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INFLUENCE OF ANTIOXIDANTS ON DNA INTEGRITY, MITOCHONDRIAL FUNCTION AND FERTILIZING POTENTIALS OF CRYOPRESERVED BUFFALO SPERMATOZOA

(With One Table, 6 Figures and 2 Photoes)

By

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دراسة تأثير بعض مضادات الأكسدة علي قابليه حيامن الجاموس للتجميد وسلامة الحامض النووي للحيامن وكفاءتها الاخصابية معمليا وحقليا

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تهدف الدراسة الحالية إلي دراسة تأثير التجميد وكذالك تأثير اضافة بعض مضادات الاكسدة الي ممدد السائل المنوى علي قابلية حيامن الجاموس للتجميد وعلي الحامض النووى للحيامن وكذا قدرتها الاخصابية معمليا ومعدل العشر حقليا. تم تجميع عينات السائل المنوي من ستة طلائق جاموسي وبعد تقيم السائل المنوي معمليا تم تمديده في ممدد التريس فقط (المجموعة الضابطة) أو المضاف الية تركيزات مختلفة من بعض مضادات الأكسدة مثل الجلوتاثيون المختزل؛ كاتاليز؛ حمض الفا ليبوك وسوبر أوكسيد ديسميوتيز. بعد تبريد وتجميد الحيامن الجاموسي بالنظام الفرنسي تم تقيمه من حيث نسبه الحركه الأماميه والحيويه وتشوهات القلنسوه وكذا اثر التجميد على الميتوكوندريا والحامض النووي للحيامن وكذالك قدرته الاخصابيه معمليا وحقليا. حيث أوضحت نتائج الدراسة الحالية أن تجميد حيامن الجاموس في ممدد التريس (المجموعة الضابطة) أدي إلي تأثير سلبي كبير علي الحركه الأماميه والحيويه وتشوهات القلنسوه (٤٣,٣٣±٣,٣٠ % ، ٨٦,٦٧±٤,٤٢ و٢٧,٣٣±٣,٨٥% علي التوالي) وكذالك على وظائف الميتوكوندريا (1,88±0.05) كما أدي الي ارتفاع تشظي الحامض النووي (٤,٤٢±٧١,٦٧%) وكذالك قلل قدرته الاخصابيه معمليا وحقليا (٤٦,٥٨ و ٥١,٦٧% علي التوالي) بينما أظهرت نتائج الدراسة الحالية أن إضافة مضادات الأكسدة إلي ممدد التريس لة تأثير ايجابي كبير علي تحسن وظائف السائل المنوي الجاموسي المجمد بناء علي التركيز المضاف به الي الممدد. حيث أوضحت نتائج الدراسة الحالية أن إضافة

١٠ملليمول من الجلوتاثيون المختزل أو ١٠٠ وحدة/مللي من كاتاليز أو ١٥ ملليمول من حمض الفا ليبوك أو ٥٠ وحدة/مللي من سوبر أوكسد ديسميوتيز إلي الممدد قد أدي إلي زيادة معنوية كبيرة في نسبه الحركة الأماميه للمني بعد الاساله (٢,٨٩ ±٦٥,٠٠ ، ١,٦٦ ±٦٦,٦٧ ، ١,٦٦ ± ٦١,٦٧ و٣,٣٤±٦٣,٣٣% علي التوالي) وحيويه المني(١٠,١٢±١٦٧,٥٠ ، ٣,٠١±١٦٦,٦٧ ، ٤,٦٥ ±١٥٠,٨٣ و١٠,١ ±١٦٨,٣٣ علي التوالي) كما أدي إلي انخفاض معنوي كبير في نسبه تشوهات القلنسوة (٤,٠٥±١١,٦٦ ، ٢,٩١ ± ١١,٣٣ ، ٣,٩٣±١٢,٣٣ و٢,٣٤±١٠,٣٣% علي التوالي) كما حسن من وظائف الميتوكوندريا حتي بعد مرور ثلاث ساعات من الاساله (٠,٠٥±٣,٤٢ ، 0.16±٣,٢٩ ، ٠,٠٨±٢,٨١ و ٠,٠٨±٣,٣١علي التوالي) كما حافظ علي سلامة الحامض النووي للحيامن (٤,٣٣±٣٧,٦٧ ، ٢,٦١ ±٣٦,٣٣ ، ٥,٠٥ ±٤٣,٣٣ و ٥,٠٤ ±٣٦,٠٠% علي التوالي). كما أوضحت نتائج الاخصاب المعملي إن إضافة ١٠ملليمول من الجلوتاثيون المختزل أو ١٠٠ وحدة/مللي من كاتاليز أو ٥٠ وحدة/مللي من سوبر أوكسد ديسميوتيز إلي الممدد قد أدي إلي زيادة معنوية كبيرة في النمو إلي طور البلاستوسيست (١١,٨٦ ،١١,٥٤ و ١٤,٢٠ % علي التوالي) ، كما أظهرت نتائج التلقيح الحقلي أن إضافة ١٠ملليمول من الجلوتاثيون المختزل أو ١٠٠ وحدة/مللي من كاتاليز أو ٥٠ وحدة/مللي من سوبر أوكسد ديسميوتيز إلي الممدد أدي الي زيادة كبيرة في معدل العشر بعد الجس (٦٢,١١،٦٢,٧٩ و ٦٧,٧٤ % علي التوالي). ومن خلال نتائج الدراسة الحالية يمكن أن نستنتج أن للتجميد تأثير سيئ علي محتوي الحامض النووي والقدرة الاخصابية للسائل المنوي الجاموسي في حين أن إضافة مضادات الأكسدة الجلوتاثيون المختزل ،كاتاليز حمض الفا ليبوك أو سوبر أو كسيد ديسميوتيز إلي ممدد التريس يلعب دورا هاما وكبيرا في تحسن وظائف السائل المنوي الجاموسي المجمد بناء علي التركيز المضاف به إلي الممدد ويبدو ذلك من خلال قدرتها علي مقاومة التأثير الضار لعمليات الأكسدة أثناء التجميد علي سلامة الحامض النووي للحيامن.

