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EFFECTS OF AMPHORA COFFEAEFORMIS ALGA EXTRACT ON MORPHOLOGY AND ANTIOXIDANT ENZYMES OF CRYOPRESERVED BUFFALO SPERMATOZOA

By

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ABSTRACT

Cryopreservation induces sub lethal damage to the spermatozoa, thereby reduce their fertile life. There are some biochemical additives that may enhance buffalo semen freezability; Amphora coffeaeformis is one of these biochemical semen additives. Till now, the exact effects of Amphora coffeaeformis on buffalo semen processing outcomes haven't been discovered. The current study aimed to clarify Amphora coffeaeformis roles during buffalo semen cryopreservation. Cryopreserved semen was assessed for post- thawing motility, viability, acrosomal integrity, antioxidant enzymes concentration, lipid peroxidation and ultrastructure changes. Current results indicated that addition of 100 µl/ml Amphora coffeaeformis to semen extender significantly (P<0.05) improved post-thawing motility, viability and acrosomal integrity (63.33±9.28 %, 133.33±9.40 and 12.33± 2.02 %, respectively) compared with control (43.33±6.01 %, 74.16± 10.93 and 24.67±2.03 %, respectively). Moreover, 100 µl/ml Amphora coffeaeformis significantly increased (P<0.05) total antioxidant capacity (TAC), superoxide dismutase (SOD), glutathione peroxidase (GPx) and ascorbic acid concentrations (0.58 ± 0.05 mµ/ml, 95.00 ± 4.16 U/ml, 87.61 ± 5.20 U/L and 58.33 ± 2.16, respectively) with respect to the control (0.15 ± 0.06 mµ/ml, 31.25 ± 1.65 U/ml, 51.73 ± 2.32 U/L and 22.14 ± 7.84, respectively). Furthermore, 100 µl/ml Amphora coffeaeformis significantly decreased (P<0.05) lipid peroxidation of the cryopreserved spermatozoa compared with the control semen (9.04 ± 0.69 vs 24.58 ± 1.60 nmol/ml). Likewise, at this concentration. Ultra-structure examination illustrated that addition of 100 µl/ml Amphora coffeaeformis to semen protected the plasma membrane, acrosomal region and mitochondria and maintained the ultrastructure integrity of the cryopreserved

spermatozoa compared with the control spermatozoa .In conclusion the addition of 100 µl/ml Amphora coffeaeformis to the freezing extender improves fine structure integrity through enhancing the antioxidant defense of buffalo sperm cells and decreasing the rate of lipid peroxidation.Therefore, amphora coffeaeformis as a natural antioxidant may reduce cryodamage and improve sperm cryopreservation quality.

Key words:

Amphora coffeaeformis, cryopreservation, buffalo semen, ultrastructure, antioxidant activity.

INTRODUCTION

Infertility and reduced fertility are one of the major problems in veterinary practices especially for valuable animals. Male infertility represented about 30% to 50% of infertility cases **(Aliabadi *et al.,* 2013).** Semen cryopreservation is one of the most effective and acceptable methods to maintain male fertility potential. Long-term sperm storage, sperm banking and the possibility of storing the sperm of proven bull are the main objectives of using cryopreservation method. Unfortunately, despite many advantages of sperm cryopreservation, this method entries some hazardous that reduces sperm fertilizing capacity through morphological damage, DNA damage and generally impaired sperm motility and viability **(Singer *et al.,* 1980).** Several researchers found that cryopreservation decreases sperm motility parameters, normal morphology and increases programmed sperm death **(Badr *et al.,*2010).** Poly unsaturated fatty acids (PUFA) are a basic component of mammalian sperm cells, which contributing the major skeleton of its membrane structure, integrity, metabolism and their ability to penetrate and fertilize the oocytes through many of physicochemical modifications **(Guthrie and Welch, 2012).** In this respect, it had been found that buffalo sperms were prone more to lipid peroxidation compared with bull sperm because it is rich in polyunsaturated fatty acids (PUFA) **(Garg *et al.,* 2009).** Lipid peroxidation during in-vitro handling and sperm storage, are probably the primary causes of this fertility dysfunction **(Cecil and Bakst 1993).** Oxidation is one of the major causes of frozen semen deterioration **(Ukeda *et al.,* 2002).** Besides, in the semen cryopreservation lipid peroxidation is an important deteriorative reaction during processing and storage. Therefore, it is extremely necessary to optimize cryopreservation method since it is an effective step in improving male infertility. Adding substances that prevent damage to sperm, to cryopreservation extender can improve sperm cryopreservation condition. These substances include commercial antioxidant supplements that are capable of preventing sperm damage and destruction through removing

