AI and embryo transfer department,
Anim. Reprod. Res. Instit., Al-Haram (P.O.B. 12556), Giza, Egypt.

INFLUENCE OF ANTIOXIDANTS ON FREEZABILITY, IN VITRO FERTILIZING POTENTIAL AND CONCEPION RATE OF BUFFALO SPERMATOZOA

(With 7 Tables)

By

M. R. BADR, MAHA, S. ZIADA, G.M. DARWISH and A.A. NASRA,

(Received at 27/9/2003)

تأثير مضادات الأكسدة على قابلية حيامين الجاموس للتجميد ومعدل الإخصاب معملياً وحقلياً

مجدى رمضان بدر ، مها سليمان زيادة ، جمال مصطفى درويش
عبد الخالق عطية نصرة

لقد وجد أن تكون جزيئات الأكسجين الشاردة أثناء تصنيع السائل المنوى المجمد لـــها تـــأثير سلبى قوى على حيوية الحيوانات المنوية ولذلك فإن منع هـــذه الظـــاهرة بإضافـــة مضـــادات الأكسدة قد يلعب دوراً كبيراً فى تحسين حيوية الحيوانات المنوية وكذا قدرتـــها الإخصابيـــة . وجدير بالذكر أن العلاقة بين مستوى مضادات الأكسدة وحيوية الحيامين الجاموسى وقدرتـــها الإخصابية مازالت غير كاملة الدراسة ولذلك صممت هذه التجارب لدراسة تأثير المســـتويات المختلفة لعدة أنواع من مضادات الأكسدة على قابلية حيامين الجاموس للتجميد وقياس قدرتـــها الإخصابية باستخدام تكنولوجيا الإخصاب خارج الرحم وكذا تلقيح عدد من الإناث فـــى الحقـــل لتقدير نسبة الحمل. تم تجميع عينات السائل المنوى من ستة طلائق جاموســـى وتخفيفـــه فـــى مخفف التريس المضاف اليه عدة تركيزات من عدة أنواع من مضادات الأكسدة وتم تجميده فى النيتروجين السائل . وقد تم تقدير الحركة الفردية للحيوانات المنوية قبل التجميد وبعـــده وكـــذا تقدير الحيوية بعد الإسالة وتم قياس بعض الإنزيمات مثل AST و AKP التى تـــدل علـــى صحة غشاء الحيوانات المنوية . وتم استخدام بعض المعالجات لتجربة الإخصاب خارج الرحـم وكذلك تم فى تجربة أخرى تلقيح عدد ١٣٨ أنثى فى الحقل لتحديد القدرة الإخصابيـــة لحيـــامن الجاموس المجمدة والمعالجة بمضادات الأكسدة . وقد أظهرت هذه الدراسة أن إضافة مضادات الأكسدة الى مخفف التريس بتركيزات منخفضة تحسن من الوظائف الحيوية للحيوانات المنويـــة وأظهرت أيضا أن إضافة السلينيوم وفيتامين أ و ج وهـــ مجتمعة وبتركيزات منخفضة تحســـن من معدل الحركة الفردية(٥٨,٣٣ %) والحيوية بعد الإسالة (١٣٤,١٦) وتحسن من مستوى الإنزيمات الـــ AST ، AKP ومعدل الإخصاب المعملى (٥٨,٣٣ %) و معدل النمو إلـــى

طور البلاستوسيست (١٧,٣٣%) وكذلك نسبة الحمل فى الإناث الملقحة فى الحقل (٦٩,٤٣ %) مقارنة بالمعاملات الأخرى . ولذلك تظهر هذه الدراسة بقوة أن إضافة مضادات الأكسدة مجتمعة وبتركيزات منخفضة تلعب دوراً حيوياً فى تحسين الوظائف الحيوية للحيوانات المنوية فى الجاموس ولذلك توصى هذه الدراسة بإضافة مضادات الأكسدة لمخففات السائل المنوى الجاموسى لما لها من أهمية عظمى فى تحسين القدرة الاخصابية للسائل المنوى الجاموسى المجمد .

SUMMARY

Growing evidence suggests that the generation of reactive oxygen species. (ROS) during semen processing and their detoxification by antioxidants plays a very important role in sperm functions. However, the relationship between the level of antioxidants and the buffalo sperm functions remains poorly understood. Therefore, the present study was designed to investigate the influence of different antioxidant concentrations supplemented extender on semen freezability and fertilizing ability. Semen was collected from six buffalo bulls using artificial vagina. Above averaged semen quality (motility > 70% and concentration 800.00 x 10^6 sperm cells / ml) was pooled together. Pooled semen was extended in Tris based extender supplemented with different antioxidant concentrations, frozen over liquid nitrogen vapor and stored in liquid nitrogen. Frozen – thawed buffalo semen treated with low or high antioxidant concentrations were used to assess its in vitro fertilizing potential. Antioxidants treated buffalo spermatozoa with superior post thawing quality were used for artificial insemination (AI) to record the conception rate. Low antioxidant concentrations improved sperm functions. Antioxidants in combination, selenium + vitamin A + C + E (Selenium - ACE) at low concentrations had a beneficial effect on post thaw sperm motility (58.33 %), viability index (134.16), AST (101.33 U/ml) , AKP (10.33U/l) , in vitro fertilization rate (58.33%) , in vitro embryonic development to blastocyst stage (17.33%) and conception rate (69.43%). The present results strongly suggests that the addition of selenium -ACE at low concentrations to the extended buffalo semen improves post thaw sperm quality, in vitro fertilizing potential and conception rate.

Key words: buffalo, frozen semen, antioxidant, fertilizing poteontial.

INTRODUCTION

Improvement of semen cryopreservation requires a better understanding of the properties of the currently used extender. Excessive generation of reactive oxygen species (ROS) during or following semen cryopreservation, increases lipid peroxidation, that reduce sperm activity and alter sperm functions (Askari *et al.*, 1994 and Bilodeau *et al.*,2002).Buffalo spermatozoa contain comparatively more unsaturated fatty acids than other species which make them more vulnerable to lipid peroxidation (Singh *et al.*,1989).Addition of antioxidants to the media brought beneficial effects in preventing loss of motility , inhibiting lipid peroxidation (Kim and Parthasarathy,1998) and improving semen preservation (Sarlos *et al.*,2002). Vitamin E (-tocopherol and its derivatives), the predominant chain – breaking lipid-soluble antioxidant in animal cells, is believed to be the primary free radicals scavenger in mammalian cell membranes (Chow, 1991) and prevents lipid peroxidation (Cerolini *et al.*, 2000), thereby preserving spermatozoa metabolic activity and cellular viability (Beconi *et al.*, 1993).