SUMMARY

The objective of the current study was to evaluate the effect of cryopreservation and fortification of freezing extender with some antioxidants on the DNA integrity and the fertilizing potentials of cryopreserved buffalo spermatozoa. Buffalo semen was collected, evaluated and extended in Tris-based extender supplemented with different concentrations of reduced glutathione (GSH), catalase (CAT), alpha lipoic acid (ALA) and superoxide dismutase (SOD). Semen was examined post-thawing to evaluate, freezability, mitochondrial function, DNA integrity and the natural and assisted fertilizing potentials. The main findings emerging from the present study were that cryopreservation decreased ($P<0.01$) significantly sperm freezability, mitochondrial activity, DNA integrity and fertilizing potentials of frozen-thawed buffalo spermatozoa. Meanwhile, in vitro provision of freezing

extender with GSH, catalase, ALA and SOD had a beneficial effect on the function of the cyropreserved buffalo spermatozoa, in a dose dependent trend. Fortification of semen extender with 10 mM GSH, 100 U/ml catalase, 15 mM ALA or 50 U/ml SOD increased ($P<0.01$) significantly post-thawing progressive sperm motility (65.00±2.89, 66.67±1.66, 61.67±1.66 and 63.33±3.34%, respectively); viability indices (167.50±10.12, 166.67±3.01, 150.83±4.65 and 168.33±10.1, respectively) and maintained the acrosomal integrity (11.66±4.05, 11.33±2.91, 12.33±3.93 and 10.33±2.34%, respectively). Moreover, improved the mitochondrial function (3.42±0.05, 3.29±0.16, 2.81±0.09 and 3.31±0.08, respectively); DNA integrity (37.67±4.33, 36.33±2.61, 43.33±5.05 and 36.00±5.04%, respectively). Additionally, in vitro provision of freezing extender with 10 mM GSH, 100 U/ml catalase or 50 U/ml SOD increased in vitro embryo development to the blastocyst stage (11.86, 11.54 and 14.20 %, respectively) and augmented the natural pregnancy rate (62.11, 62.79 and 67.74%, respectively). In conclusion, cryopreservation promotes DNA fragmentation in buffalo spermatozoa that can be counteracted by the addition of GSH, catalase, ALA and SOD. These antioxidants appear to play an important role in sperm antioxidant defense strategy in a dose dependent trend and could be of significant benefit in improving the freezability, DNA integrity and the natural and assisted fertilizing capacity of the cryopreserved buffalo spermatozoa.

Key words: *Semen, artificial insemination, cryopreservation*

INTRODUCTION

Semen cryopreservation and artificial insemination (AI) offer many advantages to the livestock industry, particularly in conjunction with genetic evaluation and selection programs (Johnson *et al.*, 2000). However, the biggest obstacle to exploiting cryopreserved semen of many species is damage of sperm membrane structures during freezing and thawing, which leads to fewer viable and motile cells post-thawing (Hammerstedt *et al.*, 1990). Consequently, fertility following AI is poorer than with fresh semen in most species (Holt, 2000). Cryopresevation of spermatozoa is associated with an increase in reactive oxygen species (ROS) generation and lipid peroxidation (Linfor and Meyers, 2002 and Zalata *et al.*, 2004); thereby, cryopreservation of spermatozoa may subject sperm cells to oxidative stress and potential

DNA damage (Sanocka and Kurpisz, 2004). Spermatozoa are sensitive to oxidative stress because they lack cytoplasmic defenses (Saleh and Agarwal, 2002). Excessive ROS formation by spermatozoa during the cryopreservation process has been associated with a detrimental effect on sperm DNA (Tominaga *et al.*, 2004) and sperm fertilizing potentials (Ball *et al.*, 2001). It is difficult to block the OS-induced injury to cells because ROS are continuously produced by cellular aerobic metabolism (Aitken and Krausz (2001)). However, oxidative DNA damage in mammalian sperm suspensions can be counteracted by the addition of seminal plasma that contains the predominant source of antioxidant protection (Potts *et al.*, 2000). Unfortunately, sperm preparation for cryopreservation involves the removal of the seminal plasma and consequently the main source of sperm protection. Moreover, the process of cryopreservation is associated with an alteration in antioxidant defense systems; including a decrease in intracellular glutathione (Bilodeau *et al.*, 2000 and Gadea *et al.*, 2004) and superoxide dismutase (SOD) content (Nair *et al.*, 2006). So, one obvious way to overcome the detrimental effects of ROS on the cryopreserved sperm performance could be the addition of antioxidant compounds to the freezing extender to block or prevent oxidative stress. A variety of antioxidants has been examined to either scavenge ROS directly or counter the effects of ROS toxicity in the semen of mammalian species (Rossi *et al.*, 2001). Therefore, the goal of the present study was to evaluate the effect of cryopreservation and the fortification of freezing extender with some antioxidants as reduced GSH, catalase, alpha lipoic acid (ALA) and superoxide dismutase (SOD), on buffalo semen freezability, mitochondrial function, DNA integrity, natural and assisted fertilizing potentials of the cryopreserved buffalo spermatozoa.

MATERIALS and METHODS

Semen collection and cryopreservation:

Semen samples were collected from six buffalo bulls of proven fertility, with the use of an artificial vagina. Only semen samples of at least 70 % initial motility and 800.00X10^{6}sperm cells/ml were used. Immediately after collection, semen samples were pooled and diluted at a 1:8 ratio at 30°C with a commercial Tris-egg yolk–based extender (Optidyl®; Bio-Vet France). Diluted semen samples were split into 13 portions as follows: control (extender only); GSH (5, 10 and 20 mM); catalase (100, 200 and 300 U/ml); ALA (10, 15 and 20 mM) and SOD

(50, 100 and 200 U/ml). Immediately after dilution, the extended semen of each treatment was evaluated then, it was cooled to 5°C over 60 minute in a cold cabinet. The cooled semen was loaded into 0.25 ml French straws (IMV, l'Aigle, France) sealed with polyvinyl acid and then lowered into liquid nitrogen vapor inside foam box. The straws were then immersed into liquid nitrogen and stored for at least two weeks before analysis. Frozen semen was thawed by plunging straws into a 37°C water bath for 30 seconds. Classical semen quality tests (motility, viability and acrosomal integrity) were assessed according to Mohammed *et al.* (1998) after thawing.