oxygen free radicals **(Sarica *et al.*, 2007).** However, it is suspected that those antioxidants are responsible for some side effects on semen quality **(Lindenschmidt *et al.*, 1986).** Antioxidants are involved in the oxidation mechanism by scavenging free radicals, chelating catalytic metals and by acting as oxygen scavengers **(Büyükokuroğlu *et al.*, 2001).** As a natural antioxidant source, plants have an ability to absorb the sun's radiation for generating high levels of oxygen as secondary metabolites of photosynthesis. Oxygen is easily activated by ultra violet (UV) radiation and heat from the sunlight to produce toxic ROS. Therefore, plants produce various antioxidative compounds to protect them from harmful effects of ROS **(Aruoma 1998).**Currently; microalgae are receiving more attention as nutraceuticals and health foods in the market. Several microalgae, such as Chlorella sp., Spirulina sp. and amphora sp. are grown commercially for the production of algal products such as β-carotene, lutein and phycocyanin. In addition, the antioxidative activity of phycocyanobilin from Spirulina platensis was evaluated against oxidation of methyl linoleate in a hydrophobic system or with phosphatidylcholine liposomes **(Hirata *et al.*, 2000).** Phycocyanobilin effectively inhibited the peroxidation of methyl linoleate and produced a prolonged induction period. Another microalga, Aphanizomenon flos-aquae (Cyanophyta) was reported to contain high amounts of phycocyanin,a photosynthetic pigment with antioxidant and anti-inflammatory properties **(Benedettia *et al.*, 2004).**The objective of this study was to investigate the antioxidant properties from benthic diatoms, amphora coffeaeformis in order to understand the usefulness of these algae in buffalo semen cryopreservation. Therefore, the present study aimed to investigate the effect of Amphora coffeaeformis extract on buffalo semen cryopreservation, ultrastructure changes and biochemical activities, in order to provide suitable natural antioxidant source for preventing the risks of chemical antioxidants.

MATERIAL AND METHODS

Amphora coffeaeformis extraction:

Axenic culture of the isolated alga *amphora coffeaeformis* was done by incubated alga with growth medium contains 14 ppm N as urea. Growth was early done with 250 ml washing bottles till 14 L fully transparent Plexi-glass cylender. Growth condition and nutrition were employed as early described **(El-Sayed *et al.*, 2015).**When growth reached the maximum, algal broth was centrifuged by cooling centrifuge; washed by deionized water and re-centrifuged to remove all accompanied nutrients **(Hassan *et al.*, 2015).**

Solution (1) 5gm of dried alga were dissolved in 100ml distal water. Solution (2) 5gm of dried alga were dissolved in 100ml hot distal water. Filtration both solution and mixed both solution these mixture used in current study.

Diluent Preparation:

The cryoprotective extender used in the current study was composed of 2.42 g Tris, 1.48 g citric acid, 1.00 g fructose, 6.6 mL glycerol, 20 mL egg yolk, 25 mg gentamicin and 50,000 IU penicillin; all of these components were dissolved in 100 mL deionized water and supplemented with different concentrations of amphora coffeaeformis extract.

Semen Collection:

Semen samples were obtained randomly from six evaluated buffalo bulls (aged 3 to 5 y) kept at the Animal Reproduction Research Institute farm (Cairo, Egypt). Two consecutive ejaculates were collected from each bull weekly for successive eight weeks using an artificial vagina. The ejaculates were pooled to eliminate variability between the evaluated samples. The semen samples were assessed for volume, sperm concentration and percentage of motile spermatozoa. The ejaculates with at least 70% motility, 800×10^6 sperm cells/ml and >85% normal sperm morphology were used for the present study. All experiments were done with at least 3 replicates for each group.

