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OPTIMIZING CRYOPRESERVATION TECHNIQUES FOR DANDARAWI ROOSTER SPERM: EFFECTS OF CRYOPROTECTANTS AND SUCROSE ON MOTILITY, FERTILITY, HATCHABILITY, AND ULTRASTRUCTURE

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Abstract: Cryopreservation is pivotal for conserving genetic diversity. This study aimed to optimize cryopreservation techniques for Dandarawi rooster sperm through two experiments. Experiment I explored the optimal levels of two cryoprotectants, with or without sucrose, in two trials. Semen from 20 Dandarawi males was pooled, diluted, and divided into five aliquots. In trial 1, one aliquot remained fresh (control), while others were frozen in straws using glycerol (GLY) at concentrations of 8% and 11%, with or without 5% sucrose. In trial 2, one aliquot served as a control, while the others were frozen in pellets using a cryo-diluent containing 5% or 7% dimethylacetamide (DMA), with or without sucrose. Danadarawi hens (n=10 per group) were inseminated with fresh (control) or frozen-thawed sperm, with six repetitions of semen collection and insemination. In Experiment II, pooled semen was similarly divided. Aliquot 1 served as a control, while aliquots 2 and 3 were frozen in pellets with 5% DMA cryo-diluent, with and without 5% sucrose, respectively. Aliquots 4 and 5 were frozen in straws with 8% GLY, with and without 5% sucrose. Transmission electron microscopy (TEM) evaluated spermatozoa for cryodamage. GLY level did not significantly affect motility, fertility, or hatchability, but sucrose notably improved hatchability with 8% and 11% GLY. DMA did not significantly impact motility, although 7% DMA demonstrated the highest fertility. Sucrose enhanced hatchability with 5% DMA but not with 7% DMA. TEM analysis revealed higher levels of acrosomal damage in spermatozoa frozen with GLY compared to DMA, with sucrose consistently mitigating acrosomal injuries. Moderate damage was observed in the tail, sparing the nucleus and midpiece. In conclusion, the choice of freezing method and the inclusion of sucrose significantly influenced sperm freezing outcomes, demonstrating their critical roles in preserving sperm quality. Sucrose was particularly effective across all treatments, notably in protecting the acrosome region.

Keywords: Dandarawi chickens, dimethylacetamide, glycerol, sperm ultrastructure, disaccharides

INTRODUCTION

Unlike mammalian sperm, avian spermatozoa face unique challenges in cryopreservation due to their distinctive morphology, including a filiform shape, long tail, and condensed nucleus (Donoghue and Wishart, 2000; Long, 2006). Freezing and thawing processes lead to significant reductions in the number of viable sperm capable of fertilization in the sperm storage tubules (Pierson et al., 1988; Tajima, 2013). Moreover, the success of freezing protocols can vary widely between species (Blesbois and Brillard, 2007; Blesbois, 2011; Blesbois, 2012; Tajima, 2013), necessitating species-specific cryodiluents and freezing methods to maintain acceptable post-thaw viability and fertility (Siudzińska and Łukaszewicz, 2008).

Differences in frozen/thawed sperm motility and fertility outcomes among commercial poultry species are well-documented in the literature (Çiftci and Aygün, 2018). These variations are influenced by factors such as diluent type, sperm packaging, freezing/thawing method, number of sperm cells used, temperature curve, and cryoprotectant concentration (Long et al., 2010). Glycerol (GLY) and dimethylacetamide (DMA) are commonly used cryoprotectants in avian sperm cryopreservation, although they can adversely affect fertilizing ability (Abouelezz et al., 2017). GLY, if not reduced to less than 1% in the cryodiluent before insemination, can act as a contraceptive (Hammerstedt and Graham, 1992; Abouelezz et al., 2015a; Abouelezz et al., 2015b). DMA has been noted for its toxicity to spermatozoa, necessitating rapid freezing and immediate insemination post-thawing (Abouelezz et al., 2015a, 2015b, 2017).

Sucrose, a disaccharide sugar, is added to freezing diluents to enhance cell dehydration and reduce internal ice crystal formation due to its high molecular weight, which prevents diffusion across sperm cell membranes (Froman et al., 1999; Purdy, 2006). Moreover, at low hydration levels, sucrose interacts with phospholipid membranes, stabilizing sperm membranes (Hincha et al., 2006).