Evaluation of mitochondrial function of frozen-thawed spermatozoa:

Functional integrity of sperm mitochondria was assessed using a modified mitochondrial reduction assay according to Mosmann (1983). For each treatment of the frozen-thawed semen samples, three wells were used. A 200 µl of semen sample and 10 µl of freshly prepared Tetrazolium salt (MTT, Sigma) [3-(4, 5-dimethylthiazol-2-VL)-2, 5-diphenyl tetrazolium bromide] stock solution were placed in ELISA plate. The reaction was left under 5% CO_2 at 37°C for four hours in darkness and then lyses buffer was added over night. The rate of reduction was determined using ELISA reader (versa max, USA); at wave length 470 nm and the reading was expressed at optical density. This assay was performed for three successive hours post-thawing to obtain three optical densities for each treatment.

Assessment of sperm DNA integrity:

The DNA status of individual cells was determined by the neutral single cell gel electrophoresis (comet) assay according to (Boe-Hansen 2005). For this assay, frozen-thawed spermatozoa were diluted in phosphate buffer saline (PBS) then embedded in agarose, followed by cell lysis, DNA decondensation, electrophoresis, neutralization, and DNA staining with 50 µl of 20 µg/ml ethidium bromide (Sigma). The cells were then visualized by fluorescent microscopy. Intact nuclei in the comet assay appeared to have compact and brightly fluorescent heads; in contrast, strand breaks in damaged cells allow DNA migration during electrophoresis, and a tail of DNA could be seen behind the head, giving the appearance of a comet (Hughes *et al.*, 1996). After subjecting spermatozoa to the comet assay, sperm nuclei were imaged with epifluorescent microscope (Green filter: N2.1 with Exitation filter: BP 515-560, Dichromatic Mirror: 580, Suppression filter: LP 590, Germany). Images of sperm nuclei were digitized with Leica (DFC 280,

Cambridge, UK) camera. One hundred (100) sperm nuclei were scored for each sample. Sperm comets were visually scored according to Collins *et al.* (1995) into five grades from grade 0, no comet (no damage) to grade 4, large comet (extensive damage). The individual grade scores for 100 spermatozoa from each treatment were converted into a composite score by multiplying the number of sperm nuclei by the corresponding numerical score. Thus, the composite score could range from 0 (all undamaged) to 400 (all maximally damaged).

Evaluation of in vitro fertilizing potential of the treated buffalo semen:

Semen in vitro fertilizing ability was assessed using in vitro fertilization technology, according to Totey *et al.* (1992). Immature buffalo oocytes were collected from fresh ovaries just after slaughter at a local abattoir. Cumulus-oocyte complexes (COCs) were collected by aspiration of medium-sized (2-8 ml) ovarian follicles and were washed twice in modified Dulbecco's phosphate-buffered saline. Only oocytes with a homogeneous ooplasm and a complete and dense cumulus cells were selected for in vitro maturation. The selected oocytes were cultured in TCM-199 medium (Earl's salt, Sigma Chemical CO., St. Louis, Mo., USA) supplemented with 10% heat inactivated fetal calf serum (FCS, Gibco, 30 K-0351), 10 μg/ml Luteinizing hormone, 5 μg/ml follicle stimulating hormone and 1 μg/ml estradiol-17β. The oocytes were cultured for 24 hour at 39°C in an atmosphere of 5% CO_2 in air with maximum humidity.

Three straws from each treatment were thawed in a water bath at 37°C for 30 sec. The most motile spermatozoa were separated by swim up technique in the fertilization medium, modified Tyrode's Albumin-Lactate-Pyruvate (TALP) containing 6 mg/ml bovine serum albumin (BSA), for 1 hour (Parrish *et al.*, 1988). The uppermost layer of the medium containing the most spermatozoa was collected and washed twice by centrifugation at 2000 rpm for 10 minutes. The sperm pellet was resuspended in the fertilization TALP medium containing 10 μg/ml heparin. After appropriate dilution, 2 μl of sperm suspension was added to the fertilization drops, containing the matured oocytes, at a final concentration 2 $X10^6$ sperm cell/ml. Gametes were co-incubated in the fertilization drops under sterile mineral oil for 18 hour at 39°C in an atmosphere of 5% CO_2 in air with maximum humidity. At the end of gametes co-incubation, some inseminated oocytes were examined for signs of fertilization and the other oocytes were in vitro cultured for further embryo development. The inseminated oocytes were freed from

cumulus cells and attached spermatozoa by gentle pipetting and then cultured in TCM-199 medium with Hepes modification for seven days at 39°C in an atmosphere of 5% CO_2 in air with maximum humidity. The proportional of cleaved oocytes was recorded 48 hour after insemination and those developed to the morula and blastocyst stages were recorded at 5-7 day post-insemination.

Fertility Study:

A preliminary fertility trial was performed in a private farm to compare between control semen and antioxidants treated semen at their best concentration, as shown from freezability, mitochondrial function and DNA integrity, results. Buffaloes (3.5 - 4 years) were randomly assigned to one of five treated groups: group 1 (120 buffaloes) was inseminated using control semen; group 2 (95 buffaloes) was inseminated using GSH treated semen, group 3 (86 buffaloes) was inseminated using catalase treated semen, group 4 (98 buffaloes) was inseminated using ALA treated semen and group 5 (93 buffaloes) was inseminated using SOD treated semen. Pregnancy diagnosis was performed 45 days post-insemination by transrectal palpation.

Statistical analysis:

All data were analyzed by using Costat Computer Program (1986) Cottort Software, and were compared by the least significant difference least (LSD) at 1% and 5% levels of probability. The results were expressed as means ± S.E.M. Pearson correlation coefficients were used to calculate the relationships between DNA integrity and sperm quality tests. In vitro fertilization rate, embryo development and total pregnancy rate were analyzed by chi-square analysis (X^2).

