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Quantitative Ultrastructure Evaluation of Egyptian Buffalo Bull Frozen-Thawed Spermatozoa under the Effect of Honey

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Abstract **Original Research Article**

Enormous damage to spermatozoa occurs during handling and/or deep cold storage. In the meantime, detection of the alternations of spermatozoa in routine semen analysis is very subjective and depends upon the skill of the technician in the lab. This work aimed to verify the value of electron microscopic as well as single cell electrophoresis assays in evaluating the changes in spermatozoa extended with honey (0.5, 1.0, 2.0 and 4.0 %) in skim milk extender. Frozen-thawed extended buffalo semen samples were evaluated for motility, membrane integrity (via hypo-osmotic swelling test), ultrastructure configuration (via electron microscope) and DNA integrity (via comet assay). Data showed that the addition of honey decreased the number of spermatozoa which showed ruptured plasma and acrosomal membranes, improved sperm motility and kept DNA integrity. Milk extender is good for improving spermatozoa cryo-resistance especially with the addition of honey 2.0%. Further studies are mandatory to declare how honey works to stabilize spermatozoa against cryopreservation.

Keywords: Buffalo, Freezing, Honey, Spermatozoa, Ultrastructure and comet assay.

INTRODUCTION

Recent surveys revealed that the analysis of cryopreserved semen which depends only on; concentration, morphology, viability, and motility are not highly accurate enough for the prediction of spermatozoa quality [1]. Therefore, additional investigations are still mandatory for the evaluation of spermatozoa fertility potential.

An assessment of spermatozoa by the electron microscope has been proven to be the suitable implement for estimating the exact degree of damage and very early stages of damage in spermatozoa ultrastructural changes in general, and specifically in the fine structure of organelles, which are involved in fertilization [2].

DNA quality of sperm is largely acknowledged as a valuable tool for the estimate of sire fertility [3]. The integrity of sperm DNA is an important sperm quality parameter concurs with the intact nuclear chromatin material and consequently successful accomplishment of fertilization [4].

The cryo-processing, as well as, the preservation of semen at very low temperature greatly compromise the bull sperm quality [2] verified the role of transmission electron microscopy for the detection of the extent of damage in the fine structures and organelles of spermatozoa after freezing.

Freezing process induces changes in the composition of lipid, and affect the integrity and permeability of acrosomal and plasma membrane [5], and mitochondria [6] due to the reorientation of the normal structure of the membrane-bound phospholipids under the effect of low temperature [7, 8].

The leading target of an extender to keep the survival of cell and to maintain the intact plasma membrane. The fertility is positively related to the proportion of spermatozoa with an intact plasma membrane on cryo-preserved semen [1]. Semen extenders which contain active components, such as casein micelles in milk, are able to prevent the removal of phospholipids and cholesterol from the plasma membrane and keep it intact [9]. The extender for cryopreservation should contain enough salts to balance osmolality and source for energy supply, such as

fructose before freezing in order to prevent cell injury [10].

The current work aimed to demonstrate, quantitatively, the ultrastructural changes and DNA integrity of spermatozoa in the presence or absence of honey in frozen-thawed buffalo extended semen.

MATERIALS AND METHODS

Animal care

All experiments applied in animals were under rules and guidelines of animal care of Benha University, Egypt. All efforts were made to keep bulls intact and without suffering.

Semen sample collection and extension

Semen samples from four proven fertile buffalo bulls, 4-6 years old, kept at Teaching Farm, Fac. Vet. Med., Benha University, Egypt, were collected with an artificial vagina (IMV Model, France) maintained at 42-45 °C, for a period of 8 weeks. Ejaculates were kept in the water bath for 10 minutes at 37°C before evaluation and extension. Semen was assessed microscopically for motility (at 100× and 400×) and concentration with Neubauer haemocytometer. Semen samples of good quality (i.e. viability ≥ 70-75% and conc. ≥ 800 $\times 10^6$/ml) were pooled and allocated into five aliquots in replicate for further processing. Extenders were prepared by adding 0 (control), 0.5, 1.0, 2.0 and 4.0 ml of honey per 100 ml of 10% of heat treated (92-95 °C for 10 minutes) skimmed milk (SKIMZ®, Candia). Antibiotics (Penicillin 1000 IU/ml and Streptomycin 1000 µg/ml) were added to the extenders at room temperature. Extended semen was slowly cooled to 5° (approximately for 2 h), equilibrated for 2 h, and frozen in the nitrogen vapor before being dipped and stored in the nitrogen tank till assay.