Fertility assessment is crucial in evaluating freezing protocols as it directly reflects the success of cryopreservation techniques (Donoghue and Wishart, 2000; Blesbois et al., 2008). Laboratory measures such as motility, viability, and membrane integrity often overestimate post-thawed sperm fertilizing capacity, leading to lower observed fertility rates (Long, 2006; Mocé et al., 2010).

Sperm morphology is a critical indicator of semen quality (Malmgren, 1997) and plays a key role in assessing male fertility (Bertschinger et al., 1992). While light microscopy provides a general view of sperm structure, electron microscopy (EM) offers detailed insights into spermatozoa morphology, making it invaluable for diagnosing fertility issues in avian species (Visco et al., 2010). Transmission electron microscopy (TEM) has proven effective in accurately distinguishing between severe hypo-motility and complete absence of sperm motility (Visco et al., 2010).

During freezing/thawing, sperm cells are subjected to osmotic, chemical, and mechanical stresses (Hammerstedt et al., 1990), which damage plasma membranes, mitochondria, and acrosomes (Bakst and Sexton, 1979), thereby reducing sperm viability and functionality and lowering fertility rates by 30-60% compared to untreated sperm (Westfall and Harris, 1975; Bakst and Sexton, 1979). Structural abnormalities such as thickened, irregular, or disrupted plasmalemma, conical acrosome, and exposed mitochondrial sheath in the midpiece are common after cryopreservation (Gee et al., 1985). Approximately 60% of chicken spermatozoa experience irreversible organelle destruction post-thawing (Bakst and Sexton, 1979). Glycerol removal can cause spermatozoa to coil within the plasma membrane, displacing the acrosome and compromising membrane

integrity (Bakst and Howarth, 1977; Westfall and Howarth, 1977). Careful adjustment of glycerol levels is essential to prevent acrosome damage (Maeda et al., 1990).

The objectives of the current study were as follows:

1) Determine the optimal levels of glycerol (GLY) and dimethylacetamide (DMA) as cryoprotectants, and evaluate the effectiveness of sucrose as an osmoregulatory agent in cryopreserving Dandarawi rooster sperm cells. 2) Assess the extent of damage to spermatozoa frozen using GLY or DMA as cryoprotectants. 3) Investigate whether the addition of sucrose as an osmoregulator protects frozen/thawed spermatozoa.

MATERIALS AND METHODS

We confirm that all experimental procedures involving animals were conducted in strict accordance with the animal ethics guidelines of the Faculty of Agriculture, Assiut University. All efforts were made to minimize discomfort and stress to the animals throughout the study.

Experimental birds:

The experimental birds were obtained from the Poultry Research Farm, Faculty of Agriculture, Assiut University, Assiut governorate, Egypt. At 26 weeks of age, birds were housed in individual cages of 30× 40× 40 cm under the photoperiod 16 h light: 8 h dark, and fed a commercial diet containing 17% crude protein, 2750 kcal/kg diet, 2.80% crude fat, 3.13% crude fiber, 3% Ca, and 0.68% P. During the semen collection period, twenty adult males and fifty females were selected and used in consecutive trials of sperm freezing and artificial insemination (AI).

Semen collection and preparation:

At sexual maturity, twenty roosters were selected according to their response to the process of semen collection, ejaculate volume and sperm motility. Roosters were trained for semen collection before sampling. At 28 weeks old, semen was collected from males by the abdominal massage technique according to Burrows and Quinn (1937). Semen was collected carefully to avoid contamination by blood or faeces. In each experiment, semen was collected six times with 3 day intervals between each two consecutive collection times. Ejaculates were pooled in each collection time in falcon tubes (15 ml) and diluted immediately with Lake and Ravie extender 1 : 1 (v : v) comprising glucose (0.8 g), sodium glutamate (1.92 g), magnesium acetate tetrahydrate (0.08 g), polyvinylpyrrolidone [relative molecular mass (M_r) = 10 000] (0.3 g), potassium acetate (0.5 g), dissolved in distilled water up to 100 ml; pH =7.08, and osmolality 343 mOsm/kg (Lake and Ravie, 1984). Then semen was transported to the lab and kept at 5˚C until use. Lake and Ravie diluent was prepared in the laboratory and all chemicals, cryoprotectants, and sucrose were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Panreac Quemica S.A. (Barcelona, Spain).