RESULTS

Data presented in table 1 revealed that, fortification of freezing extender with antioxidants improved the freezability of buffalo semen compared to the control semen in a dose-dependent trend. Addition of 10 mM GSH, 100 U/ml catalase, 15 mM ALA or 50 U/ml SOD to semen extender, appeared to be the best concentrations that improved ($P<0.01$) significantly the post-thawing sperm motility (65.00±2.89, 66. 67±1.66, 61.67±1.66 and 63.33±3.34%, respectively); viability indices (167.50±10.12, 166.67±3.01, 150.83±4.65 and 168.33±10.1, respectively) and maintained acrosomal integrity (11.66±4.05, 11.33±2.91, 12.33±3.93 and 10.33±2.34%, respectively) compared to the control semen.

Table 1: Effect of antioxidants addition to the freezing extender on the buffalo semen freezability.

Treatments	Dilution motility	Post-thawing motility	Viability index	Acrosomal integrity
GSH 5 Mm	76.67±6.01^{a}	56.67±3.34^{abcd}	148.33±4.41^{ab}	14.33±4.05^{d}
GSH 10 Mm	78.33±1.67^{a}	65.00±2.89^{a}	167.50±10.12^{a}	11.66±4.05^{d}
GSH 20 mM	71.67±4.42^{a}	43.33±1.66^{d}	96.67±8.21^{c}	25.66±2.90ab^{c}
CAT 100 U/ml	75.00±2.89^{a}	66.67±1.66^{a}	166.67±3.01^{a}	11.33±2.91^{d}
CAT 200 U/ml	76.67±6.02^{a}	53.33±6.68ab^{cd}	130.00±5.01^{b}	16.33±2.61^{cd}
CAT 300 U/ml	73.33±6.02^{a}	45.00±5.78^{cd}	99.17±11.68^{c}	26.67±1.46^{ab}
ALA 10 Mm	71.67±3.34^{a}	50.00±5.78b^{cd}	126.67±12.03^{b}	17.66±1.86^{bcd}
ALA 15 mM	73.33±1.67^{a}	61.67±1.66^{ab}	150.83±4.65^{ab}	12.33±3.93^{d}
ALA 20 mM	70.00±2.89^{a}	45.00±2.89^{cd}	95.83±7.13^{c}	28.33±3.48^{a}
SOD 50 U/ml	81.67±1.6^{a}	63.33±3.34^{a}	168.33±10.1^{a}	10.33±2.34^{d}
SOD 100 U/ml	78.33±4.42^{a}	58.33±1.67^{abc}	139.17±6.51^{b}	12.33±3.17^{d}
SOD 200 U/ml	71.67±1.67^{a}	46.66±6.69^{cd}	103.33±8.83^{c}	25.33±1.20^{abc}
Control	71.67±6.02^{a}	43.33±3.30^{d}	86.67±4.42^{c}	27.33±3.85^{ab}
Over all mean	74.62±1.09	53.72±1.65	129.17±5.04	18.43±1.31

GSH: Reduced glutathione CAT: Catalase SOD: Superoxide dismutase ALA: Alpha lipoic acid
Values with different superscript letters in the same columns are significantly different at least (P<0.05).

Data regarding the effect of antioxidants addition to the freezing extender on the mitochondrial function of the cryopreserved semen are illustrated in Fig. 1. In vitro provision of buffalo semen extender with antioxidants significantly (P<0.01) increased the mitochondrial function of the frozen-thawed buffalo semen in a dose dependent trend. The present data showed that, addition of 10 mM GSH, 100 U/ml catalase, 15 mM ALA or 50 U/ml SOD, to the semen extender were the best concentrations that enhanced (P<0.01) significantly the mitochondrial activity of the frozen-thawed buffalo spermatozoa after 3 hour of thawing (3.42±0.05, 3.29±0.16, 2.81±0.09 and 3.31±0.08, respectively) compared to the control semen (1.88±0.05). The current results showed strong positive correlation between mitochondrial activity and in vitro fertilization rate and pregnancy rate (R=0.97 and 0.82, respectively, P<0.01).

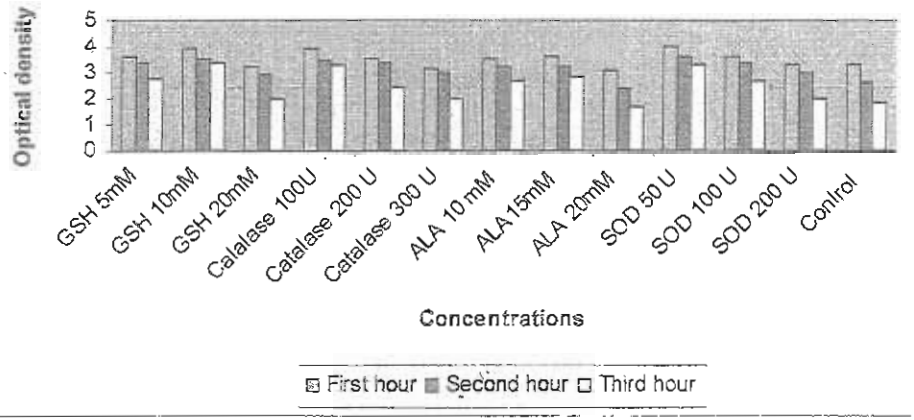


Fig. 1: Effect of antioxidants addition to the freezing extender on the mitochondrial activity of the cryopreserved buffalo spermatozoa.