Transmission electron microscopy

Thawed (45 °C for 30 sec.) frozen semen was centrifuged at 800 ×g for 5 min. The supernatant was discarded, and the pellet was washed once with 0.1 M KPO_4 buffer, pH 7.2. The washed pellets were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, and post fixated for 2 h in 1% osmium tetroxide buffered to pH 7.4 at 4°C. The pellets were dehydrated in serial grades of ethyl alcohol and embedded in epoxy resin embedding medium for sectioning. Semi-thin sections (0.5 µm in thickness) were prepared for the histological examination after toluidine blue staining. The ultrathin sections were cut by Ultracut microtome, stained with lead citrate and uranyl acetate [11]. The samples for ultrastructure observation were prepared in the Egyptian Army Veterinary Hospital, Nasr city, Egypt, and examined by a Hitachi H- 7650 transmission electron microscope (80KV) in Huazhong Agriculture University, Wuhan, Hubei province, China.

Comet assay (single cell electrophoresis assay)

Damage to spermatozoa DNA in triplicate samples per trial was assessed using the comet assay as formerly mentioned [12] with the slight modifications. Straws were thawed at 37°C for 30 seconds, washed twice with calcium and magnesium free PBS (pH 7.4), and the sperm pellet was reconstituted in PBS (pH 7.4). 20,000 spermatozoa in 10 µl PBS were admixed with 90 µl 1% low melting gel, layered on the first dried agarose layer prepared by coating slides with 300 µl 1% normal melting gel and left to solidify at 4˚C for 30 min. For cell lysis, slides were immersed in vertical coupling jar filled with lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl; pH10, 1X triton, and 40 mM Dithiothreitol) for 1 hour at room temperature. Proteinase K (10 µg/ml) was added thereafter and the slides were incubated overnight at 37°C. Slides were washed with distilled water and loaded with unwinding alkaline solution (1 mM EDTA, 0.05 M NaOH; pH 12.1) for 15 min. Slides were washed with 1 X TBE (pH 7.4) twice for 5 min each and then placed onto horizontal electrophoresis tank side by side. The tank was filled with neutral electrophoresis buffer (1X TBE; pH 8.0) and the electrophoresis was conducted for 20 min at 25 volts and 10 mA. The slides were drained and flooded with absolute ethanol for 10 min in order to precipitate the DNA. Each gel was stained with 50 µl of ethidium bromide (20 µg/ml) and visualized using 40× objective of Leica epifluorescent microscope (Green filter: N2.1 with Excitation filter: BP 515-560, Dichromatic Mirror: 580, Suppression filters: LP 590). The images for the sperm nuclei were digitalized with true chrome retina screen camera version 4.2 build 5001 (copyright Tucson photonics co. Ltd). A minimum of 100-200 sperm cells nuclei per sample was measured using image analysis software TnTek CometScore™ freeware (Ver. 1.5) to obtain % of DNA in the head, % of DNA in the tail, tail length and tail moment length.

Head DNA (%) is the intensity of the head compared with the intensity of the whole comet. Tail DNA (%) is the intensity of the tail compared with the intensity of the whole comet and calculated as tail DNA/cell DNA intensity × 100. Tail length is the distance of DNA migration from the center of the edge of the head to the end of the tail. Tail moment length is the distance from the center of the head to the center of the tail and calculated as Tail DNA % × length of tail

Statistical analysis

The data are presented as mean (± SE). The data on comet assay were analyzed with One-way ANOVA and *Post-hoc* Dunnett's multiple comparison tests using SPSS (Ver. 16). P value was set at < 0.05 to define statistical significance.