Semen Processing:

After the evaluate on of semen quality, the fresh semen samples were pooled and then split into 4 equal portions and diluted at 30°C with Tris-based extender supplemented with different concentrations of amphora coffeaeformis extract (50, 100, 200 and 400 µl/mL) Vs. Tris-based extender only (control) to obtain 120×10^6 sperm/ml . The fresh semen samples were transferred to pre warmed tubes. Semen was cooled from 37 to 5°C throughout 60 min in a cold cabinet. The cooled semen was loaded into 0.25 ml polyvinyl chloride straws (IMV, L'Aigle, and France), horizontally placed in a refrigerator and kept at 4°C for 1 h. These straws were then placed 6 cm above the liquid nitrogen surface where the temperature was approximately −120°C. After 15 min, they were immersed directly into liquid nitrogen (−196°C) for storage. The straws were stored at least for 24 h before evaluation **(Badr *et al.*, 2010).** Frozen semen straws were thawed in water bath at 37°C for 30 sec. Post-thawing sperm motility; viability and acrosomal integrity were assessed.

1-Assessing of sperm quality post-thawing:

I- a- Analysis of Sperm Motility.

The percentage of linear motile sperm was examined visually. For each treatment, 3 straws were thawed separately by immersion in a water bath at 37°C for 30 sec. The sperm samples were evaluated at 37°C by phase contrast microscope equipped with a warm stage at 200× magnifications.

I-b- Assess of Acrosomal Membrane Integrity.

Acrosomal integrity was assessed using silver nitrate stain in a procedure as described by **Chinoy *et al.* (1992).**

II- Ultrastructure analysis of the cryopresreved spermatozoa:

The ultrastructure changes occurred for the cryopreserved buffalo spermatozoa were evaluated by transmission electron microscopy (TEM) according to **Boonkusol *et al.* (2010).** Straws from each treatment were washed three times by centrifugation at 1000 rpm for 5 min using PBS (Phosphate Buffer Saline). The frozen-thawed semen was prefixed for 2-3 h with PBS containing 2.5% glutaraldehyde, washed three times by centrifugation at 1000 rpm with PBS (pH 7.4) for 5 min at 4°C and post-fixed in 1% osmium tetraoxide for 1-2 h at 4°C. Spermatozoa were dehydrated in in ascending grades of ethanol and embedded in epon resin. Semi-thin sections were prepared from the blocks, stained using toluidine blue, and demonstrative areas of semi-thin section were chosen. Ultrathin sections of 50 - 60 nm thickness were cut using the Leica EM UC6 ultramicrotome. Following this, uranyl acetate and lead citrate were used to stain the spermatozoa. Randomly fields were examined by a transmission electronic microscope (JEOL-EM-100 S at 80 Kv at National Reacerch Center - Electron Microscopy Unit) and photographed for further analysis.

III- Assessing of antioxidant activity of the frozen semen:

III-1-Total Antioxidant Capacity (TAC).

TAC of the frozen-thawed semen was estimated using a commercial kit (Antioxidant Capacity Assay Kit, Cayman Chemical Co. Ann Arbor, MI, and USA) according to **Cortassa *et al.* (2004).** The reaction was measured spectrophotometrically at 532 nm.

III- 2-Superoxide Dismutase (SOD).

The SOD activity was measured according to **Flohe and Otting (1984).** Briefly, each semen sample was diluted 1:5 with phosphate buffer saline (PBS) (pH 7.0). The SOD activity was measured at 560 nm on a spectrophotometer and expressed as units per milliliter.

III- 3-Glutathione peroxidase (GPx).

The GPx content of sperm was measured using the method of **Sedlak and Lindsay (1968).** The semen samples were precipitated with 50% trichloracetic acid (vol/vol) and then centrifuged at 1,000 ×g at 22°C for 5 min. The GPx activity was measured at 412 nm on a spectrophotometer. The values of GPx were expressed as units per liter.

III-4- Ascorbic Acid (Vitamin C).

The concentrations of Ascorbic Acid (Vitamin C) were measured using the principle of **Das *et al.* (2009)** and the concentrations were expressed in µg/10^8 leucocytes.

III -5-Lipid Peroxidation (MDA).

The concentrations of Malondialdehyde (MDA), as indices of the LPO in the sperm samples were estimated. An aliquot (500 µl) of semen from each sample was centrifuged at 800 ×g for 10 min, sperm pellets were separated and washed by resuspending in PBS and recentrifuging (three times). After the last centrifugation, 1 ml of deionized water was added to spermatozoa and they were snap-frozen and stored at -70°C till further analysis. The samples were thawed before the lipid peroxidation assay.The concentrations of Malondialdehyde (MDA), as indices of the LPO in the sperm samples, were measured using the thiobarbituric acid reaction according to the method of **Placer *et al.* (1966).** The MDA concentrations were expressed in nmol/ml.