Data regarding the effect of antioxidants addition to the freezing extender on the DNA integrity of the frozen-thawed buffalo spermatozoa are illustrated in Figs. 2 and 3. The present data indicated that, semen cryopreservation increased ($P<0.01$) significantly the DNA damage and the composite score (71.67±4.42% and 127.67±17.04, respectively) as shown in photograph, 1. However, the increase in DNA fragmentation and composite score associated cryopreservation was significantly reduced ($P<0.01$) by the addition of GSH, catalase, ALA and SOD in a dose dependent manner. Addition of 10 mM GSH, 100 U/ml catalase, 15 mM ALA or 50 U/ml SOD to the semen extender were the best antioxidant concentrations that maintained the DNA integrity (37.67±4.33, 36.33±2.61, 43.33±5.05 and 36.00±5.04%, respectively) and significantly ($P<0.01$) decreased the composite score of the frozen-thawed semen (66.00±9.06, 65.67±8.30, 87.33±5.82 and 64.67±5.51, respectively), compared to the control frozen semen. The current results showed a significant negative correlation between DNA damage and post-thawing motility ($R=-0.95$, $P<0.01$), viability ($R=-0.97$, $P<0.01$), mitochondrial function ($R=-0.95$, $P<0.01$). There was also a strong negative correlation between DNA damage and the in vitro fertilization rate and pregnancy rate ($R=-0.96$ and -0.77, respectively, $P<0.01$).

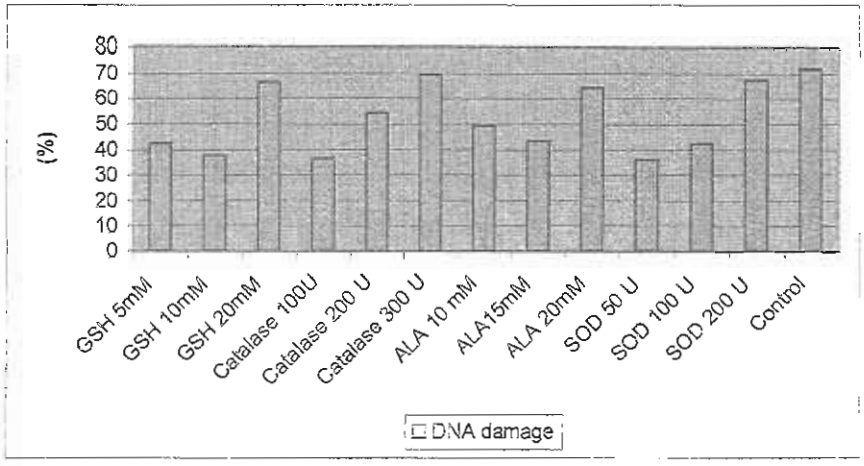


Fig. 2: Effect of antioxidants addition to the freezing extender on the DNA integrity of the cryopreserved buffalo spermatozoa.

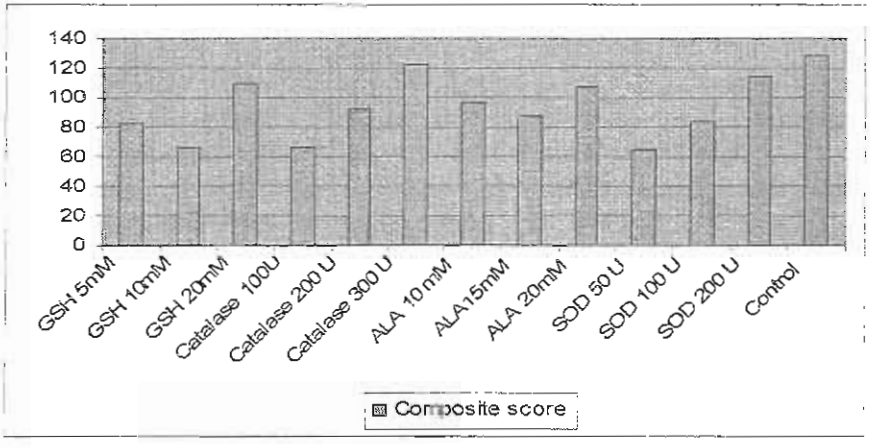


Fig. 3: Effect of antioxidants addition to the freezing extender on the DNA composite score of the cryopreserved buffalo spermatozoa.

Data regarding the effect of replenishing of semen extender with antioxidants on the in vitro fertilizing potentials and embryo development are presented in Figs. 4 and 5. The current results revealed that, addition of antioxidants to the freezing extender had a positive effect on the in vitro fertilization rate in a dose-dependent manner ($P < 0.05$) compared with the control semen. When 10 mM GSH, 100 U/ml catalase or 50 U/ml SOD was added to the freezing extender, a higher proportion of decondensed sperm

heads was observed inside the oocyte (65.06, 67.95 and 68.67%, respectively) compared with the control semen (46.58 %).

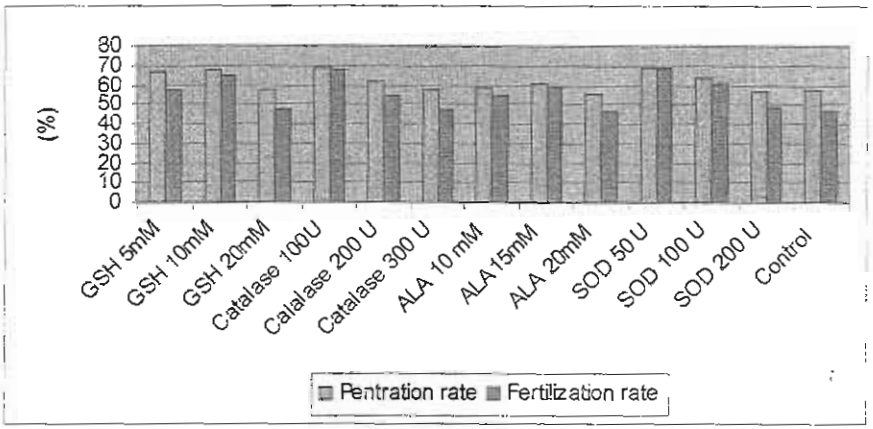


Fig. 4: Effect of antioxidants addition to the freezing extender on the in vitro fertilizing ability of the cryopreserved buffalo spermatozoa.