The sperm concentration was evaluated in pooled samples using a hemocytometer (Neubauer chamber). Motility percentage was subjectively assessed in 1:40 (v/v) diluted fresh as well as frozen/thawed samples under 400 × magnification (Microscobe version: Olympus IX53; Olympus America INC., 3500 Corporate Parkway, Center Valley, Pennsylvania 18034-0610, U.S.A.). Sperm motility rate was expressed as the percentage of total motile sperm (0 to 100%).

Experimental design:

Experiment I

Two separate trials were carried out. In each trial, after measuring the sperm concentration of the pooled semen, specimen was divided into five aliquots and extended using either Lake & Ravie diluent to obtain a sperm concentration of 1000 × 10^6 cell/mL and served as a control or was extended using one of the cryo-diluents mentioned below to obtain a sperm concentration of 1200 × 10^6 cell/mL.

In trial (1), the first aliquot was stored at 5ºC for approximately 3 hours before performing the AI (control). The remaining four aliquots

were extended with Lake and Ravie extender containing different levels of glycerol and sucrose to give final concentrations of 8% and 11% glycerol in combination with either 0% or 5% sucrose. In trial (2), the same design was followed except that the cryoprotectant glycerol was replaced by DMA at final concentrations of 5% and 7%. The cryo-diluents were mixed with the semen in each experiment at 5°C and left for 10 minutes for equilibration before freezing (Santiago-Moreno et al. 2011).

Freezing procedures

Glycerol-in-straw method:

The semen samples containing glycerol were frozen following the gradual freezing on nitrogen vapor in two steps. The samples were loaded in French straws (250 µL) which were kept at 10 cm then at 5 cm above the surface of liquid nitrogen for 10 and 5 minutes, respectively. Thereafter, straws were plunged into liquid nitrogen (−196°C) and stored until thawing.

DMA-pellets method:

After equilibration, the semen samples containing DMA were pipetted and plunged directly into the liquid nitrogen drop by drop from a height of approximately 11 cm above the surface of the liquid nitrogen, using a micro-pipette which produces a drop size of approximately 70 µl to form the pellets.

Thawing methods:

After two hours of storage, the frozen pellets containing DMA were thawed by placing them in a water bath at 60°C for 20 to 30 seconds (Chalah et al., 1999). The frozen samples in straws containing GLY were thawed in a water bath at 5°C for 3 minutes. For glycerol removal, subsequently, the thawed samples underwent progressive dilutions using Lake and Ravie medium at 5°C to a final dilution of 1 : 4 (v/v) in six dilution steps according to the method reported by (Mocé et al., 2010). The dilution steps were as follows; 1: 0.07, 1: 0.18, 1: 0.32, 1: 0.6, 1: 1.25 and 1: 1.58 (v/v) in which two-minute intervals were given after each dilution to allow GLY efflux from the cells. The samples were then centrifuged at 500 × g for 10 min and the supernatant solution was discarded and the pellet was re-suspended in Lake and Ravie medium until the same volume of the sample before freezing was reached.

Egg collection and incubation:

In both trials, fertility and hatchability rates were estimated from the results of performing six consecutive AIs. Three-day intervals were given between each two consecutive inseminations. A total of fifty females, ten females per treatment group, were intravaginally inseminated either by 100 µl/hen of fresh semen (control; 100 × 10^6 sperm), or by 250 µl/hen of the post-thawed semen (300× 10^6 sperm). All inseminations were performed between 14:00 and 16:00 h (Burrows and Quinn, 1939).

All produced eggs were collected after 24 hours from the first insemination until the third day of the last AI, and were set in the incubator (37°C and 55- 65 % relative humidity). Eggs were candled on the 7^{th} day of incubation to determine fertile eggs. Eggs not showing embryonic development were cracked to identify blastodermal development.

Fertility and hatchability percentages were calculated as follows;

Fertility % = (Number of fertile eggs/ total number of set eggs) × 100.

Scientific hatchability % = (Number of hatched chicks/ total number of fertile eggs) × 100.

Commercial hatchability % = (Number of hatched chicks/ total number of set eggs) × 100.

Experiment II

The design of experiment II was made based on the obtained results from experiment I. From the same twenty Dnadarawi roosters, pooled semen was collected and divided into five aliquots. The first aliquot was kept at 5°C and served as control (fresh semen). Aliquots 2 and 3 were frozen using 5 % DMA with and without 5 % sucrose, respectively. While aliquots 4 and 5 were frozen using 8 % GLY with and without 5 % sucrose, respectively. Sperm motility of

fresh and frozen/thawed semen was assessed and samples given sperm motility scores higher than 95 % for fresh and 30 % for frozen/thawed semen were used.