RESULTS

A summary of cellular alterations examined by light and transmission electron microscopes of cryopreserved spermatozoa extended with milk-honey

extenders is presented in table 1 and table 2, respectively.

Table-1: Motility and membrane integrity of buffalo semen extended with at different levels of honey as assessed by light microscope

Item	Honey level (%) in milk extender				
	0	0.5	1.0	2.0	4.0
Motility	43.75±2.39	45.00±2.04	46.25±4.26	48.75±3.15	53.75±3.14
Membrane integrity	73.67±2.12	78.67±3.92	75.67±7.45	80.33±1.85*	74.33±3.36

* denotes significant differences (P< 0.05) as compared with control.

Table-2: Spermatozoa abnormalities rates in buffalo semen extended with at different levels of honey milk as seen by electron microscope

Item (%)		Honey level (%) in milk extender				
		0.0	0.5	1.0	2.0	4.0
Normality		50.0	37.5	52.5	67.5	65.0
Abnormality		50.0	62.5	47.5	32.5	35.0
	Plasma membrane rupture	20.0	35.0	30.0	17.5	15.0
	Acrosomal membrane rupture	10.0	15.0	5.0	5.0	5.0
	Acrosome dispersion	5.0	5.0	5.0	5.0	2.5
	Nuclear membrane damage	5.0	5.0	5.0	2.5	10.0
	Abnormal sperm tail	10.0	2.5	2.5	2.5	2.5

Spermatozoa cryopreserved in control milk extender showed slight rupture of the plasma membrane in the head region and dispersion of the acrosome content (Fig. 1A,B), but marked abnormal arrangement in the peripheral fibrils of the mid- and principle pieces. Comet assay analysis revealed that the DNA % in the head was comparatively high, but DNA% in tail and tail moment was low (Fig. 3 and 4).

Spermatozoa stored in milk extender with honey 0.5% showed rupture of plasma membrane over the head and tail regions, while others showed the dispersion of the acrosome content after rupturing of the plasma membrane and acrosomal membrane (Fig. 1C,D). DNA% in the head and tail length were slightly low, but DNA% in tail and tail moment were slightly high as compared with control (0% honey) (Fig. 3 and 4).

Spermatozoa extended in milk-honey 1% presented ruptured plasma membrane all over the sperm cell parts (Fig. 2A-C). However, the tails of spermatozoa showed the normal arrangement of the peripheral fibrils and filaments of the axial complex. DNA% in head slightly decreased, but DNA% in the tail ($p<0.05$), tail length and tail moment slightly increased as compared with control (Fig. 3 and 4).

In milk-honey 2.0%, spermatozoa showed rupture of plasma membrane over the head region and mid-piece, but the normal principle and end pieces of the sperm tail. Some spermatozoa showed a disturbance in the mitochondria in the form of pale staining in few numbers of mitochondria (Fig. 2D-F). Comet assay analysis was similar to that of spermatozoa extended with milk-honey 1.0% (Fig. 3 and 4).

In milk-honey 4.0% extender, spermatozoa still showed rupture of plasma membrane over the head and tail regions, dispersion of acrosome and nuclear damage, although and normal arrangement of mitochondria and peripheral fibrils and axial filament complex in the tail region (Fig. 2G-I). Comet assay analysis showed that the DNA % in head significantly ($p<0.05$) decreased, but DNA % in tail ($p<0.001$), tail length ($p<0.01$) as well as tail moment ($p<0.05$) significantly increased as compared with milk-honey 0% (Fig. 3 and 4).