Vitamin C, the major water-soluble antioxidant, is the first line antioxidant that proved to inhibit lipid peroxidation and scavenge free radicals (Machlin and Gabriel, 1980). Combination of both ascorbate and - tocophrol in sperm preparation medium reduced significantly H_2O_2 – induced DNA damage in a dose-dependent manner (Donnelly *et al.*, 1999 and Brennan *et al.*, 2000).

Beta-carotene is a powerful antioxidant, that works synergistically with - tocopherol to prevent lipid peroxidation and offers effective antioxidant activity in tissues with low oxygen concentration (Kozicki *et al.*, 1981).

Selenium is an essential trace element and has a powerful antioxidant activity. Recent evidence suggested that, selenium supplementation increased the antioxidant glutathione peroxidase activity thus decreasing the ROS leading to an increase in male fertility (Smith *et al.*, 1979 and Castellini *et al.*, 2002).

As a step towards elucidating the relationship between antioxidants supplementation and fertilizing ability of buffalo semen, the objectives of the present study were to investigate the effect of supplementation of buffalo extender with different antioxidant concentrations on freezability of buffalo spermatozoa, in vitro fertilizing potential and conception rate of treated frozen semen.

MATERIAL and METHODS

Extender preparation:

Tris based extender was prepared according to Steinbach and Foote (1967). Different antioxidant concentrations were added to the Tris based extender as follow; extender alone (control); vitamin E (1,2 and 3mg/ ml); vitamin C (3,6 and 9 mg / ml); vitamin A (50,100 and 150 IU); selenium (2.5,5 and 10 µg / ml); combination of more than one antioxidant:-

Vitamin A + vitamin E; vitamin A + vitamin C; vitamin A + selenium; vitamin C + vitamin E; vitamin C + selenium; vitamin E + selenium; vitamin A+ vitamin C+ vitamin E + selenium all previous antioxidants combination were at the lowest concentrations mentioned before. Lastly, vitamin A +vitamin C + vitamin E + selenium, at the highest concentrations used in this study.

Semen collection and preservation:

Semen samples were collected from six buffalo bulls, kept at the Animal Reproduction Research Institute farm, Al-Harm. Only semen samples of at least 70% initial sperm motility and $800.00x10^6$ sperm cells /ml were pooled, divided into 21 portions and diluted at a 1: 8 ratio at 30C°with Tris based extender containing different antioxidant concentrations. The diluted semen samples were cooled to 5C° throughout 45 minutes, equilibrated for 2h at 5C° and then looded into 0.25-ml PVC mini straws (IMV, L' Aigle, France). The straws were then subjected to deep freezing in liquid nitrogen vapor for 15 minutes, immersed and stored in liquid nitrogen containers according to Mohammed *et al.* (1998).

Evaluation of semen freezability:

After 24 hours, frozen semen samples were thawed at 35 C° for 30 sec. Sperm motility was evaluated after dilution and post thawing. Viability indices were recorded for thawed samples that were incubated at 37 C° for 3 h according Milovanov (1962). Aspartate Amino Transferase (AST) and Alkaline Phosphatase (AKP) enzymes of diluted and thawed semen samples were measured spectrophotometrically according toTietz (1976).

Evaluation of fertilizing potential of the treated semen in vitro:

Semen samples frozen in Tris extender containing low or high antioxidant concentrations were used for evaluating of their fertilizing ability.

In vitro oocyte maturation:

Oocytes were collected, selected and matured in vitro in TCM-199 medium for 24 h according to Badr *et al.* (2003).

In vitro sperm preparation:

Three straws from each treatment were thawed in a water bath at 35 C° for 30 sec and semen was layered under 1ml of modifid Tyrode's medium (Sp.TALP) in 1.5 ml test tubes. After 1 h, 400 µl of the uppermost layer of the Sp. TALP medium was collected and washed twice by centrifugation at 2000 rpm for 10 minutes and then resuspended in 1ml of fertilization medium; modified Tyrode's medium (F.TALP) supplemented with 100 µg/ml heparin according to (Parrish *et al.*, 1988).

In vitro fertilization

In vitro matured oocytes having fully expanded cumulus cells were cultured in 50-µl drop of the respective F-TALP medium. They were inseminated with the treated frozen – thawed semen at a final concentration of $2x10^6$ motile sperm cells /ml. Gametes were incubated under sterile paraffin oil for 18 h at 39 C° in 5 % Co_2 in humidified air. After oocyte-sperm incubation, some of oocytes were fixed in acetic acid-ethanol (1:3) and stained with 1% aceto–orcein stain and examined under a phase contrast microscope (x400) for evaluating their fertilization rate. Fertilization was considered to be occurred when male pronucleus or enlarged sperm heads were present. Groups of the inseminated oocytes were then freed from cumulus cells and attached spermatozoa by gentle pipetting and cultured in TCM-199 medium with Hepes. The proportion of cleaved oocytes was recorded 48 h after insemination and those developed to the morula and blastocyst stages were recorded at 5-to7- days post insemination.

Assessment of conception rate:

This trial was carried out to detect whether the improvement of post-thawing motility and viability due to semen treatment with antioxidants would be reflected in improvement in the fertility rate. Semen samples with the highest post thawing motility, viability and fertilizing potential were used to inseminate 138 buffalo-cows for evaluating of the conception rate. Semen straws were randomly distributed among AI centers and herds in El-Gharbia fields. The

percentage of buffaloes that did not return to estrus within 56-d after AI was used as a measure of conception rate.