Preparing samples for transmission electron microscopy:

In experiment II, semen samples were fixed in 5 % cold phosphate buffered glutaraldehyde in the ratio of 1: 5 (v : v) and kept for 24 – 48 hours at 5˚C. Thereafter, samples were gently centrifuged and the pellets were washed 3 – 4 times in cacodylate buffer (pH 7.2) for 20 minutes each, then fixed in 1% osmium tetroxide (O_4S_4) for two hours before rewashing (four times) by cacodylate buffer. Afterwards, samples were processed through ascending grades of ethyl alcohol (30, 50, 70, 90, and 100%) and finally embedded in epon – araldite mixture according to (Bozzola and Russell, 1991). Semi thin sections with a thickness of 0.5 – 1 micron were prepared using ultramicrotome (Reichert ultracuts – Leica –Wild M3Z) and photographed by se30 Olympus camera. Thereafter, ultrathin sections in thickness of 500 -700 nm. were made using Leica AG ultra-microtome and contrasted in uranyl acetate and lead citrate. Finally, samples were examined by JEOL instrument 1200 electron microscope at 80 kilo electron-volt and photographed by CCD digital camera (Model XR- 41).

Statistical analysis

For experiment I, the motility, fertility and hatchability rates as affected by freezing, cryoprotectant level and sucrose treatments were statistically analyzed. The motility values (n=6) were transformed by arcsine before analysis and analyzed by ANOVA following the model

$$\mathbf{x_{ij} = \mu + A_i + e_{ij}}$$

Where: x_{ij}= the motility values, μ= the overall mean, A_i= the effect of GLY, DMA or sucrose treatment, and e_{ij}= the residual (j = 1–6). The Duncan's multiple range test (Duncan, 1955) was used to compare between means wherever significant differences were found. Finally, the Chi-square test was used to assess the association between the treatments, fertility and hatchability rates. All calculations were made using SAS software (SAS institute, 2009).

RESULTS

Experiment I

Trial 1:

The effects of using 8% and 11 % GLY with and without 5 % sucrose on sperm motility, fertility and hatchability percentages are shown in Table 1. Significant reductions in total sperm motility ($P < 0.0001$) were observed in frozen treatment groups when compared to that of the control group. The reduction was more pronounced in the GLY-5% sucrose treatments ($P < 0.05$). The post-thawed sperm motility was comparable in 8% and 11% GLY treatments. The results showed that the fertilizing ability of frozen-thawed spermatozoa in all treatments was significantly ($P<0.05$) lower than that of the control group. Similarly, hatchability percentages were significantly ($P<0.01$) lower in frozen/thawed semen treatments than in the control. Also, GLY level (8% and 11% GLY) had no significant effect on hatchability percentages. When sucrose was absent in the cryo-diluent, none of the eggs succeeded to hatch. However, 9% and 13.5% of the eggs produced healthy chicks when sucrose was present in the 8% and 11% GLY treatments, respectively.

Trial 2:

The effects of using 5% and 7% DMA with and without 5% sucrose on sperm motility, fertility and hatchability percentages are shown in Table 2. Similar to the results obtained in experiment one, it was observed that the total sperm motility, fertility and hatchability percentages ($P < 0.0001$) of the treatments were lower than those of the control; in addition, neither DMA level nor the presence of sucrose had an effect on sperm motility ($P > 0.05$). Although hens inseminated with frozen/thawed semen using 7% DMA had a higher fertility rate (31.14%) than those of the other frozen treatments ($P < 0.05$), none of these fertile eggs succeeded to hatch. Hatched chicks were obtained only from the 5% DMA + 5% sucrose treatment.

Experiment II:

The presence of sucrose in the dilution medium has significantly contributed to protecting the acrosome of frozen/thawed spermatozoa from cryo-damages. Approximately 66.7% of spermatozoa frozen using 8% GLY alone exhibited abnormal and damaged acrosomes, more than double the ratio observed in spermatozoa frozen using 5% DMA treatment (30%). No damages were observed in the acrosome region of unfrozen spermatozoa. Additionally, the acrosomes of spermatozoa frozen using sucrose in their cryo-medium remained intact.

About 15% of spermatozoa frozen using GLY alone showed excessive granulated nuclear material. This abnormality was less frequent in other freezing treatments and was absent in unfrozen spermatozoa.

Almost all unfrozen spermatozoa appeared to have a normal midpiece, while abnormalities ranged from 18% to 52% in frozen/thawed spermatozoa using DMA and GLY with or without sucrose.

