25 ± 1.38 Vs 75.67 ± 3.82). Figure 2 demonstrate both maximal level (treatment 14, 18.25±4.95) and its minimal one (treatment 4, 67.15±1.67).

DISCUSSION

Zinc and tocopherol have been recognized for several decades as indispensable for normal male fertility (Chaney, 1992). It was clear from Table (3) that in vivo supplementation of zinc was significantly ($p<0.05$) increased the sperm motility after dilution Mann and Lutwak Mann (1981) preponderated the debates that the presence of zinc in the seminal plasma influence the motility and survival of mammalian spermatozoa, a fact that proved by Nour (1985) in buffalo semen and Misra and Fridovich (1972) and Swarup and Sekhon (1976) in bull semen. However Pitts et al. (1966) did not found any relationship or specific effects of zinc on sperm motility in bull semen.

In the present study, in vivo supplementation of zinc improved post thawing buffalo sperm motility, similar results was found by Salantiu et al. (1981) and Si et al. (1990) they proved that addition of zinc to the bull ration increased the post thawing sperm motility.

In view of the present results addition of zinc sulfate to buffalo semen diluent proved to improve the post thawing motility, although there is no available literature concerning in vitro effect of zinc on post thawing sperm motility, yet its importance on post thawing motility could be inferred from the results proved by Pangawkar et al. (1988) they indicated that the freezability of bull semen was related to the high content of zinc in the seminal plasma. On the other hand, Bhavsar et al. (1989) failed to speculate any correlation between zinc concentration in the seminal plasma and post thawing motility.

The function of zinc in sperm motility was due to its effect on the mechanical properties of the accessory fiber (Garbers, et al., 1971 and Baccetti et al., 1973) by regulating the metabolism of cyclic guanosin monophosphate (Santos Sacchi, et al., 1980) and adenosin triphosphate (Hidirglou and Knipfel, 1984), also a zinc ion dependant protein was isolated from the seminal plasma which was believed to be one of many factors that inactivate the plasmatic inhibitor of sperm motility (Strzezek et al., 1987). It was clear from Table (3) that in vivo supplementation of vit. E did not affect semen motility after dilution. The present results in line with Kozicki et al. (1978) they found that supplementation of vit. A, D3 and E had no significant effect on bull semen motility, while Shavkun

et al. (1979) obtained a higher figure for semen quality and volume after supplementation of vit. A, D, E, B12 and cobalt sulfate.

Post thawing motility was proved to be highly significant ($p<0.01$) than control after in vitro and/or in vivo supplementation of vit E. Also, Shubina (1982) succeeded to increase the suitability of bull semen for freezing after in supplementation of vit. E. While Kozicki, et al. (1981) found non significant effect of vit. A, D, E and C on bull semen freezability.

Concerning the effect of vit. E on post thawing motility AL-Khanak and AL-Hanak (1989) improved it by adding tocopherol in glucose saline diluent as antioxidant. While AL- Khanak et al. (1992) highlighted vit.E as a cryoprotective than vit. A or D2.

Mann and Lutwok Mann (1981); Putnam &Combsen (1987) and McDowell (1989) they reported the significant importance of tocopherol for sperm motility. Vit. E involved in the normal phosphorylation reaction, especially of high energy phosphate compound (ATP), which had an important role for bovine sperm motility and metabolic activity.

Post thawing buffalo sperm viability was significantly improved after in vivo and/ or in vitro supplementation of zinc and tocopherol. In vivo supplementation of zinc sulfate was resulted to give better survival rate to bull spermatozoa (Shubin and Shubina, 1977). When alpha tocopherol was fed to the bull, sperm resistance was increased (Maksimov et al., 1965 and Sampath & Anrlth kumar, 1975). In line with the present study Stolbov and Rimanova (1983) increased bull sperm survival after addition of vit. E to lactose diluent.

As matter of fact, mammalian spermatozoa are particularly susceptible to peroxidative damage by viture of their high content of polyunsaturated fatty acids and their relative paucity of cytoplasmic enzymes for scavenging reactive oxygen species (superoxide amino, hydrogen peroxide and hydroxyl radicals) that initiate lipid peroxidation of sperm membrane which lead to defective sperm function (Aitken and Clarkson, 1987). The importance of zinc as co- factors for superoxide dismutase activity (Keen and Graham, 1989) emphasized the fundamental role of zinc in the antioxidant defense system of living cells by catalyzing the dismutation of superoxide anions to hydrogen peroxide and oxygen which subsequently protect the polyunsaturated fatty acids (abounding in sperm phospholipids) from oxidation by superoxide anion (Abu-Errish et al., 1978). On the other hand, dl-α- Tocopherol was detected to be involved in the formation of biological membranes including the

mitochondrial membranes (McDowell, 1989) which contain the main machinery for trapping, conserving and supply sperm energy (Mann and LuMann, 1981).