Statistical analysis:

The statistical analysis were performed using a computerized statistical analysis system (SAS, 1985). Data were subjected to analysis of variance (ANOVA) to clarify the effect of antioxidants on motility, viability, enzyme activities and conception rate. In vitro fertilization rate and embryos development were analyzed using Chi-square analysis (x^2). The pearson correlation test was used to calculate the correltion between sperm parameters measured and conception rate.

RESULTS

Data regarding the effect of different antioxidant concentrations on sperm motility and viability indices of cryopreserved buffalo semen are presented in Table 1.

The percentage of motility immediately after dilution was significantly ($P<0.05$) increased when semen extender was supplemented with low concentrations of selenium – ACE (81.66 %) in comparison to 9 mg/ml vitamin C (66.66 %), vitamin A + vitamin E (66.66%) and vitamin A + vitamin C (65.02 %) supplementation.

Selenium–ACE supplementation to semen extender, at low concentration increased significantly ($P< 0.01$) the post-thawing sperm motility (58.33 %) compared to other used antioxidant concentrations, except selenium – ACE at high concentrations.

The data outlined in Table 1, showed that, the lowest percentage of reduction in post-thawing sperm motility than those after dilution motility were recorded with low concentration of selenium – ACE and 2.5µg/ml selenium supplemented semen extender (28.55 and 31.94 %, respectively) compared to control samples (38.73 %) and other antioxidant treatments.

The viability index of cryopreserved buffalo semen was significantly increased ($P<0.01$) with low concentrations of selenium – ACE supplementation to semen extender (134.16) as compared with control (85.83) and all other antioxidants supplementation, except that of selenium at 2.5 µg/ml. The lowest viability indices in the present study were recorded with 9 mg/ml vitamin C (39.16), vitamin C + vitamin E (40.01) and vitamin C + vitamin A (36.66).

The results regarding the effect of different antioxidant concentrations on enzyme activities of cryopreserved buffalo semen are

persented in Table 2. Supplementation of buffalo semen extender with low concentrations of selenium – ACE significantly (P<0.01) decreased AST performance after dilution and post-thawing (68.66 and 101.33U/ml, respectively) as compared with all high antioxidant concentrations. Alkaline Phosphatase activity (AKP) after dilution was significantly (P<0.05) reduced with low concentrations of selenium – ACE supplemented semen extender (8.01 U/L) compared to vitamin C + selenium (15.33 U/L) and vitamin E + selenium (15.33 U/L) supplementation. Similarly, low concentrations of selenium – ACE decreased significantly (P<0.01) the post-thaw alkaline phosphatase activity (10.33 U/L) compared to all high antioxidant concentrations and other antioxidant combinations except with vitamin E + vitamin C and selenium + A C E at high levels.

As shown in Table 3, the rate of male pronucleus formation was significantly higher in the oocytes that inseminated with frozen – thawed buffalo semen treated with low concentration of selenium – ACE (58.33 %; P<0.05) than when they inseminated with 3mg/ml vitamin C treated semen (42.86%) or with control (43.06 %). However, the in vitro penetration rates did not differ among different treatments and control.

The cleavage rate and in vitro embryo development to compacted morulae and blastocysts (Table 4) were significantly improved (p <0.05) in oocytes inseminated with low concentration of selenium –ACE treated semen (54.67, 26.67 and 17.33 %, respectively) in comparison to control semen (32.61, 10.87 and 4.35 %, respectively) and 3mg/ml vitamin C treated semen (30.77, 9.62 and 3.85 %, respectively). There was no significant difference between vitamin E, vitamin A, selenium and selenium- ACE regarding the cleavage rates or morulae and blastocysts developmental rates.

As shown in Table 5, the rates of in vitro sperm penetration were significantly decreased (p <0.05) when oocytes were inseminated with frozen semen treated with high antioxidant concentrations compared to those inseminated with control semen (57.41 %). The rate of male pronucleus formation were significantly (P< 0.05) reduced when oocytes were inseminated with frozen–thawed buffalo semen treated with high concentration of vitamin C and vitamin A (19.57% and 20.51 %, respectively) compared to those inseminated with control semen (40.74 %). Similarly, high concentrations of vitamin C and A decreased significantly (P < 0.05) the cleavage rates (18.52 and 19.69 %, respectively) compared to control group (3[illegible].21%; Table 6). However,

there were non significant differences among groups regarding the developmental rates to morula and blastocyst stages; Table 6.

The results regarding the influence of different antioxidants supplementation to semen extender on the conception rate are presented in Table 7. After 56-d of AI insemination, Selenium–ACE at low concentrations increased significantly ($P < 0.05$) the conception rate (69.43 %) compared to control (45.78 %). There is a positive correlation at ($P < 0.05$) between conception rate and viability index.

DISCUSSION

The results in the current study emphasize that addition of appropriate concentrations of antioxidants can play an important role in improving freezability and fertilizing potential of buffalo spermatozoa .The beneficial effects of low concentrations of antioxidants on sperm freezability recorded in the present study are entirely consistent with *Witting (1980) ; Affranchino et al .(1991) ; Beconi et al.(1993) ; Sarlos et al .(2002) and Khalifa (2002*) and on in vitro buffalo embryonic development with *Goto et al . (1993) and Takahashi et al .(1993*).The ability of antioxidants to increase sperm function , enzyme activity and in vitro fertilizing potential of spermatozoa exquisitely dependent on the inhibition of the damaging effect of lipid peroxidation (*Aitken et al., 1998 ; Kim and Parthasarathy , 1998 and Dalvit et al . 1998*) . Lipid peroxidation induces structural alterations, particularly in the acrosomal region of the sperm cell that cause the plasma membrane to loose its ability to act as a permeability barrier. Therefore, lipid peroxidation induces a fast and irreversible loose of motility, a deep change of sperm cytosolic enzymes, affecting the semen preservation, sperm – oocyte fusion and in vitro embryo development (*Jones and Mann, 1977; Alvarez and Storey , 1984 ; Halliwell et al ., 1992 ;Johnson and Nasr-Esfahani , 1994 ; Parinaud et al ., 1997 and Iwata et al ., 1998*).