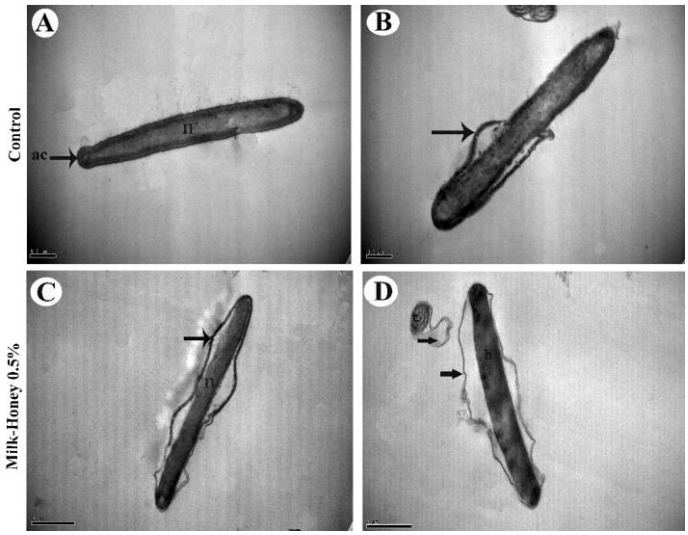

Fig-1: A representative micrographs of buffalo spermatozoa extended with milk extender supplemented with 0 % (A,B) Scale bar indicated 0.5µm and 0.5 % (C,D) Scale bar indicated 1µm. acrosome (ac), nucleus (n), head of spermatozoa (h), tail (t) and ruptured plasma membrane (arrows)

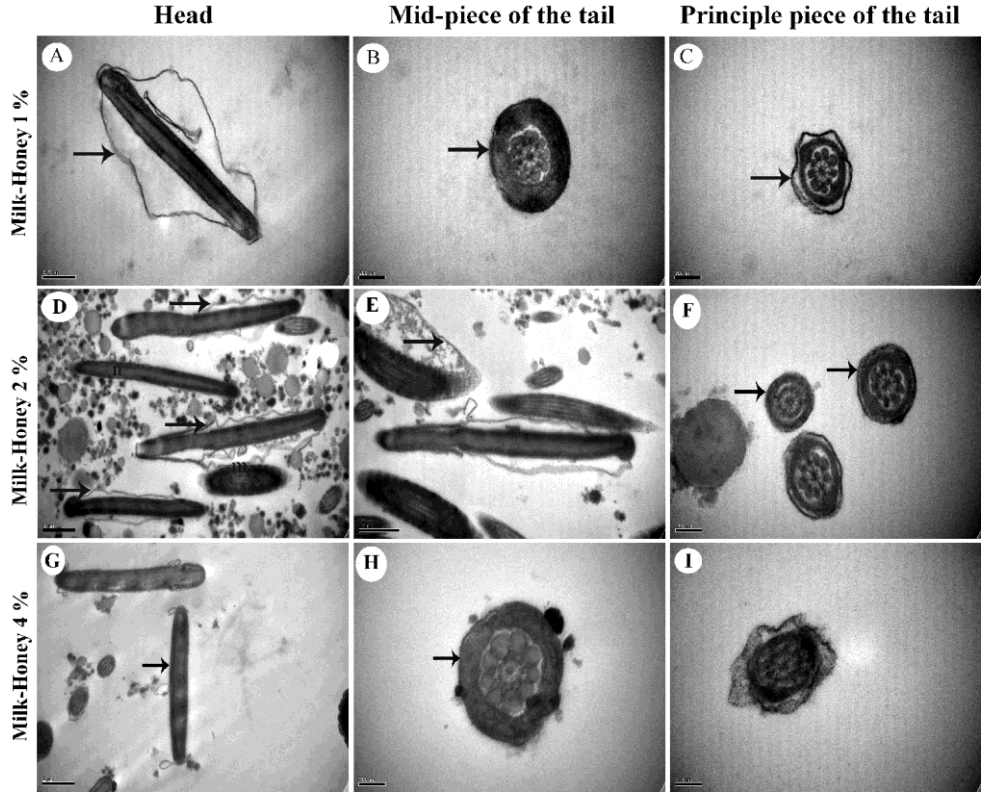

Fig-2: A representative micrographs of buffalo spermatozoa extended with milk extender supplemented with 1.0 % (A-C), 2.0 % (D-F), and 4.0 % (G-I) of honey. Plasma membrane (arrows), nucleus (n) and mitochondria (m)