It was clear from the displayed results in Table (3) that the inclusion of zinc sulfate and/or alpha tocopherol in tris based diluent before freezing of semen from dietary zinc and tocophrol supplemented buffalo bulls, resulted in highly significant reduction of the percentage of defected acrosomes. Brandis and Granach (1990) obtained the same results in bull after supplementation of rations with vit. C, D3, E, zinc copper, cobalt iodine manganese and carotene. However Kozicki et al. (1981) observed a significant increase in the percentage of acrosomal abnormalities in the bull semen after daily doses of vit. A, D, E and C for 9 weeks. Beconi et al. (1993) studied the effect of natural antioxidant (vit. E) on the various thermal treatment during cryopreservation of bovine semen and obtained greater percentage of intact acrosome . The greater percentage of intact acrosome accompanied with in vivo and /or in vivo supplementation of zinc and vit. E appears to be due to the antioxidant capacity of vit. E (Combs et al., 1975) and the importance of zinc as a co-factor in stabilizing the biological membranes (Mann and Lutwak Mann, 1981).

The sperm penetration distance was proved to be highly significant in treatment 4,13 and 14 only which focusing the importance of adding tocopherol to semen diluent. The highly significant increase of post thawing sperm motility as well as the sperm penetration distance in the present study had proved the speculation that recorded the significant positive correlation between them (Okuda et al., 1988 and Suttiyotin et al., 1995).

The present study confirmed the protective action of zinc and /or vit. E on the plasma cell membrane of buffalo spermatozoa after freezing and thawing (Table 4) as indicated from the minimum rate of extracellular enzyme (Aspartate aminotransferase and acid phosphatase) released (Fig. 1 & 2). These two enzymes were known to be intracellular enzymes which were taken as a measurement of cellular injury (Stallcup, 1965). Zinc was known to have a critical role in the stabilizing of biomembranes by binding the sulphhydrl groups forming mercaptides, thus membrane – bound zinc alters the fluidity and stabilization of membranes and prevent in part the oxidation of essential sulpfhydryl groups via keletion (Keen and Graham, 1989). Since buffalo spermatozoa were fond to be rich in polyunsaturated fatty acids which considerably favored the occurrence of

lipid peroxidation and might lead to a damage to sperm membranes (Guraya &Sidhu, 1975; Jain & Anand, 1976 a&b and Singh et al., 1989). While vit. E was act as antioxidant and anti- free radical agent (Newberne and Conner, 1989). In the presence of oxygen, spermatozoa were linked to form a highly reactive free radicals (superoxide anion & hydrogen peroxide radicals (which was found to induce oxidation of the polyunsaturated fatty acids content of the sperm membrane leading to an irreversible damage of the spermatozoa (Macleod, 1949; Vandemark et al., 1949 and Mann & Lutwak Mann, 1975). Vitamin E was known to scavenge the superoxide anion by increasing superoxide dismutase enzyme activity and sequentially reduce lipid peroxides (Beconi et al., 1993). Furthermore Pfeifer and McCay (1971) and Combs et al. (1975) claimed that vit. E acts as intracellular antioxidant by interacted with the selenium containing glutathione peroxidase to prevent the oxidative breakdown of biological membrane by hydroperoxides. In view of the present study it was concluded that in vitro and /or vivo supplementation of Vit.E and /or zinc proved to protect the plasma membrane of buffalo spermatozoa during freezing and thawing.

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Table 1: Zinc and vitamin E content in the feedstuffs fed to buffalo-bulls (on DM basis):

Type of basal ration	Ingredients	Zinc (ppm)	Vitamin E (ppm)
Basal ration 1	Berseem	48.70	61.60
	Concentrate mixture	79.00	0.71
Basal ration 2	Darawah	31.20	24.02
	Concentrate mixture	79.00	0.71

ppm : part per million.
DM: dry matter.