Moreover, the results presented in Fig. 5 clarified that, addition of antioxidants to the freezing extender prior to freezing significantly increased ($P < 0.05$) the cleavage rate, the morula and the blastocyst development, in a dose dependent manner. Addition of 10 mM GSH, 100 U/ml catalase or 50 U/ml SOD to the freezing extender, significantly ($P< 0.05$) increased the cleavage rate (50.58, 46.55 and 48.21%, respectively), the morula (18.64, 19.23 and 19.64%, respectively) and the blastocyst development (11.86, 11.54 and 14.20%, respectively) compared to the control semen (36.99, 6.85 and 2.74%, respectively) as shown in photograph, 2. The current results demonstrated that, addition of ALA to the freezing extender had a non beneficial effect on the in vitro fertilizing ability and embryo development of the cyopreserved buffalo spermatozoa. Moreover, addition of higher antioxidant concentrations to the semen extender resulted in a detrimental effect on the fertilizing potential and embryo development in vitro.

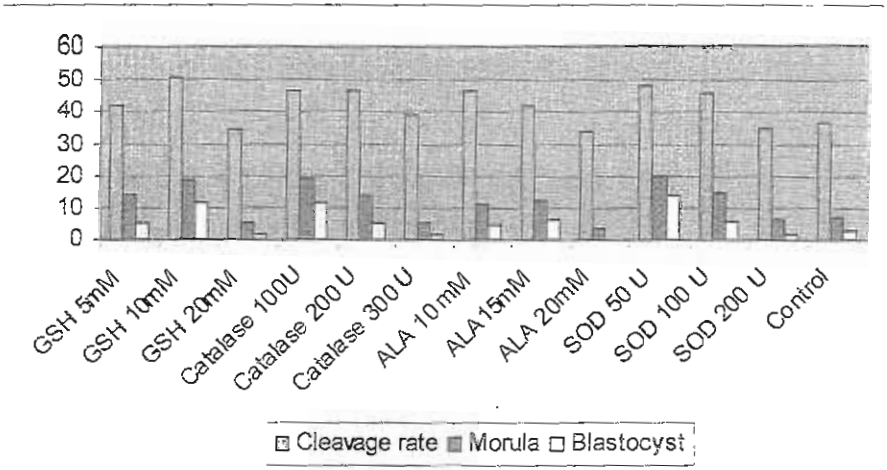


Fig. 5: Effect of antioxidants addition to the freezing extender on the embryo development in vitro.

Data regarding the effect of fortification of semen extender with antioxidants on the pregnancy rate is demonstrated in Fig. 6. The results showed that addition of 10 mM GSH, 100 U/ml catalase or 50 U/ml SOD to the semen extender significantly ($P< 0.05$) augmented the pregnancy rate (62.11, 62.79 and 67.74%, respectively) compared with the control semen (51.67%). However, the pregnancy rate among the antioxidants treated groups were not statistically significant ($P > 0.05$).

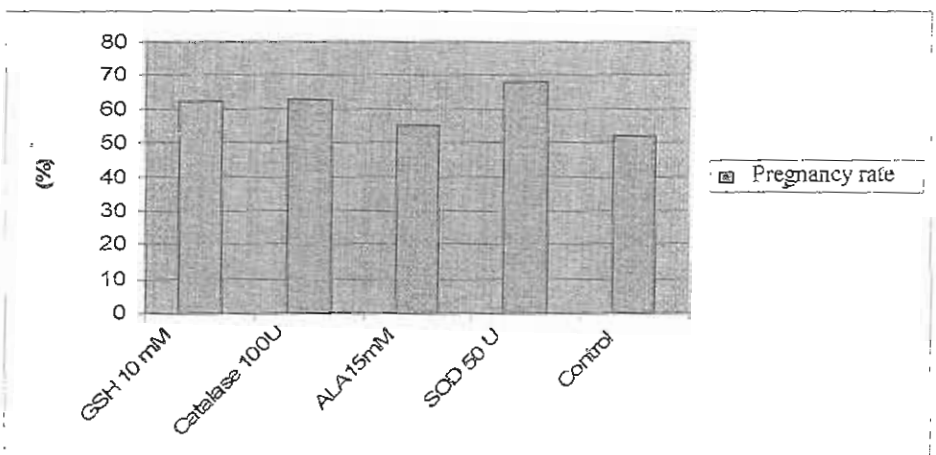
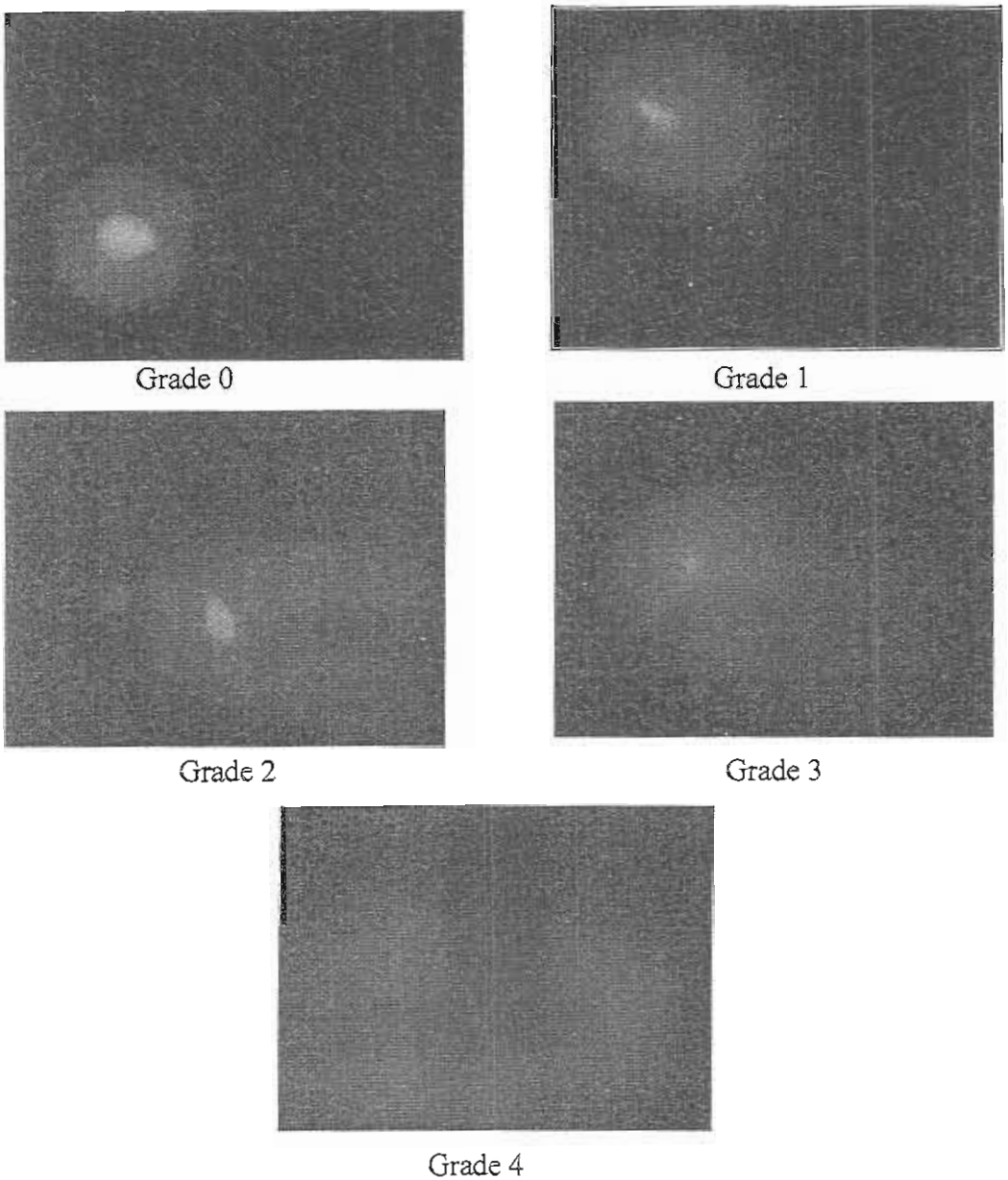
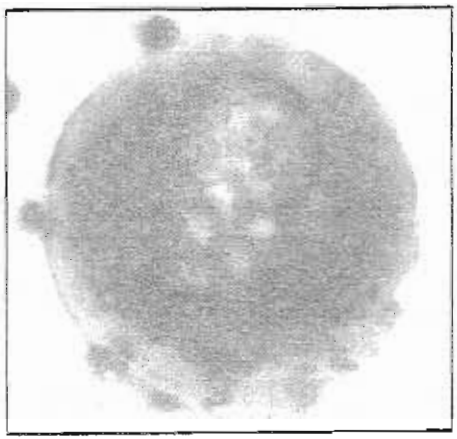