Statistical Analysis:

All data were analyzed by using Costat Computer Program (1986), Version 3.03 copyright Cottort Software and were compared by the Least Significant Difference least (LSD) at 5% levels of probability. The results were expressed as means ±SE. The mean values of the percentages of motile sperm,acrosome-intact sperm, enzyme activity and embryo development were compared using Duncan's multiple range test by oneway ANOVA procedure, when the F-value was significant ($P < 0.05$).

RESULTS

The results presented in (Table 1) revealed that, addition of amphora coffeaeformis extract to the freezing extender improved the freezability of buffalo bull spermatozoa compared with the control semen, in a dose-dependent trend. Addition of 100 µl/ml amphora coffeaeformis extract to semen extender, appeared to be the best concentration that increased significantly ($P<0.05$) the post-thawing sperm motility, viability index and maintained acrosomal integrity (60.00±2.85 %, 164.17±15.31 and 13.67± 1.85 %, respectively) compared with the control

semen (45.42±8.66 %, 69.17± 10.85, 22.33±2.40 %, respectively). However, high concentration of amphora coffeaeformis extract (400 µl/ml) significantly reduced (P<0.05) post-thawing sperm motility, viability index and increased acrosomal abnormalities (30.00± 5.78 %, 70.00±11.56and 25.33±2.03 %, respectively).

Table (1): Effect of amphora coffeaeformis extract addition to freezing extender on post-thawed buffalo semen characteristics.

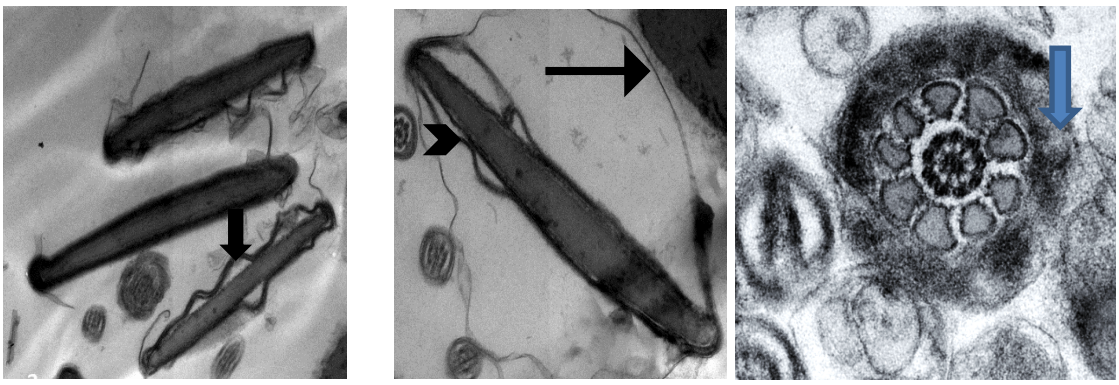
Treatment	Post-thawing motility (%)	Viability index	Acrosomal integrity (%)
Control	**35.00±2.89 ^{c}**	**69.17± 10.85 ^{bc}**	**22.33±2.40 ^{a}b**
amphora 50 µl/ml	**56.67± 3.33 ^{ab}**	**155.00±12.34 ^{a}**	**18.33±1.76 ^{bc}**
amphora 100 µl/ml	**60.00±2.85^{a}**	**164.17±15.31 ^{a}**	**13.67± 1.85^{c}**
amphora 200 µl/ml	**45.00±5.78^{bc}**	**95.00±5.21^{b}**	**20.33±2.40 ^{bc}**
amphora 400 µl/ml	**31.67± 3.33 ^{c}**	**51.67±6.01 ^{c}**	**28.67±2.03 ^{a}**

Three replications of the experiment were conducted. Results are presented as mean ± SEM Values with different superscripts in the same columns are significantly different at least (P<0.05).