Selenium addition to the semen extender has an important role in improving buffalo bull sperm freezability and in vitro fertilizing ability. This improvement of the sperm function may probably be due to increase glutathione peroxidase activities, which will give protection against spontaneous lipid peroxidation (*Smith et al., 1979; Kendall et al., 2000 and Castellini et al. 2002*). In spite of glutathione's effectiveness in preserving sperm motility , *Lindemann et al. (1988)* have found that mature bull spermatozoa have a little amounts of this common biological antioxidant. Moreover, *Sigel et al. (1980)* indicated

that, the motility of bovine spermatozoa was increased in vitro as the selenium level increased up to 1 µg/ml and decreased thereafter.

Vitamin E have a positive influence on buffalo bull semen freezability , in vitro fertilizing potential and fertility rate in a dose – dependent manner .. The main biological function of vitamin E likely is to scavenge free radicals, inhibit lipid peroxidation and provide sperm, with dose – dependent, protection against ROS which induce DNA damage (*Brezezinska – Slebodzinska et al., 1995; Marin – Guzman et al.,1997; Donnelly et al ., 1999; El-Sheltawi et al., 1999 and Verma and Kanwar , 1999*) .Vitamin E increased sperm motility , viability and further embryo development through its prevention of an oxidative reduction in the level of the major poly unsaturated fatty acids in membranes (*Lucy , 1972 ; Chow , 1991 ; Brezezinska – Slebodzinska et al ., 1995 and Cerolini et al .,2000*) as well as the protective effect it exerted on the plasmatic membrane of the bovine spermatozoa during deep freezing and post- thawing incubation (*Beconi et al., 1991 ; Marin-Guzman et al .,1997 and Verma and Kanwar, 1999*) .

Vitamin C improved buffalo bull sperm function in a dose – dependent manner.The positive influence of vitamin C on sperm quality are in agreement with (*Verma and Kanwar , 1999 ; Sakagmi et al., 2000 and Khalifa, 2002*). Addition of ascorbate to sperm preparation medium did provide sperm with complete protection against ROS, decreasing the susceptibility to lipid peroxidation at high oxygen tension in a dose dependent manner (*Beconi et al., 1993 and Donnelly et al., 1999*). A complex relationship between vitamin E and vitamin C was existed by (*Machlin and Gabriel 1980*). They proved that, vitamin C regenerated vitamin E molecules that have undergone free radical attack and may also spared vitamin E from oxidation.Vitamin E and C combination in semen extender was expected to improve freezability and fertilizing ability of buffalo spermatozoa, more than vitamin E alone, by elevating the antioxidant action of vitamin E. Instead, our results showed that sperm function worsened when vitamin C was added. Similar observation was reported recently with human semen (*Hughs et al., 1998*) and bovine spermatozoa (*Olsen and Seidel , 2000*) . Both vitamins are strong reducing agents and may act as prooxidants by maintaining iron and other metals in a reduced states thus promoting lipid peroxidation (*Yamamoto and Niki,1988*).

Selenium–ACE addition to semen extender at low concentrations have an important role in improving buffalo bull conception rate. The positive effects of selenium – ACE on conception rate are in agreement with (*Foote et al., 2002 and Khalifa ,2002*). The observed improvement

found in semen quality of treated frozen–thawed buffalo semen may be responsible for increased conception rate of cryopreserved buffalo semen in the field .The results from the current study indicate that selenium and antioxidations addition to buffalo semen extender can affect buffalo semen quality, but the greater effect seemed to be from selenium .The interaction of vitamin A, C and E with selenium at appropriate concentration may inhibit lipid peroxidation and therefore , improved buffalo sperm freezability and fertilizing potential.

On the other hand, it was observed from the present study that supplementation of buffalo semen extender with high concentrations of antioxidant were associated with a dramatic decline in the functional and fertilizing potential of buffalo spermatozoa. The dramatic effects of high antioxidant concentrations on sperm function and fertilizing ability are entirely consistent with previous studies (*Kozicki et al., 1981; Alabi et al., 1985; Dalvit et al., 1998; Verma and Kanwar, 1999 and Donnelly et al., 1999*) who reported abrupt fall in sperm motility, viability, in vitro fertilizing potential and concomitant increased lipid peroxidation with high concentrations of antioxidant supplementation. Exposure of spermatozoa to high level of antioxidants can rapidly overwhelm the spermatozoon's defenses and induce alteration in the fluidity of sperm plasmatic membrane (*Aitken et al.,1998 and Dalvit et al., 1998*) . Such severely damaged spermatozoa could not participate in fertilization, because collateral peroxidative damage to the plasma membrane would seriously impair the capacity of these cells for movement and sperm – oocyte fusion (*Aitken and Fisher, 1994*).

In conclusion, the present study provides evidence that selenium – ACE at appropriate concentrations enhance buffalo bull semen freezability, in vitro fertilizing potential, buffalo embryonic development in vitro and conception rate, presumably by affording protection from reactive oxygen species and lipid peroxidation.

REFERENCES

Affranchino , M.A . ; Trinchero , G . D .; Schang , L .M . and Beconi , M.T. (1991) : Bovine spermatozoa as lipoperoxidation inhibitor. Com.Biol .; (Bs. Aires) 9 : 261-274.

Aitken, R.J. and Fisher, H.M. (1994): Reactive oxygen species generation and human spermatozoa: the balance of benefit and risk . Bioessays; 16: 259-267.

Aitken, R.J.; Gordon, E.; Harkiss, D.; Twigg, J.P.; Milne, P.; Jennings, Z. and Irvine,D.S. (1998): Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa . Biol. Reprod. ; 59 : 1037-1046.

Alabi, N.S.; Whanger, P.D. and Wu, A .S. H. (1985): Interactive effects of organic and inorganic selenium with cadmium and mercury on spermatozoal oxygen consumption and motility in vitro. Biol. Repord.; 33 : 911-919.

Alvarez, J.G. and Storey, B. T. (1984): lipid peroxidation and the reactions of superoxide and hydrogen peroxide in mouse spermatozoa. Biol. Repord.;30 (4):833 -841.

Askari, H .A.; Check, J. H.; Peymer, N. and Bollendorf, A. (1994): Effect of natural antioxidants tocopherol and ascorbic acids in maintenance of sperm activity during freeze-thaw process. Arch. Androl.; 33 (1): 11 – 15.