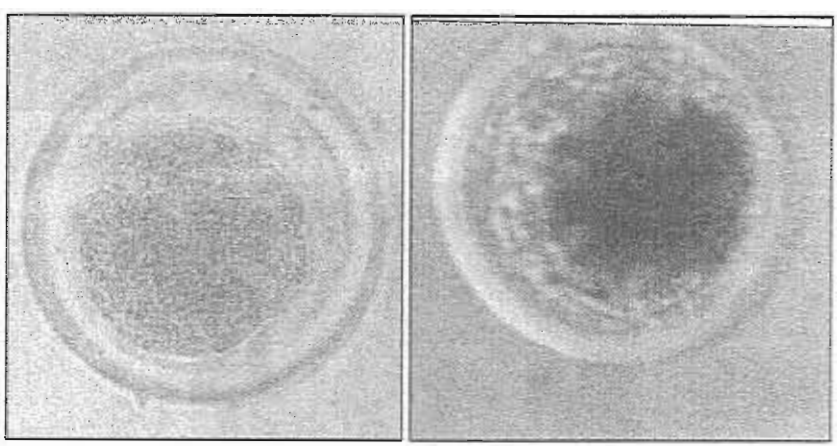
Fig. 6: Effect of antioxidants addition to the freezing extender on the pregnancy rate.



Photograph 1: Epiflourescent images of buffalo spermatozoa after the single cell gel electrophoresis (comet) assay and staining by ethedium bromide. Relative changes in DNA fragmentation are represented by an increasing amount of DNA present in the comet tail and are scored from grade 0 (no comet tail) to grade 4 (maximum damage). Magnification 40 ×.



(a)



(b) (c)

Photograph 2: (a) Fetilized oocyte showing male pronucleus formation, (b) compact morula stage and (c) blastocyst stage that resulted from insemination of matured buffalo oocytes with antioxidant-treated semen.

DISCUSSION

Reactive oxygen species (ROS) generation during sperm processing (cryopreservation/thawing), accompanied by low scavenging and antioxidant levels in sperm-processing media will induce a state of oxidative stress (Hellstrom *et al.*, 1994). The authors further added that, high levels of ROS (superoxide, hydroxyl, hydrogen peroxide, nitric oxide, peroxynitrile) endanger sperm motility, viability, and function by interacting with membrane lipids, proteins and mitochondrial DNA. These observations may explain the results of the current study, that emphasized that, cryopreservation of buffalo spermatozoa is associated with damage of sperm function affecting those processes required for the successful fertilization. These results are in consistent with Bilodeau *et al.* (2000). The significant decrease in the cryopreservsed sperm motility, viability and acrosomal integrity may be attributed to extensive chemical-physical damage to the extracellular and intracellular membranes of the sperm that resulted in changes in the lipid phase transition and/or increased lipid peroxidation during cryopreservation (Ball *et al.*, 2001). After cryopreservation, the production of ROS leads to increased lipid peroxidation (Zalata *et al.*, 2004) which leads to a significant loss of sperm motility (O'Connell *et al.*, 2003). Moreover, the link between ROS and reduced motility may be due to a cascade of events that result in a decrease in axonemal protein phosphorylation and sperm immobilization, both of which are associated with a reduction in membrane fluidity (de Lamirande and Gagnon, 1995).