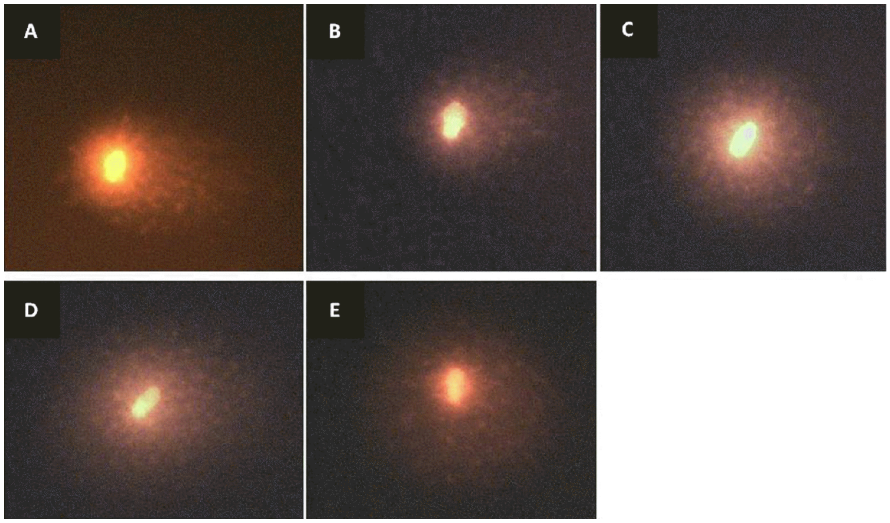

Fig-3: Representative images of single cell electrophoresis assay (comet assay) detecting DNA damage of buffalo spermatozoa frozen in milk extenders supplemented with different levels of honey. A: control (0%), B: 0.5%, C: 1.0%, D: 2.0%, and E: 4.0%

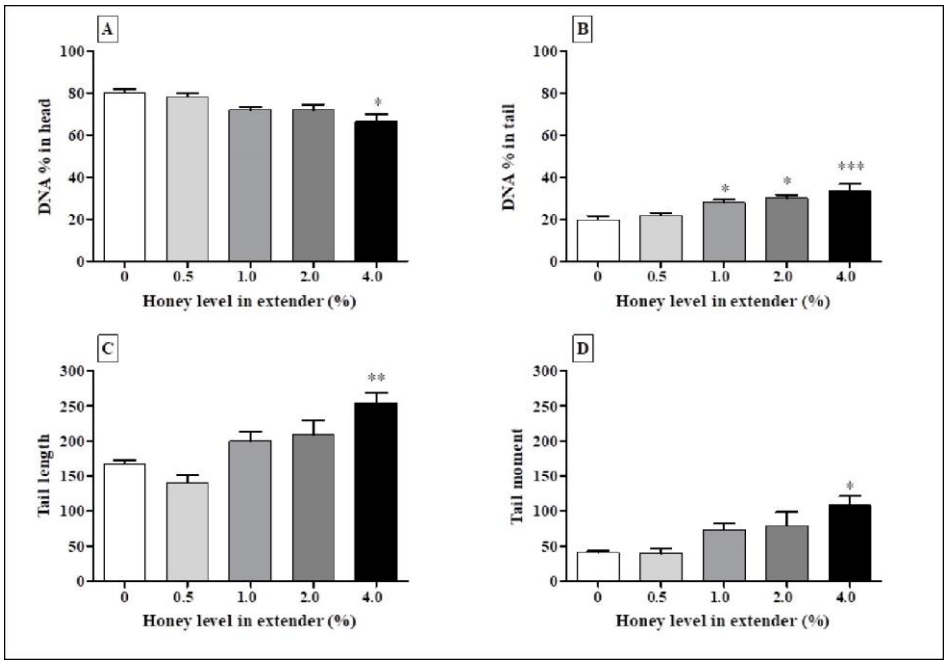

Fig-4: Comet assay of frozen-thawed Egyptian buffalo bull spermatozoa under the effect of different levels of honey in milk extender. *, ** and * denoted considerable differences as compared with control extender (0% honey) at p< 0.05, 0.01 and 0.001, respectively**