Badr, M.R.; Darwish, G.M.; Sosa, G.A.; Agag, M.A. and El- Azab, A.I. (2003): Factors affecting the technology of in vitro fertilization and embryo production in buffaloes: I - Post-slaughtering time, follicular size, methods of collection and quality of oocytes. Egyptian Soc. Anim. Reprod. Fert. 15 th Annual Congress, 93-102.

Beconi , M.T.; Affranchino, M.A.; Schang , L.M. and Bearlegui , N.B. (1991) : Influence of antioxidants on SOD activity in bovine sperm . Biochem. Int.; 23 (3): 545-453.

Beconi, M.T.; Francia, C.R.; Mora, N.G. and Affranchino, M.A. (1993) :Effect of natural antioxidants on frozen bovine sperm preservation .Theriogenology;40:841-851.

Bilodeau, J.F.; Blanchette, S.; Cormier, N. and Sirard, M.A.(2002): Oxygen species – mediated loss of bovine sperm motility in egg yolk Tris extender : protection by pyruvate , metal chelators and bovine liver or oviductal fluid catalase . Theriogenolgy ; 57 (3) :1105 – 1122.

Brennan, L.A.; Morris, G.M.; Wasson,G.R.; Hannigan, B.M. and Barnett, Y.A.(2000): The effect of vitamin C or vitamin E supplementation on basal and H_2O_2 induced DNA damage in human lymphocytes . Br. J. Nutri .; 84 (2) : 195 – 202.

Brezezinska- Slebodzinska, E.; Slebodzinski, A.B.; Pietras, B. and Wieczorek, G. (1995): Antioxidant effect of vitamin E and glutathionine on lipid peroxidation in boar semen plasma. Biol. Trace Element Res.; 47 : 69-74.

Castellini, C.; Lattaioli, P.; Bosco, A. D.and Beghelli, E. (2002): Effect of supranutritional level of dietary alpha-tocopherol acetate and selenium on rabbit semen. Theriogenology ; 58(9):1723-1732.

Cerolini, S.; Maldjian , A.; Surai , P. and Noble , R. (2000) : Viability , susceptibility to peroxidation and fatty acid composition of boar semen during liquid storage. Anim. Reprod. Sci.; 58 (1-2): 99-111.

Chow, C.K.(1991): Vitamin E and oxidative stress . Free Radical Biol. Med .; 11:215 – 232.

Dalvit , G.C.; Cetica , P.D. and Beconi , M.T. (1998) : Effect of alpha-tocopheral and ascorbic acid on bovine in vitro fertilization . Theriogenology ; 49 (3) : 619-627.

Donnelly, E.T.; McClure , N. and Lewis , S.E. (1999) : The effect of ascorbate and alpha-tocopherol supplementation in vitro on DNA integrity and hydrogen peroxide-induced DNA damage in human spermatozoa. Mutagenesis; 14 (5): 505-512

El-Sheltawi , M.A.F.;Abd El Malak , M.G.;Abdel Malak ,G. and Khalifa,T.A.A. (1999): Impact of zinc and tocopherol on functional competence of cryopreserved buffalo spermatozoa . Assiut Vet . Med . J .; 42 :(83)-291-309 .

Foote , R.H . ; Brackett , C.C. and Kaproth , M.T. (2002) : Motility and fertility of bull sperm in whole milk extender containing antioxidants . Anim . Reprod . Sci . ; 71 (1-2) :13-23.

Goto ,Y . ; Noda ,Y.; Mori,T. and Nakano ,M . (1993) : Increased generation of reactive oxygen species in embryos cultured in vitro .Theriogenology ; 15: 69 – 75 .

Halliwell , B.; Gutteridge , J.M.C. and Cross, C.E. (1992) : Review article : free radicals , antioxidants and human disease : where are we now ? Lab. Clin . Med.; 119:598-620.

Hughs, C.M.; Lewis , S.E.M.; Mckelvey – Martin , V.J. and Thompson , W. (1998) : The effects of antioxidant supplementation during percell preparation on human sperm DNA integrity . Hum . Reprod . ; 13 : 1240- 1247.

Iwata , H.; Akamatsu , S.; Minami , N. and Yamada , M. (1998) : Effects of antioxidants on the development of bovine IVM /IVF embryos in various concentrations of glucose . Theriogenology ;50(3) : 365-375.

*Johnson ,M.H. and Nasr-Esfahani , M.H. (1994):*Radical solutions and cultural problems : could free oxygen radicals be responsible for the impaired development of mammalian embryos in vitro ? Bioessays ;15 : 69 -75.

Jones , R. and Mann , T. (1977) :Toxicity of exogenous fatty acid peroxides towards spermatozoa . J.Reprod. Fertil .; 50: 255-260.

Kendall , N . R . ; McMullen ,S.; Green , A . and Rodway , R.G. (2000) : The effect of zinc , cobalt and selenium soluble glass bolus on trace element status and semen quality of ram lambs . Anim . Reprod .Sci.,62: 277-283.

Khalifa , T.A.A. (2002) : Effect of some antioxidants on viability of preserved buffalo and ram semen . Ph .D. Thesis , Fac. Vet. Med. , Cairo University.

Kim ,J.G. and Parthasarathy , S.(1998): Oxidation and the spermatozoa. Semin Reprod . Endocrinol .; 16 (4):235-239 .

Kozicki , L. ; Silva , R.G . and Barnabe , R . C. (1981) : Effects of vitamins A , D_3 , E , and C on the characteristics of bull semen . Zbl . Vet .Med . A , 28:538-546.

Lindemann , C.B.; O'Brien , J . A . and Giblin , F. J . (1988) : An investigation of the effectiveness of certain antioxidants in preserving the motility of reactived bull sperm models. Biol . Reprod . ; 38 : 114-122 .

Lucy , J . A. (1972) : Functional and structural aspects of biological membranes :
A suggested structural role for vitamin E in the control of membrane permeability and stability . Ann . N . Y . Acad . Sci.; 203 : 4-11.

Machlin , L . J . and Gabriel , E .(1980) :Interactions of vitamin E with vitamin C , vitamin B 12 , and Zinc. In :Levander , O . A .and Cheng , L . , (eds.) Micronutrient Interactions : Vitamins , Minerals and Hazardous Elements . Ann. N.Y. Acad . Sci . ; 355 : 98-107 .