Sperm DNA integrity is important for the success of natural or assisted fertilization, including normal development of the embryo (Lopes *et al.*, 1998). The current results revealed that cryopreservation is associated with a drastic effect on DNA integrity and mitochondrial function. This may be true particularly in light of a recent report by Baumber *et al.* (2003); Sanocka and Kurpisz (2004) and Slowińska et al. (2008) who showed that sperm DNA integrity and mitochondrial function deteriorated after cryopreservation. Sperm DNA damage induced by cryopreservation may be attributed to the oxidative stress, which is detrimental to sperm DNA integrity (Fraser and Strzeżek, 2005). Recently, Ward and Ward (2004) provided evidence that mammalian spermatozoa contain a mechanism by which they can digest their own DNA when exposed to stressful environment. Thus, increased DNA damage of the cryopreserved spermatozoa could be attributed to the activation of endonucleases released from the deteriorated plasma

membrane of the frozen-thawed spermatozoa (Fraser and Strzeżek, 2005). The highly significant inverse associations between DNA damage and post-thawing sperm motility and viability indices indicating that as the percentage of buffalo spermatozoa with good motility declined, susceptibility to chromatin denaturation rose. These results are supported by other studies with bull (Januskauska *et al.,* 2001). In addition, Januskauska *et al.* (2001) reported negative relationships between DNA damage and sperm viability and speculated that abnormal chromatin structure might impede the survival of bull spermatozoa during freezing and thawing. Also, there was a strong negative correlation between DNA damage and the percentage of spermatozoa with dysfunctional mitochondria in the cryopreserved buffalo semen. This may explain that sperm motility is associated with healthy mitochondria and that mitochondrial damage may result in a reduction in sperm movement (Donnelly *et al.,* 2000). Moreover, a strong inverse association has been observed in the present study between DNA damage and the capacity of sperm to fertilize. These results are in accordance with Rosenbusch (2000), who indicated that human spermatozoa that have incomplete chromatin condensation fertilize a very low percentage of ova in vitro or fail to fertilize, even after direct injection of spermatozoa into the ovum. Therefore, the consistent association found in the current study between DNA integrity and sperm parameters, suggest that detection of DNA deterioration by comet assay might be a useful index for the assessment of sperm function and fertility.

With reverence to the effect of cryopresevation on the fertilizing potentials of fozen-thawed buffalo spermatozoa in vitro, the present study clarified that cryopreservation of buffalo spermatozoa decreased significantly the in vitro embryo development. These results are in accordance with Host *et al.* (2000) and Aitken and Krausz (2001). and this may be attributed to intrinsic generation of excess ROS that resulted in increased oxidative stress and increased DNA fragmentation. Sperm DNA damage induced by oxidative stress will be associated with collateral peroxidative damage to the sperm plasma membrane, consequently the plasma membrane loses the fluidity and integrity that is necessary for sperm-oocyte fusion and can therefore compromise the fertilization potential of the sperm or resulting in a low rate of embryonic development and early pregnancy loss (Ahmadi and Ng 1999 and Sakkas *et al.,* 2002).

The current results clarified that, addition of antioxidants to the freezing extender prior to cryopreservation significantly improved the freezability and the natural and assisted fertilizing potential of the frozen-thawed buffalo spermatozoa. This protective effect on sperm function was dose dependent. These results are in consistent with Gadea *et al.* (2005) and Kawakami *et al.* (2007). GSH could be an important regulator of the scavenging system and one of the most important non-enzymatic antioxidants in sperm cells (Griveau and Le Lannou, 1997) as, GSH probably affects plasma membrane lipid packing and sulfhydryl group content in membrane proteins in sperm (Gadea *et al.*, 2005). Therefore, the beneficial effect of GSH on the sperm freezability and fertilizing potentials may be attributed to its ability to protect sperm against oxidative damage and reduction of oxidative stress-induced DNA oxidation and DNA fragmentation (Agarwal and Said, 2003 and Funahashi and Sano, 2005). Moreover, GSH has a likely role in sperm nucleus decondensation and may alter spindle microtubule formation in the ovum and protect ova and embryo during in vitro fertilization, thus affecting the outcome of pregnancy (Gadea *et al.*, 2004 and Sikka, 2004).

The advantageous effect of SOD on the sperm function may be attributed to the protection of cells from the toxic effect of superoxide anions via dismutates (O_2^-) to form O_2 and H_2O_2 and also could counteract the NADPH-induced oxidative stress in sperm (Mennella and Jones, 1980). The main function of SOD is to protect spermatozoa against spontaneous O_2 toxicity and lipid peroxidation (Alvarez *et al.*, 1987). A significant correlation has been found between the malondialdehyde production and SOD activity was found in the bovine spermatozoa (Beconi *et al.*, 1991). The presence of high SOD endogenous content delays the onset of lipid peroxidation and the loss of motility as the SOD level is positively correlated with the sperm motility (Alvarez *et al.*, 1987).

The useful effect of catalase on semen freezability and fertilizing potentials that observed in the current study may reside in its ability to convert H_2O_2 to water and oxygen. This might cause inhibition of anaerobic glycolysis with a subsequent reduction in fructose utilization by spermatozoa (O'flaherty *et al.*, 1997). It is well recognized that H_2O_2 is highly toxic to mammalian spermatozoa and the oxygenation of semen is deleterious to sperm viability (Jeulin *et al.*, 1989). Therefore, the existence of an efficient hydrogen peroxide scavenging mechanism in spermatozoa is necessary for sperm survival

The favorable effect of ALA on the sperm freezability may be due to, alpha lipoic acid fights free radical damage in both fatty and watery regions of cells and helps to recycle other antioxidants in what is described as "antioxidant synergism" (Selvakumar *et al.*, 2006).

However, the results of the present study clarified that, inclusion of increasing levels of antioxidants in the semen extender induced a drastic effect on sperm function. These results are in accordance with Aitken *et al.* (1998) who indicated that, high levels of antioxidants are associated with impaired sperm function and this may be due to the spermatozoa become more susceptible to the cytotoxic effect of H_2O_2 or the removal of the O_2 which is an important mediator of normal sperm function.

In conclusion, cryopreservation promotes DNA fragmentation in buffalo spermatozoa. DNA fragmentation can be counteracted by the addition of antioxidants, GSH, catalase, ALA and SOD. The addition of these antioxidants to the freezing extender appear to play an important role in sperm antioxidant defense strategy in a dose dependent trend and could be of significant benefit in improving the freezability, DNA integrity and the natural and assisted fertilizing capacity of the cryopreserved buffalo spermatozoa

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