Table (2): Frequency distribution of zinc and vitamin E (α-tocopherol) concentration in buffalo seminal plasma.

Zinc			Vitamin E (α-tocopherol)		
Class interval (ppm)	Number of casses	Percentage	Class interval (ppm)	Number of casses	Percentage
1.0	2	2.3	21.2	1	1.1
1.1-2.1	6	6.8	21.3-30.0	12	13.7
2.2-4.0	45	51.1	30.1-40.0	39	44.3
4.1-6.0	29	33.0	40.1-50.0	23	26.1
6.1-7.9	4	4.5	50.1-60.9	12	13.7
8.0	2	2.3	61.0	1	1.1

Table 3: Sperm motility (%), viability index, abnormal acrosome (%) and sperm penetration distance (mm) after supplementation with zinc and/or tocopherol (Mean ± SE)

Sperm function tests	Treatments															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Sperm motility after dilution (%)	76.21 ± 1.09	76.21 ± 0.88	77.27 ± 0.89	77.42 ± 1.00	81.25 ± 2.27*	78.13 ± 2.30	76.56 ± 1.69	81.25 ± 2.06*	76.88 ± 2.49	81.56 ± 1.87*	82.50 ± 2.50*	76.88 ± 2.66	79.69 ± 1.96	81.88 ± 2.66*	78.13 ± 2.82	79.69 ± 1.61
Postthaw sperm motility (%)	33.64 ± 2.13	40.61 ± 2.38 *	39.70 ± 1.79 *	44.55 ± 2.43 **	52.50 ± 4.53 **	57.50 ± 2.50 **	55.00 ± 2.42 **	59.38 ± 2.74 **	57.50 ± 2.50 **	57.50 ± 1.71 **	57.50 ± 2.50 **	52.50 ± 3.13 **	54.38 ± 1.57 **	55.00 ± 3.27 **	52.50 ± 2.50 **	53.57 ± 1.80 **
Viability index (%)	120.62 ± 2.52	130.0 ± 3.86 *	130.3 ± 3.86 *	133.8 ± 5.05 *	134.4 ± 7.67 *	141.3 ± 8.10 *	135.2 ± 4.74 **	15.50 ± 9.32 **	160.6 ± 8.63 **	156.6 ± 5.04 **	161.9 ± 7.44 **	149.7 ± 9.77 **	156.41 ± 4.41 **	163.1 ± 3.39 **	143.8 ± 4.20 **	166.4 ± 4.92 **
Abnormal acrosome (%)	19.79 ± 1.07	16.00 ± 0.90	15.30 ± 0.70	13.91 ± 0.64	15.38 ± 1.48	14.63 ± 1.13	14.38 ± 1.10	11.63 ± 1.35 **	11.75 ± 1.33 **	10.75 ± 0.66 **	10.13 ± 1.04 **	8.00 ± 1.64 **	10.38 ± 1.13 **	10.38 ± 1.71 **	11.75 ± 1.29 **	10.13 ± 0.84 **
Sperm pene-ration distance	25.79 ± 1.30	29.55 ± 1.49	29.64 ± 2.10	34.99 ± 1.88 **	26.50 ± 4.25	28.00 ± 2.86	27.63 ± 2.33	32.00 ± 3.67	32.88 ± 3.22	31.38 ± 2.45	31.50 ± 3.36	31.38 ± 3.76 **	35.56 ± 1.59 **	41.25 ± 5.61	30.13 ± 4.15	33.94 ± 2.08

Means significantly differed from control (* P<0.05 and ** P<0.01)

Treatmemnts: refere to Treatment Groups in Materials and Method.

Table 4: Effect of zinc and/or tocopherol supplementation on the rate of increase (%) in extracellular activities of aspartate aminotransferease and acid phosphatase immediately after thawing (mean ± SE)

	Treatments															
Enzyme leakage	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Aspartate transamina-se	46.79 ±3.92	39.82 ± 3.54	25.54 ± 3.04 **	25.88 ± 2.91 **	17.25 ± 2.71 **	17.13 ± 0.55 **	19.25 ± 1.23 **	15.00 ± 1.13 **	17.88 ± 0.91 **	14.63 ± 1.06 **	19.88 ± 2.15 **	14.88 ± 0.77 **	14.31 ± 0.77 **	18.25 ± 1.25 **	14.38 ± 0.94 **	15.56 ± 0.73 **
Acid phosphata-se	75.67 ± 3.82	59.42 ± 3.49 **	68.30 ± 2.46	67.15 ± 1.67 *	50.38 ± 3.31 **	53.00 ± 0.94 **	38.63 ± 3.60 **	67.38 ± 3.19	53.88 ± 2.51 **	69.25 ± 1.38	43.25 ± 2.35 **	58.88 ± 2.02 **	23.88 ± 1.27 **	18.25 ± 4.95 **	42.38 ± 5.50 **	39.13 ± 0.21 **

Means significantly differed from control (* P<0.05 and ** P<0.01)

Treatmemnts: refere to Treatment Groups in Materials and Method.