The ratio of intact tails decreased significantly (to 13%) due to freezing/thawing procedures using GLY or DMA without sucrose compared to unfrozen treatment (92%). However, the addition of sucrose helped maintain tail integrity, increasing the ratio to 30% and 33% for DMA and GLY treatments, respectively.

Figure 1 illustrates the ultrastructure of spermatozoa from aliquot 1 (fresh semen) examined via transmission electron microscopy. Longitudinal sections of the sperm head are shown in micrographs (a) and (b), revealing an intact acrosome as a complete vesicle located at the anterior base of the nucleus. Micrograph (c) presents a cross-section of a nucleus, while (d) and (e) depict cross-sections encompassing the nucleus, acrosome, and midpiece. The nuclear material appears uniformly dense, and the membranes surrounding the acrosome, nucleus, and midpiece are continuous.

Micrograph (f) displays cross-sections of the midpiece and endpiece, while micrograph (g) focuses on the principal piece. Throughout these micrographs, the plasmalemma tightly adheres to the organelles, maintaining a continuous membrane, and the nucleus shows dense, homogeneous content without distinct chromatin granules or vacuoles. Micrograph (h) provides a longitudinal section of a sperm tail, revealing a regular, striated plasmalemma tightly enclosing an intact fibrous sheath without irregularities.

Figures 2 to 7 exhibit cross and longitudinal sections of spermatozoa frozen using 5% DMA with or without 5% sucrose. In Figure 2, micrographs (a) and (b) show longitudinal sections revealing damage such as swollen mitochondria separated from the midpiece due to plasma membrane rupture in spermatozoa frozen using 5% DMA. Micrograph (c) displays longitudinal sections indicating membrane damage around the midpiece area.

Figure 3, micrographs (a) and (b), displays cross-sections of spermatozoa nuclei frozen using 5% DMA, where the nucleus appears swollen, and in some cases, the nuclear membrane shows tears resulting in cytoplasm leakage (micrograph b).

Figure 4 illustrates tail damage in spermatozoa frozen using 5% DMA alone (micrographs a, b, and c). Micrograph (a) presents cross-sections of tails with ruffled plasma membranes. Completely broken and partially torn tails are visible in micrographs (b) and (c), respectively.

Figure 5, micrographs (a) to (d), displays cross and longitudinal sections of spermatozoa frozen using 5% DMA with 5% sucrose. Micrograph (a) shows a cross-section of a bent sperm with swollen mitochondria and a continuous, ruffled plasma membrane. Micrograph (b) reveals a break between the nucleus and midpiece, while the nucleus remains dense, homogeneous, and enclosed by an intact membrane. Micrograph (c) depicts a bent sperm with an intact, continuous plasma membrane. Micrograph (d) shows the principal piece with a ruffled plasma membrane on the left section and a midpiece

containing swollen mitochondria with ruptured plasmalemma on the right section. Damage appears less severe compared to spermatozoa frozen using DMA alone.

Figure 6, micrographs (a) and (b), illustrates cross and longitudinal sections of nuclei of spermatozoa frozen using 5% DMA with sucrose. The nuclear material appears dense and homogeneous, with the membrane showing an intact but irregular surface.

Figure 7 depicts tail damage in spermatozoa frozen using 5% DMA with 5% sucrose (micrographs a and b). Both micrographs show longitudinal sections with visibly ruffled plasma membranes, suggesting that sucrose provided partial protection against complete tail breakage and loss of plasma membrane around the nucleus and midpiece.

Figures 8 to 13 present cross and longitudinal sections of spermatozoa frozen using 8% GLY with or without 5% sucrose. In Figure 8, micrographs (a), (b), and (c) demonstrate damage in the acrosome, nucleus, and midpiece of spermatozoa frozen using 8% GLY alone. Micrograph (a) shows a partial tear in the acrosome region. Micrograph (b) presents a bent spermatozoon with a cross-section revealing a distended plasmalemma around the nucleus. Micrograph (c) displays a longitudinal section with a gap between the base of the nucleus and the midpiece, along with swollen mitochondria.

Micrographs (a), (b), (c), and (d) in Figure 9 show cross and longitudinal sections of nuclei, midpieces, and endpieces of spermatozoa frozen using 8% GLY alone. The midpiece exhibits the most damage, including absence of plasmalemma and swollen, displaced mitochondria (micrographs a to d). Additionally, micrographs (a) and (b) depict a nucleus with a distended plasmalemma.