Effectofamphora coffeaeformis extract supplementation during buffalo semen cryopreservation on its ultrastructure properties:

Transmission electron microscopic examination of the frozen thawed buffalo sperm cells in the control group revealed major pathological changes in the numerous of spermatozoa localised predominantly within the acrosome and postacrosomal region of a sperm head. In many cells the acrosome and equatorial segment of a head were damaged. Variable forms of structural abnormalities of frozen–thawed spermatozoa were observed. In sagittal sections through the heads of thawed spermatozoa, the plasma membrane often was seen to be swelling in the region of acrosome Fig. (1A). in most cases plasma membrane was waved and cracked, formation of large space between the plasma membrane and outer acrosomal membrane (OAM) segmentation of the outer acrosomal membrane and the nucleus content is not homogenous in the electron density Fig. (1B). the middle piece Showed severe degeneration marked vacuolation in the mitochondria Fig. (1C) with complete absence of the

transverse cristae in few cases. While, the frozen thawed buffalo semen treated with 100µl/ml amphora coffeaeformis extract illustrated a well-defined, intact plasma membrane and intact outer and inner acrosomal membranes Fig. (2A) and Fig.(2 B), Transverse sections of Middle piece the axoneme is surrounded externally by nine coarse longitudinal fibers and the mitochondrial helix homogenous mitochondria content and high-quality mitochondrial dense electron spaces with appeared transverse cristae Fig. (2C) and Fig. (2D). Observations of numerous transverse sections through the spermatozoal tails revealed the normal axial 9+2 fiber pattern. The coarse and fine fibers of the axoneme have been clearly illustrated. Meanwhile the frozen thawed buffalo semen treated with with 400µl/ml amphora coffeaeformis extract showed similar pathological changes recorded in frozen thawed control group in addition Swelling of acrosomes with formation of irregular space between IAM and OAM and between OAM and plasma membrane (fuzzy appearance of acrosome with loss of unevenly distributed electron dense material) Fig. (3 A). Distorted mitochondrial cristae and vesiculation of plasma membrane and OAM Fig. (3B), Cysts and disintegration of plasmalemma were observed over the acrosome. The presence of space between inner acrosomal membrane (IAM) and nucleus was also observed. In some cases complete detachment of the acrosome was showed. The cross sections through the middle piece revealed that, the mitochondrial matrix is thinned and dense deposits of firmly granulated material were found regularly within mitochondrium (fig. 3C). Examination of transverse sections of the tail region showed the coarse and fine fibers of the axoneme were seen. Cysts and disintegration of plasmalemma were observed over the acrosome. The presence of space between inner acrosomal membrane (IAM) and nucleus was also noted.



A B C

Fig. (1, A): Electron micrograph for a sagital section in the sperm head from a frozen-thawed semen of control group showing swollen plasma membrane (PM) (arrows) with intact outer

acrosomal membrane (X 14000). (B) Electron micrograph for a sagital section in the sperm from a frozen-thawed semen of control group illustrating sperm with waved and cracked plasma membrane (black arrow), segmentation of the outer acrosomal membrane (arrowhead) (OAM) and swollen (x 20000). Electron micrograph of cross section in the mid-piece region moderate vacuolation of the mitochondria that contained electrontranslucentspaces with complete absence of the transverse cristae (arrows) (X 25000).