Fig. 1: Rate of increase (%) in aspartate aminotransferase leakage from frozen thawed spermatozoa

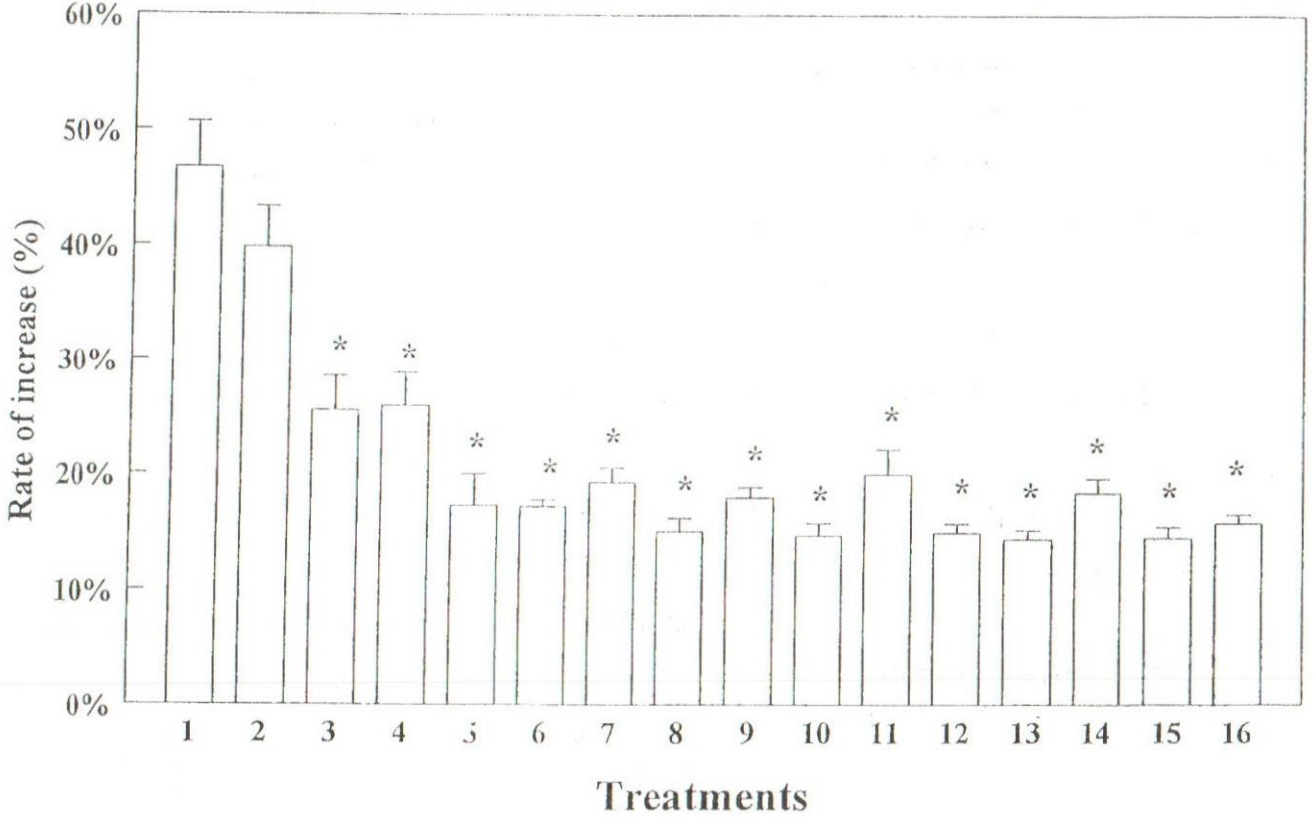

Fig. 2: Rate of increase (%) in acid phosphatase leakage from frozen thawed spermatozoa