In Figure 10, micrographs (a), (b), and (c) reveal tail damage in spermatozoa frozen using 8% GLY, with micrograph (a) showing cross-sections of tails with ruffled and distended plasmalemma. Micrograph (b) displays a longitudinal section of a tail with a ruptured plasma membrane, while micrograph (c) shows a longitudinal section of a bent spermatozoon.

Figure 11, micrographs (a) and (b), illustrates damage in the head and midpiece of spermatozoa frozen using 8% GLY with 5% sucrose. Micrograph (a) shows a sperm head with an irregular nucleus containing granulated nuclear material. Micrograph (b) displays a midpiece with absent plasmalemma, swollen, separated mitochondria, and an undamaged acrosome containing dense, homogeneous nuclear material with small chromatin granules.

Figure 12, micrographs (a) and (b), presents cross and longitudinal sections of nuclei and midpieces of spermatozoa frozen using 8% GLY with 5% sucrose. Micrograph (a) reveals longitudinal sections of two midpieces with distended plasmalemma and swollen mitochondria, while micrograph (b) shows cross-sections of intact and swollen principal pieces.

Figure 13, micrographs (a) and (b), displays longitudinal sections of tails of spermatozoa frozen using 8% GLY with 5% sucrose. The tails exhibit puffy plasma membranes but remain continuous without breakage.

DISCUSSION

Experiment 1

Regardless of the species, cryo-diluent, and the technique, cryopreservation was found to have detrimental effects on sperm vitality and quality including motility (O'Connell et al., 2002). Motility definitely affects sperm arrival to the fertilization site and their ability to compete on fertilizing the ova (Matsuzaki and Sasanami 2022). Sperm with poor motility is unlikely possess good fertilizing ability (Etches, 1996). In humans, sperm motility was decreased by 45% post freezing/thawing (Oberoi et al., 2014). In another study, sperm motility was decreased from 40.2% in fresh semen to 24.8% in post-thawing (Connell et al., 2002). In birds, reductions in Capercaillies' sperm motility by approximately 55% and 36% have been reported after in-straw and in-pellet freezing, respectively (Kowalczyk and Łukaszewicz, 2015). Similar reductions were reported in Harris hawk (Herrera et al.,

2017), Roosters and pheasant (Herrera et al., 2005).

The sperm utilizes the ATP produced by either glycolysis in the cytoplasm or oxidative phosphorylation in the mitochondria to drive the motility (Ford and Rees, 1990). Both plasma and mitochondria membranes are equally prone to damage during cryopreservation (Oberoi et al., 2014). The reduction seen in the motility of post frozen-thawed sperm has been attributed mainly to decreased or impaired mitochondrial function which consequently cut off the energy supply to the flagellum (O'Connell et al., 2002).

In agreement with the previous findings, and regardless of the cryoprotectant, we observed reductions in post-thawed sperm motility compared to the control (unfrozen sperm). The reduction in motility of frozen/thawed spermatozoa was much higher in GLY than in DMA treatments. This remarkable reduction can be attributed to the process of GLY elimination which involves repetitive dilutions and centrifugation. In addition, the reductions in sperm motility in the GLY + sucrose treatment groups were higher than that found in the GLY alone treatments. This can be attributed to excessive dehydration of the sperm cell as mentioned by Froman et al.(1999).

In the current study, the fertility obtained from frozen/thawed spermatozoa in all treatment groups in both experiments was significantly lower than that obtained from fresh sperm. These results agree with previous reports (Santiago-Moreno et al., 2011; Abouelezz et al., 2015; Abouelezz et al., 2017) which indicate a detrimental effect of cryopreservation on sperm fertilizing ability. This reduction in the fertilizing ability of frozen spermatozoa could be attributed to thermal shock during freezing/thawing, increased osmotic pressure on spermatozoa due to exposure to hypertonic and hypotonic environments resulting from ice crystal formation and melting (Pegg, 2002), cellular volumetric changes during dilution (Parks and Graham, 1992), and finally mechanical damages occur during centrifugation to eliminate GLY (Squires et al., 2004; Blanco et al., 2008).

Although decreasing GLY concentration in the cryo-medium was reported to increase the fertilizing capacity of frozen\thawed spermatozoa (Abouelezz et al., 2015b), no significant differences were observed in fertility between 8% and 11% GLY treatments. These results are in agreement with those of Blanch et al. (2014).