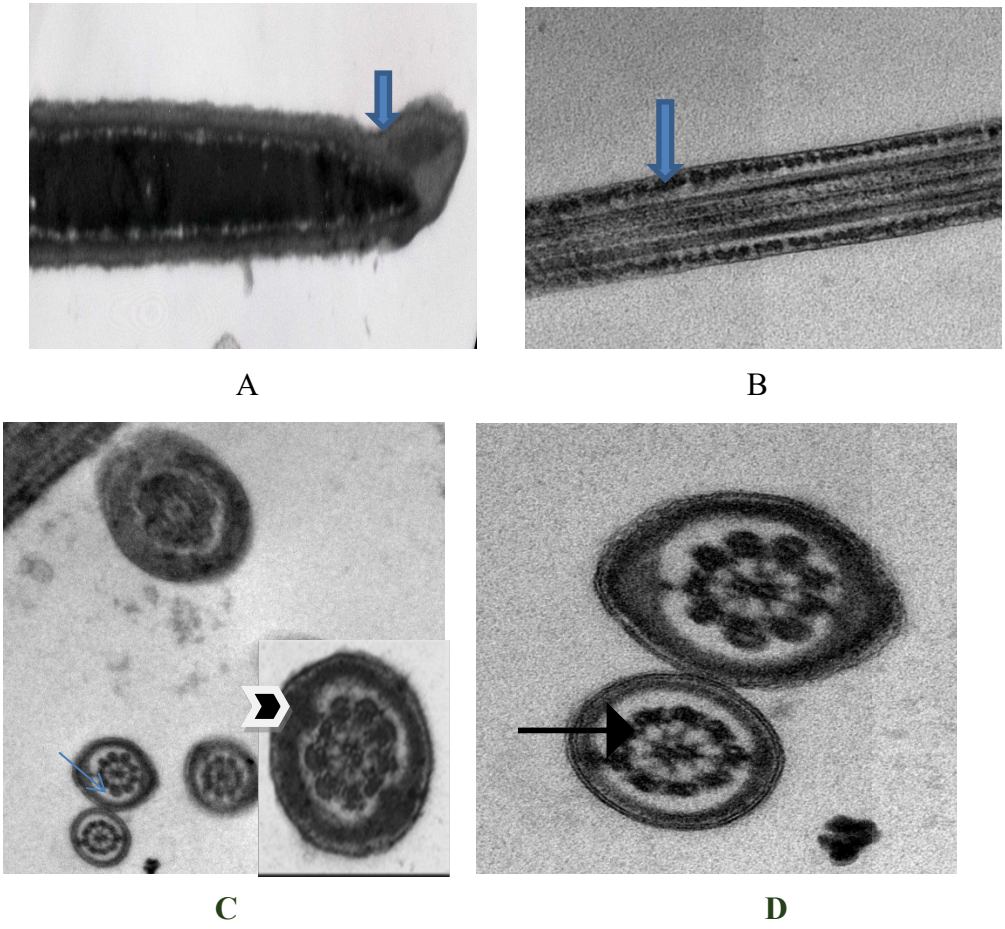
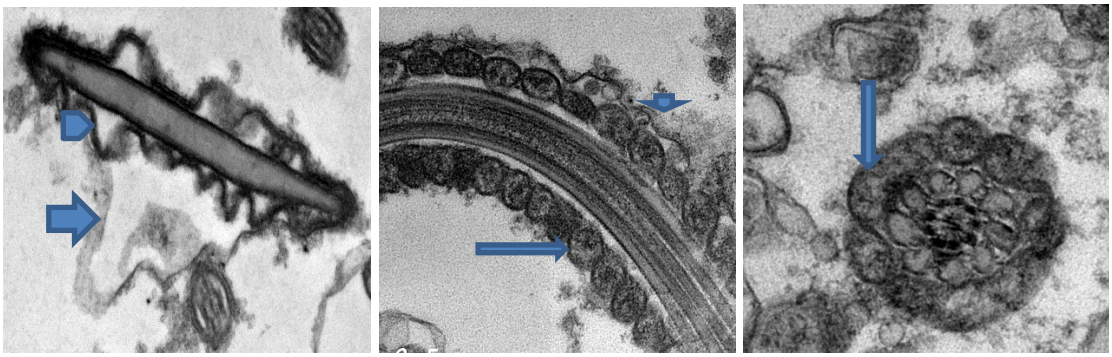


Fig. 3 (A): Electron micrograph for a sagital section in the sperm head from a frozen-thawed semen sample treated with 100µl/ml amphora,illustrating sperm with intact plasma membrane (PM) of the head (arrow) intact acrosome and the nucleus content is homogenous in the electron density. (X 14000).(B) Electron micrograph for a sagital section in the sperm middle pice from a frozen-thawed semen sample treated with 100µl/ml amphora,illustratingnormal mitochondria (arrow),longitudinal fibers(x15000). (C and D) Electron micrograph of a cross section in the mid-piece region and the tail from a frozen-thawed semen sample treated with 100µl/ml amphora, illustrating good mitochondrial dense electron spaces (arrow head) and

longitudinal fibers with intact axonemes, normal axial 9+2 fiber pattern in cross sections of sperm tails (arrow) (x 20000).