Marin - Guzman , J.; Mahan , D.C. ; Chung , Y.K. ; Pate ,J.L.; and Pope ,F.(1997) : Effects of dietary selenium and vitamin E on boar performance and tissue responses , semen quality , and subsequent fertilization rates in mature gilts . J. Anim. Sci.;75 : 2994-3003.

Milovanov , V.K. (1962) : Biology of reproduction and artificial insemination of farm animals . Monograph . Selkhoz . Lit . J. and Plakatov , Moscow.

Mohammed , K.M.; Ziada, M.S. and Darwish , G.M. (1998) : Practical trials for freezing semen of buffalo and friesian bulls : Effect of various regimens of freezing , different milk extenders and types of straws packags on post-thawing semen characters. Assiut Vet. Med . J . ; 39 (77) :70-93 .

Olsen , S.E. and Seidel ,G .E.Jr.(2000) : Culture of in vitro-produced bovine embryos with vitamin E improves development in vitro and after transfer to recipients . Biol . Reprod . ; 62 (2) : 248 – 252.

Parinaud , J . ; Lelannou , D.; Vieitez , G.; Griveau , J . F.; Milhet , P. and Richoilley , G . (1997) : Enhancement of motility by treating spermatozoa with an antioxidant solution (Sperm –Fit) following ejaculation . Hum . Reprod .; 12 (11) : 2434 – 2436 .

Parrish , J.J.; Susko-Parrish , J.; Winer , M.A. and First , N.L. (1988) : Capacitation of bovine sperm by heparin . Biol . Reprod .; 38, 1171-1180.

Sakagami, H.; Satoh, K.; Hakeda, Y.and Kumegawa, M. (2000): Apoptosis – inducing activity of vitamin C and vitamin K .Cell Mol. Biol .; 46 : 129- 143.

Sarlos, P.; Molnar, A . ; Kokai , M .; Gabor, G. and Ratky , J . (2002): Comparative evaluation of the effect of antioxidants on the conservation of ram semen. Acta.Vet. Hung.; 50 (2): 235 – 245.

SAS . 1985 : SAS User's Guide , Statistics (version 5 Ed .) . Inst . Inc. , Cary , NC.

Sigel, R.B.; Murray, F.A. ; Julein , W.E.; Maxon , A.L. and Conrad, H. R.(1980) : Effect of in vitro selenium supplementation on bovine sperm mobility . Theriogenology ; 13 : 357- 367.

Singh, P.; Chand, D. and Georgie, G.C. (1989): Lipid peroxidation in buffalo (Bubalus bubalis) spermatozoa: effect of added vitamin C and glucose. Indian J. Exp. Biol .; 27 : 1001- 1004 .

Smith , D . G.; Senger, P.L.; McCutchan, J. F. and Landa, C.A. (1979) : Selenium and glutathione peroxidase distribution in bovine semen and selenium -75 retention by the tissues of the reproductive tract in the bull .Biol .Reprod.;20:377- 383.

Steinbach,J.and Foote , R. H.(1967): Osmotic pressure and pH effects on survival of frozen bovine spermatozoa . J.Dairy Sci.;50: 205- 213 .

Takahashi , M.; Nagai , T. ; Hamano , S. ; Kuwayama , M. ; Okamura , N . and Okano , A . (1993): Effect of thiol compounds on in vitro development and intracellular glutathione content of bovine embryos . Biol . Reprod ; 49:228-232.

Tietz, N.W. (1976): Fundamentals of clinical chemistry. W. B. Saunders Company, Philadel phia.

Witting , L.A . (1980): Vitamin E and lipid antioxidants in free – radical – initiated reactions. in: Free Radicals in Biology , Pryor , W.A. (ed), Vol .IV. Academic press, Inc., New York , 1980 , pp . 295- 318.

Yamamoto, K. and Niki, E. (1988): Interaction of - tocopherol with iron: antioxidant and prooxidation effects of - tocopherol in the oxidation of lipids in aqueous dispersions in the presence of iron . Biochim . Biophy . Acta. ; 958 : 19-23.

Verma, A. and Kanwar, K. G. (1999): Human spermatozoal motility and lipid peroxidation . Indian J. Exp . Biol . ; 37 (1): 83 – 85.

Table1. *Influence of different antioxidant concentrations addition to semen extender on pre-and post freezing sperm motility and viability indices of thawed buffalo spermatozoa(Means* ± SE).