DISCUSSION

Honey is an ancient remedy for many diseases, either topically or systematically, which has lately been re-introduced by the therapists. Day-by-day increasing use of the honey in medical care by many authors declares its importance. The presented data indicated that milk extender is good at preserving the frozen semen of Egyptian buffalo bull. Moreover, the addition of honey at concentrations of 0.5 and 1.0 % did not lower the damage to plasma membrane caused due to freezing-thawing. However, the increased honey level to 2.0% in milk extender was associated with improved membrane stability against cryo-damage of buffalo bull spermatozoa. These findings are in alignment with the previously mentioned result by [13] who explained that honey allows the keeping of membrane integrity to occur during cryopreservation. Also [14] added that the use of honey as an energy source in combination with garlic as a natural antibiotic during cooling of ram spermatozoa improved the survival of ram spermatozoa significantly when stored at 4℃. A recent applied study by [15] confirmed that honey incorporation 2.0 % in skim milk extenders significantly improve spermatozoa capacity for fertilization *in vitro*.

Our results in agreement with [16], who mentioned that the process of freezing and thawing caused considerable ultrastructural changes to the

acrosomes and middle pieces including rupture of the plasma membrane and acrosomal membrane with or without dispersion of acrosomal contents and decrease of the electron density of mitochondria. It is well established that freezing and thawing induced a morphological alteration in the spermatozoa ultrastructure [17, 18]. Honey consists of high quantities (approximately 70-85% of the honey solids) of monosaccharides (fructose and glucose), and small quantities of disaccharides (maltose and sucrose), trisaccharides and oligosaccharides [19-21]. Glucose of honey is the principal constituent that crystallizes as it exists in supersaturated status. Besides, storage of honey at temperatures below 40°C decreased its crystallization [19]. Moreover, when crystallization proceeds in an organized mode a high number of minute crystals are not formed [21, 22]. The glass transition temperature of the pure honey ranged from -42 to -51 °C [23]. Taken together, our results beside aforementioned data indicated that the damage in spermatozoa in frozen-thawed semen was due to the deleterious effect of cry-processing at a low level of honey incorporated (concentrations 0.5% and 1.0 %). These results might be accused to the dissimilarity in the density or the viscosity of the extender or even the existence of large particles in bull semen [24]. The frozen stored honey its dynamic viscosity is higher than honey stored at room temperature [25].

Data of single cell electrophoresis indicated that the honey at concentration 0.5-2.0% maintained the DNA integrity of cryopreserved buffalo spermatozoa, while honey 4.0% was concomitant with the highest ($p<0.05$) DNA damage as compared with control. High concentration (4%) of honey was associated with high spermatozoa metabolic activity and ROS production that might exceed the innate antioxidant activity of honey. As some aerobic organisms produce reactive oxygen byproducts including superoxide, hydroxyl, and hydrogen peroxide [26]. The ameliorative activities of honey against DNA damage perhaps related to the antioxidant properties of honey that are advantageous in countering progressive glycation and lipid oxidation end products, which can persuade oxidative stress [27]. Natural honey contains phenolic acids, flavonoids [28], and other enzymes, peroxidase and catalase [29]. All of these active components act together to give a synergistic antioxidant effect by inhibiting the production of ROS and scavenging the superoxide anion [28].

In conclusion, honey as a natural sugar enriched source could be used as a supplement in milk extender to minimize the spermatozoa damage during freezing-thawing. However, the honey level should be 2.0% or 4.0% to avoid the interruption of the ultrastructure of the plasma membrane and DNA integrity. Electron microscope and comet assays are highly recommended supplementary or complementary tests for the routine evaluation of frozen semen.

Conflict of interest

The authors declare that they have no conflict of interest.

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Author's contributions

Mohamed Kandiel and Ahmed Reda designed the study, performed experiments, analyzed data and wrote the manuscript. Mona Hussein performed ultrastructure study, wrote and revised the manuscript. Xiaojuan Cao helped in taking the photos of ultrastructure study.

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