The current result that hatchability in both experiments was conditional by the presence of sucrose is interesting. The success, which was seen in the hatchability, without any improvement in the fertility, due to the cryo-diluent inclusion of sucrose may indicate its role in protecting the DNA integrity of the sperm which is necessary to guarantee the delivery of intact DNA to the ovum and consequently pursuing normal embryonic development. In canine, the addition of sucrose (0.25 M), as a non-penetrating cryoprotectant, to human tubular fluid (HTF) and bovine serum albumin (BSA) protected spermatozoa against DNA fragmentation and better-preserved DNA integrity during ultra-rapid freezing than HTF alone (Sánchez et al., 2011). Any defects in the genetic material transported by the sperm to the ova may result in a subsequent embryonic mortality (Twigg et al., 1998). Purdy (2006) reported on the role that sucrose plays in acquiring the spermatozoa a better survivability which may be reflected on the hatchability.

Non-penetrating compounds such as disaccharides are used by some researchers along with penetrating cryoprotectants to aid in sperm cell integrity and improve survivability during freezing/thawing processes. The non-penetrating substances are less toxic than penetrating CPAs (Aisen et al., 2002), and their addition to the cryo-medium enables decreasing permeating CPAs levels without losing their protective effects (Mosca et al., 2016). Non-penetrating CPAs prevent the formation of ice crystals by dehydrating sperm cells and

at the same time regulate the osmotic pressure faced by the cells. In addition, disaccharides such as sucrose and trehalose allow long-term storage at high subzero temperatures (Rosato and Iaffaldano, 2013).

In rabbits, the use of sucrose in bovine serum albumin-based cryo-diluents maintained the fertilizing potential of frozen spermatozoa and resulted in comparable fertility outcomes to that obtained by using fresh semen (Rosato and Iaffaldano, 2013). In birds, the role that disaccharides play in protecting spermatozoa during freezing/thawing processes seems to be species-dependent. Additionally, the concentration of disaccharides in the cryo-diluent can significantly affect their protective efficiency, influencing sperm survival and integrity during cryopreservation. Brown et al. (2018) observed that crane spermatozoa cryopreserved in a cryo-diluent containing Me2SO alone showed higher post-thaw survivability and ability to bind to inner perivitelline membrane than those frozen in cryo-diluent containing Me2SO and 100 mmol sucrose. In chickens, the inclusion of 200 mmol sucrose in ethelyne glycol containing cryo-medium did not improve the motility and viability and significantly decreased curvilinear velocity post-thawed spermatozoa compared to those frozen using cryo-diluent containing ethylene glycol alone (Miranda et al., 2018). On the other hand, turkey spermatozoa cryopreserved using 5% sucrose (146 mmol) + 5% trehalose in combination with DMA had higher post-thaw motility than those frozen using DMA alone (Blanco et al., 2011). Thananurak et al. (2019) reported that low concentrations of sucrose (1 mmol) in the cryo-diluent substantially improved chicken spermatozoa motility, survivability, membrane integrity, acrosome integrity, mitochondrial function and resulted in higher fertility outcomes compared to that obtained when sucrose was added in high concentrations (from 15 to 100 mmol). Thananurak and his colleagues (2019) suggested the beneficial effects of using low concentrations of sucrose could be attributed to the formation of strong covalent bonds between sucrose and sperm membrane molecules that results in coating the sperm surface and improving the membrane stability during freezing and thawing.

Although previous work did not evaluate the effect of using sucrose as a non-penetrating cryoprotectant on the hatchability percentage in chickens, the researchers found that sucrose inclusion in cryo-diluents in combination with penetration cryoprotectants did not give an additional benefit to post-thawed chicken sperm (Miranda et al., 2018) except when added at low inactive osmotic concentrations (1 mmol; Thananurak et al., 2019). On contrary, our results indicate beneficial effects on hatchability when sucrose was present in the cryo-diluent at the level of 146 mmol. The results here also indicate that sucrose helped embryos to hatch when it was added to lower concentrations of DMA. This observation agrees with the findings of Blanco et al. (2011) who reported that sucrose was more beneficial at lower DMA concentrations in cranes.