Treatments	concentrations / ml	Diluted sperm motility	Post-thawing sperm motility	Reduction %	Viability index
Control		73.33±1.66 **ab**	45.01±2.88 **a-d**	38.73±2.82 **b-e**	85.83 ±10.63 **c-e**
Vitamin E	1 mg	75.01 ±2.88 **ab**	46.66 ± 1.66**a-d**	37.59 ± 3.15 **c-e**	87.50 ± 7.22 **cd**
	2 mg	71.66 ± 1.67 **ab**	45.00 ±2.89 **a-d**	37.30 ± 2.86 **c-e**	70.00 ± 3.81 **c-f**
	3 mg	73.33 ± 1.66 **ab**	41.66 ±1.66 **b-e**	43.17 ±1.92 **a-e**	66.66 ± 1.66 **c-f**
Vitamin C	3 mg	76.66 ±1.66 **ab**	45.01 ±2.88 **a-d**	41.11 ±4.83 **a-e**	63.33 ±9.61 **d-f**
	6 mg	75.02 ±2.88 **ab**	33.33 ± 4.40 **d-f**	55.25 ±6.88 **a-d**	50.83 ±7.11 **ef**
	9 mg	66.66 ± 1.66 **b**	31.66 ± 4.41 **d-f**	52.74 ± 5.44 **a-e**	39.16 ± 13.09 **f**
Vitamin A	50 iu	76.66 ±3.33 **ab**	45.02 ± 2.87**a-d**	41.07 ± 4.78 **a-e**	85.01 ±9.46 **c-e**
	100 iu	78.33 ± 3.33 **ab**	41.66 ±1.66 **b-e**	46.76 ± 0.13 **a-e**	80.01 ± 8.36 **c-e**
	150 iu	78.33 ± 1.66 **ab**	36.66 ± 1.65 **c-f**	53.05 ± 3.19 **a-e**	67.51 ± 10.89 **c-f**
Selenium (Se)	2.5 µg	78.33 ± 1.66 **ab**	53.33 ± 1.67 **ab**	31.94 ± 0.69**d-e**	119.16 ± 4.62 **ab**
	5 µg	76.66 ± 1.66 **ab**	38.33 ± 4.40 **b-f**	49.72 ± 6.66 **a-c**	81.66 ±6.51 **c-e**
	10 µg	70.01 ± 2.88 **ab**	33.33 ± 3.33 **d-f**	51.86 ±6.76 **a-c**	72.50 ± 8.03 **c-f**
Vitamin E + C	1 mg + 3 mg	71.66 ± 1.66 **ab**	31.66 ± 4.39 **d-f**	56.03 ±5.12 **a-d**	40.01 ± 5.77 **f**
VitaminE+A	1 mg + 50 iu	66.66 ± 1.66 **b**	25.00 ± 2.88 **f**	62.45 ± 4.53 **a-c**	52.50 ± 3.82 **d-f**
Vitamin E+ Se	1 mg + 2.5µg	73.33 ± 4.4 **ab**	25.00 ± 2.89 **f**	65.51 ±4.76 **a**	37.50 ±3.82 **f**
VitaminC+A	3 mg + 50 iu	65.02 ± 2.88 **b**	23.33 ± 4.41 **f**	64.13 ± 6.40 **ab**	36.66 ± 3.33 **f**
VitaminC+Se	3mg +2.5 µg	71.66 ± 3.33 **ab**	31.66 ± 4.40 **df**	55.04 ± 8.51**a-d**	40.83± 6.50 **f**
VitaminA+Se	50 iu + 2.5 µg	70.02 ± 2.88 **ab**	28.33 ±4.39 **e-f**	58.87 ±7.89 **a-c**	42.51 ± 8.66 **f**
Selenium-ACE	Low levels *	81.66 ± 1.66 **a**	58.33 ± 1.66 **a**	28.55 ± 1.85 **e**	134.16 ± 7.26 **a**
Selenium -ACE	High levels**	75.02 ± 2.88 **ab**	50.00 ± 2.87**abc**	32.99 ± 5.4 **d-e**	100.66 ± 5.81 **bc**
Over all mean		73.57 ± 0.70	38.57 ± 1.41	47.80 ± 1.63	69.23 ± 3.63
Significance		P<0.05	P<0.01	P < 0.01	P < 0.01

Values are from 6 trials for each treatment .

Values with different letters in the same column were significantly different (P<0.05)and (P<0.01)

* Low levels :2.5 µg / ml selenium + 50 iu / ml vitamin A + 3 mg / ml vitamin C + 1 mg / ml vitamin E .

**High levels :10 µg / ml selenium + 150 iu / ml vitamin A + 9 mg / ml vitamin C + 3 mg / ml vitamin E .

Table 2 . *Influence of different antioxidant concentrations addition to semen extender on pre –and post freezing enzymes activity of buffalo semen(Means ± SE)* .

Treatments	concentrations / ml	AST * (U / ml)		AKP ** (U / L)	
		Diluting AST	Thawing AST	Diluting AKP	Thawing AKP
Control		73.01± 3.05 **de**	105.66± 4.69 **cd**	9.66 ± 0.87 **ab**	15.33±0.66 **a-c**
Vitamin E	1 mg	77.02±2.51 **b-e**	110.33±5.17**a-d**	10.33±0.87 **ab**	13.33±1.20 **a-c**
	2 mg	79.66±3.18 **d-e**	116.01±2.64 **a-c**	12.01±1.52 **ab**	17.01±1.52 **a-c**
	3 mg	85.00±2.88 **a-d**	121.33±1.85 **ab**	12.02±1.52 **ab**	19.33±1.45 **ab**
Vitamin C	3 mg	77.01±3.21 **b-e**	113.66±4.37**a-d**	10.66±1.20 **ab**	13.01±1.52 **a-c**
	6 mg	80.33±3.17 **a-e**	121.33±2.40 **ab**	12.02±1.73 **ab**	16.33±2.72 **a-c**
	9 mg	85.66±2.60 **a-d**	124.01±2.07 **ab**	13.00±1.52 **ab**	19.33±1.85 **ab**
Vitamin A	50 iu	76.01±3.05 **c-e**	114.66±3.17**a-d**	11.01±1.52 **ab**	15.01±1.15 **a-c**
	100 iu	89.33±2.33 **a-c**	116.33±2.96 **a-c**	12.66±0.87 **ab**	19.33±2.96 **ab**
	150 iu	89.33±2.33 **a-c**	120.66±2.60 **ab**	14.33±1.20 **ab**	19.66±2.96 **ab**
Selenium	2.5 µg	71.66±2.18 **de**	106.01±2.32 **cd**	8.66 ± 0.87 **a**	11.01±2.96 **ab**
	5 µg	76.01±2.08 **c-e**	114.33±2.90**a-d**	11.33±0.8 **ab**	15.33±1.52 **ac**
	10 µg	80.33±2.84 **a-e**	120.01±2.89 **a-c**	13.66±1.1 **ab**	20.33±1.76 **a**
Vitamin E + C	1 mg + 3 mg	84.33±3.48 **a-d**	121.33±1.85 **ab**	12.66±1.1 **ab**	17.66±2.08 **a-c**
Vitamin E+ A	1 mg + 50 iu	91.01±3.78 **a-c**	122.66±2.33 **ab**	13.0±0.87 **ab**	20.33± 0.87 **a**
Vitamin E+ Se	1 mg + 2.5µg	91.33±3.84 **a-c**	125.01±3.05 **a**	15.33± 1.55 **a**	22.33± 2.03 **a**
Vitamin C+ A	3 mg + 50 iu	91.66±4.40 **ab**	121.66±2.72 **ab**	12.33±1.2 **ab**	21.33± 1.45 **a**
Vitamin C+Se	3mg +2.5µg	94.66±3.18 **a**	124.01±2.64 **ab**	15.33± 1.45 **a**	22.01±1.52 **a**
Vitamin A+Se	50 iu +2.5µg	92.33±3.37 **ab**	123.66±2.84 **ab**	12.33±2.1 **ab**	21.33± 2.03 **a**
Selenium -ACE	Low levels*	68.66±3.53 **e**	101.33±1.85 **d**	8.01± 1.45 **b**	10.33± 0.87 **c**
Selenium - ACE	High levels **	73.33±2.03 **d-e**	108.66±3.84**b-d**	10.33±1.7 **ab**	14.01±1.45 **a-c**
Over all mean		82.22±1.13	116.79±1.02	11.93±0.45	17.31 ± 0.73
Significance		P < 0.01	P<0.01	P<0.05	P<0.01