A B C

Fig. (3 A): Electron micrograph for a sagittal section in the sperm head from a frozen-thawed semen treated with 400µl/ml amphora illustrating swollen, outer acrosomal membrane (arrow head) and plasma membrane (arrow) fuzzy appearance of acrosome; unevenly distributed electron dense material within the acrosome(x 24 000) (B). Electron micrograph of a longitudinal section in the mid-piece from a frozen-thawed semen sample treated with 400 µl/ml amphora showing distorted mitochondrial cristae (arrow).Also, swollen plasma membrane (arrowheads (x 18000). (C) Electron micrograph of cross section in the mid-piece from a frozen-thawed semen sample treated with 400µl/ml amphora showing degeneration and vaculation of mitochondrial cristae (arrow).Also,swollen plasma membrane (x 24 000). Data regarding the effect of amphora coffeaeformis addition to the freezing extender on the total antioxidant capacity (TAC), antioxidant enzymes and lipid peroxidation of the cryopreserved semen are presented in (Table 2). In vitro provision of semen extender with 100µl/ml amphora coffeaeformis significantly augmented ($P<0.05$) total antioxidant capacity (TAC), superoxide dismutase (SOD), glutathione peroxidase (GPx) and ascorbic acid concentrations (0.58 ± 0.05 mµ/ml, 95.00 ± 4.16 U/ml, 87.61 ± 5.20 U/L and 58.33 ± 2.16, respectively) compared with the control (0.15 ± 0.06 mµ/ml, 31.25±1.65U/ml, 51.73±2.32U/L and 22.14±7.84, respectively). Furthermore, 100 µl/ml amphora coffeaeformis significantly decreased ($P<0.05$) lipid peroxidation of the cryopreserved spermatozoa compared with the control semen (9.04 ± 0.69 *vs* 24.58 ± 1.60 nmol/ml).

Table (2): Effect of Amphora coffeaeformis addition to freezing extender on buffalo bull semen antioxidant activity.

Treatment	TAC (mμ/mL)	SOD (U/mL)	GPx (U/L)	vitC μg/10 8	Lipid Peroxidase (nmol/mL)
Control	0.15±0.06 [a]	31.25±1.65 [a]	51.73±2.32 [a]	22.14±7.84 [a]	24.58±1.60 [a]
amphora 50 µl/ml	0.51±0.05 [b]	41.75± 2.66	79.08±1.63 [b]	34.46±0.38 [a]	18.68±2.49 [a]
amphora 100 µl/ml	0.58 ±0.05 [b]	95.00±4.16 [b]	87.61±5.20 [b]	58.33±2.16 [b]	9.04±0.69 [b]
amphora 200 µl/ml	0.55±0.07 [b]	79.40±3.54 [b]	68.21 ±5.21 [ab]	45.09±2.60 [ab]	10.74 ±1.31 [b]
amphora 400 µl/ml	0.50 ± 0.05 [b]	68.80±3.76 [b]	70.60±6.29 [ab]	36.34±6.29 [ab]	16.36 ±3.66 [ab]

Three replications of the experiment were conducted. Results are presented as mean ± SEM Values with different superscript in the same column are significantly different ($p<0.05$). TAC: Total Antioxidant Capacity; SOD: Superoxide Dismutase; GPx: glutathione peroxidase; MDA: Malondialdyhide.

DISCUSSION

Recently many researchers are interested in finding any natural antioxidants having safety and effectiveness, which can be substituted for current and commercial synthetic antioxidants. Benthic diatoms have become good candidates for sources of natural antioxidants, as revealed by a number of recent studies **(Affan *et al.*, 2006).** Amphora coffeaeformis as benthic diatoms have different antioxidative activities and exhibited antioxidant potential against ROS and it would be a potential candidate as a natural water-soluble antioxidative source, which can be applied in food or food related industries **(Lee *et al.*, 2008).** However, there is a paucity of exploration about its protective role during buffalo semen cryopreservation. The present study revealed that addition of 100µl/ml amphora coffeaeformis extract to the semen extender enhanced post-thawing motility, viability of the cryopreserved spermatozoa and significantly reduced acrosomal deterioration. These results are demonstrated for the first time. The beneficial effect of amphora coffeaeformis on buffalo sperm cell quality appears to arise, at least partially, from its antioxidant properties which include the up regulation of the level and the elevation of the activities of antioxidant enzymes that may protect sperm membranes against toxic reactive oxygen. The current results indicated that amphora coffeaeformis resulted in a significant increase in total antioxidant capacity and antioxidant enzymes.