Values are from 6 trails for each treatment

Values with different letters in the same column were significantly different (P<0.05)and (P<0.01)

* Low levels :2.5 µg / ml selenium + 50 iu / ml vitamin A + 3 mg / ml vitamin C + 1 mg / ml vitamin E .

**High levels :10 µg / ml selenium + 150 iu / ml vitamin A + 9 mg / ml vitamin C + 3 mg / ml vitamin E .

* AST – Aspartate aminotransferase enzyme.

** AKP – Alkaline Phosphatase enzyme.

Table 3 . *Effect of low antioxidant concentrations addition to semen extender on in vitro penetration and malepronucleus formation of buffalo oocytes.*

Treatments	No. of matured oocytes	Penetration rate			Fertilization rate			Poly sperm		
		N	(%)		N	(%)		N	(%)	
Control	72	42	(58.33)	a	31	(43.06)	a	11	(15.28)	a
Vitamin E 1 mg/ml	92	55	(59.78)	a	42	(45.65)	ab	10	(10.87)	a
Vitamin C 3mg/ml	105	61	(58.09)	a	45	(42.86)	a	16	(15.24)	a
Vitamin A 50iu/ml	88	51	(57.95)	a	41	(46.59)	ab	10	(11.36)	a
Selenium 2.5µg/ml	77	49	(63.64)	a	37	(48.05)	ab	12	(15.58)	a
Selenium- ACE	120	83	(69.17)	a	70	(58.33)	b	13	(10.83)	a

Values are from 4 experiments for each treatment
a-b percentage within same column with different letters differ significantly (P<0.05) .

Table 4 . *Effect of low antioxidant concentrations addition to semen extender on in vitro buffalo embryos development.*

Treatments	No. of inseminated oocytes	Cleavage rate			Morula			Blastocyst		
		N	(%)		N	(%)		N	(%)	
Control	46	15	(32.61)	a	5	(10.87)	a	2	(4.35)	a
Vitamin E 1 mg/ml	65	29	(44.62)	ab	11	(16.92)	ab	7	(10.77)	ab
Vitamin C 3mg/ml	52	16	(30.77)	a	5	(9.62)	a	2	(3.85)	a
Vitamin A 50iu/ml	58	23	(39.66)	ab	9	(15.52)	ab	4	(6.89)	ab
Selenium 2.5 µg/ml	54	25	(46.29)	ab	11	(20.37)	ab	5	(9.26)	ab
Selenium- ACE	75	41	(54.67)	b	20	(26.67)	b	13	(17.33)	b

Values are from 4 experiments for each treatment
a-b percentage within same column with different letters differ significantly (P<0.05) .

Table 5. *Influence of high antioxidant concentrations addition to semen extender on in vitro penetration and male pronucleus formation in buffalo oocytes.*

Treatments	No. of matured oocytes	Penetration rate			Fertilization rate			Poly sperm		
		N	(%)		N	(%)		N	(%)	
Control	54	31	(57.41)	a	22	(40.74)	a	9	(16.67)	a
Vitamin E 3 mg/ml	42	15	(35.71)	b	10	(23.81)	a	5	(11.90)	a
Vitamin C 9mg/ml	46	15	(32.61)	b	9	(19.57)	b	6	(13.04)	a
Vitamin A150iu/ml	39	13	(33.33)	b	8	(20.51)	b	5	(12.82)	a
Selenium 10 µg/ml	46	17	(36.96)	b	13	(28.26)	ab	4	(8.69)	a
Selenium- ACE	55	24	(43.64)	ab	20	(36.36)	ab	4	(7.27)	a

Values are from 4 experiments for each treatment
a-b percentage within same column with different letters differ significantly (P<0.05) .

Table 6 . *Influence of high antioxidant concentrations addition on in vitro buffalo embryos development.*

Treatments	No. of inseminated oocytes	Cleavage rate			Morula			Blastocyst		
		N	(%)		N	(%)		N	(%)	
Control	58	21	(36.21)	a	9	(15.52)	a	4	(6.89)	a
Vitamin E 3 mg/ml	46	12	(26.09)	ab	4	(8.69)	a	2	(4.35)	a
Vitamin C 9mg/ml	54	10	(18.52)	b	3	(5.56)	a	1	(1.85)	a
Vitamin A150iu/ml	66	13	(19.69)	b	6	(9.09)	a	2	(3.03)	a
Selenium 10µg/ml	58	16	(27.59)	ab	5	(8.62)	a	3	(5 17)	a
Selenium -ACE	69	24	(34.78)	ab	8	(11.59)	a	5	(7.25)	a

Values are from 4 experiments for each treatment
a-b percentage within same column with different letters differ significantly (P<0.05) .

Table 7 . *Effect of different antioxidants addition to semen extender on the conception rate of buffalo bulls.*

Treatments	No.of buffaloes	No.of examined buffalos		Conception rate (%)	Conception rate Mean ± SE
		pregnant	Non pregnant		
Control	44	21	23	47.73	45.78 ± 1.91 **b**
Vitamin E 1 mg	44	27	17	61.36	56.40 ± 4.35 **ab**
Selenium- ACE low levels *	50	35	15	70.00	69.43 ± 6.55 **a**
Over all mean					57.20 ± 3.59

Values with different letters within same column were significantly different (P < 0.05) .

* Low levels :2.5 µg / ml selenium + 50 iu / ml vitamin A + 3 mg / ml vitamin C + 1 mg / ml vitamin E .