This result coincided with the interpretation that mentioned by **Jao and Ko (2002)** who reported that the benthic diatoms exhibited antioxidant potential against ROS and it would be a potential candidate as a natural water-soluble antioxidative source. Under physiological conditions, spermatozoa produce small amounts of ROS, which are needed for capacitation and acrosomal reaction **(Agarwal *et al.,* 2003).** Since buffalo spermatozoa contain large amounts of polyunsaturated fatty acids, it is extremely sensitive to lipid peroxidation **(Agarwal and Prabakaran 2005).** Normally, antioxidants such as superoxide dismutase, glutathione peroxidase, and glucose - 6 - phosphate dehydrogenase enzymes prevent damage to sperm membrane through super oxide and hydrogen peroxide anions breakdown. Superoxide anion radical generally form first and its effects can be exaggerated as it produces other kinds of cell damage inducing free radicals and oxidizing agents (Liu and Ng (2000). Although superoxide anion is itself a weak oxidant, it can be converted into the powerful and dangerous hydroxyl radicals as well as singlet oxygen, both of which contribute to the oxidative stress (**Korycka-Dahl and Richardson 1978)**. Therefore, it could be assumed that one of the most important roles of amphora coffeaeformis on semen function observed in the present study may be due to its ability to scavenge superoxide anion. Nitric oxide is a gaseous free radical, which has important roles in physiological and pathological conditions. The reactivities of the NO· and O·- 2 were found to be relatively low, but their metabolite ONOO- (peroxynitrite) is extremely reactive and directly induces toxic reactions, including SH-group oxidation, protein tyrosine nitration, lipid peroxidation and DNA modifications **(Moncada *et al.*,1991).** Therefore, the scavenging ability of NO· from the diatom extracts may help to interrupt the chain reactions initiated by excessive production of NO· that are harmful to semen function. Moreover the current results revealed that 100µl/ml amphora coffeaeformis reduced the lipid peroxidation of the cryopreserved spermatozoa. These results come in accordance with **Halliweill (1991)** who found that amphora coffeaeformis showed higher iron chelating activities. Ferrous iron can initiate lipid peroxidation by the Fenton reaction as well as by accelerating peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals. Moreover, amphora coffeaeformis demonstrated a noticeable capacity for iron binding, suggesting their ability as peroxidation protectors, which relates to their ferrous binding capacity **(Gülçin *et al.,* 2004**). Furthermore, Amphora is rich in total lipids and fatty acids with a high amount of polyunsaturated fatty acids (PUFAs) especially EPA and a high amount of essential amino acids **(lee *et al.,* 2008**). Therby the beneficial

effect of Amphora may attribute to high amount of essential amino acids content. These results are in line with the findings of **Bucak *et al.* (2008)**,who found that supplementation of the freezing extender with amino acids significantly augmented the characteristics of spermatozoa, such as motility, progressive motility ,viability and acrosome integrity. Regarding to the frozen thawed buffalo semen treated with 400µl/ml amphora coffeaeformis extract caused a significant reduction in sperm motility, acrosome integrity and severs pathological changes. The reason for these negative effects may be the increased osmotic pressure and hyper tonicity. In these studies,Ultrastructure investigation of bull sperm with Addition of extraction of Amphora coffeaeformis at concentrations 100 mg/ml was able to maintain, integrity of acrosome after freeze thaw cycle. We suggested that addition of Amphora extraction helped in increasing the osmotic resistance and reduced the mechanical stress to the cell membrane when compared to their control **Prathalingam, (2006**). Amino acid content in Amphora extraction ply important role in increasing percentage of viability spermatozoa during freezing/thawing without changing the lipid composition of plasma membrane during cryopreservation there by increasing the sperm quality after thawing **(Beirao *et al.*, 2012**). Regarding to the frozen thawed buffalo semen treated with 400µl/ml amphora coffeaeformis extract showed sever s pathological changes as**.** Distorted mitochondrial cristae and vesiculation of plasma membrane and OAM,Cysts and disintegration of plasmalemma were observed over the acrosome, this may perhaps due to the injury cause by freezing and thawing process in present of high concentration amphora extraction which lead to breakage certain high molecular Wight protein which appear as low or medium molecular Wight these molecular change may be responsible for damage in spermatozoa in these concentration **Khili (2012)**. In conclusion the use of 100µl/ml amphora coffeaeformis in the freezing extender improves fine structure integrity through enhancing the antioxidant defense of buffalo sperm cells and decreasing the rate of lipid peroxidation.Therefore,amphora coffeaeformis as a natural antioxidant may reduce cryodamage and improve sperm cryopreservation quality.

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