Experiment 2

Several detrimental consequences occur to avian spermatozoa during the process of freezing/thawing, negatively affecting their viability and fertilizing capacity. These consequences include acrosome detachment (Maeda et al., 1986), sperm head bending at the neck or midpiece region (Maeda et al., 1985), heads lying back along the tail (Maeda et al., 1986), and irregular plasma membrane (Marquez and Ogasawara, 1977; Westfall and Howarth, 1977). These distortions occur due to exposure to hyperosmotic pressure (Bakst, 1980) or cold shock (Bakst and Sexton, 1979). Damage severity was higher in GLY treatments due to the deglycerolization process performed before insemination to avoid its contraceptive effects (Hammerstedt and Graham, 1992).

Moreover, DMA has a lower molecular weight than glycerol, enabling rapid transition through the plasma membrane and

equilibration, which gives DMA an advantage as it induces less cell volumetric change (Gill et al., 1996; Sasaki et al., 2010). Adding sucrose as a non-permeating cryoprotectant with DMA or GLY provided additional protection to frozen/thawed spermatozoa. This protection extended to different parts of the spermatozoon, highlighting the important role sucrose plays in stabilizing cells under osmotic stress (Cleland et al., 2004). Sucrose was also reported to positively affect post-thaw sperm velocity, a crucial trait for fertility (Froman et al., 1999).

Different types of permeating and non-permeating cryoprotectants have varying effects on damage rates across different parts of frozen-thawed spermatozoa. GLY treatments showed more cryodamage than DMA in the acrosome, nucleus, and midpiece regions. Additionally, sucrose was effective in protecting the acrosome region against cryoinjuries, though its protective effect was less pronounced in the nucleus and midpiece. Sucrose also mitigated damage severity in the tail region of spermatozoa.

Each part of the sperm has distinct functions crucial for the fertilization process. The acrosome facilitates penetration of the outer vitelline membrane as the first step in fertilization (Asano and Tajima, 2017), while the nucleus contains dense chromatin carrying genetic information (Pesch et al., 2006). Mitochondria in the midpiece synthesize ATP essential for sperm motility (Froman and Feltmann, 1998), contributing to higher sperm survivability (Rovan, 2001). Lastly, the flagellum guides sperm to the oviduct for fertilization (Asano and Tajima, 2017), underscoring that damage to any of these parts can impair fertilizing capacity.

From observations of spermatozoa frozen using DMA or GLY with or without 5% sucrose (Figures 2 to 13), it can be concluded that the presence of sucrose in the cryo-medium decreases detrimental consequences of the freezing-thawing process, especially when GLY is used as a cryoprotectant. The non-permeating cryoprotectant sucrose allows for additional sperm cell dehydration, reducing levels of permeable cryoprotectants and their toxic effects (Sánchez et al., 2011), and aids in cell membrane stabilization (Koshimoto and Mazur, 2002). For instance, using 0.25 mol sucrose with 1% bovine serum albumin in ultra-rapid freezing of canine sperm achieved approximately 50% motility and 70% viability, with reduced DNA damage, compared to approximately 5% and 35%, respectively, without sucrose and bovine serum albumin (Sánchez et al., 2011).

Sperm DNA quality is critical for correct genetic transmission between generations (Sánchez et al., 2011) and is linked to mutagenic events (Moreno et al., 2004). Sperm with damaged genetic material can still fertilize ova, potentially causing mutations and developmental defects (Twigg et al., 1998).

Overall, cryopreservation of fowl spermatozoa results in low fertility rates deemed unacceptable for commercial use. This reduction is largely attributed to altered membrane structure and function during cooling, freezing, and thawing (Parks and Graham, 1992). During these processes, reordering of membrane lipids disrupts lipid-lipid and lipid-protein interactions essential for normal membrane function (Parks and Graham, 1992), leading to reduced sperm motility and fertilizing capacity. Controlled freezing procedures typically achieve no more than 50% motility post-thawing (Watson, 2000; Vishwanath, 2003), with fertility reduced approximately sevenfold (Sullivan, 2004).

CONCLUSION

Our results can be summarized as follows;

1- Post-thaw sperm motility, fertility, and hatchability are reduced compared to fresh sperm, regardless of the cryoprotectant employed.

2- Glycerol levels do not affect post-thaw sperm motility, fertility, or hatchability.

3- The presence of sucrose improves hatchability percentages significantly, especially in GLY treatments, without affecting sperm motility.

4- Freezing/thawing causes varying degrees of damage along the spermatozoon. GLY induces more cryodamage than DMA.
5- Sucrose provides wide protection to the acrosome and some benefits to the tail region but does not protect the nucleus and midpiece.

It can be concluded that, optimization of cryoprotectant types and conditions, including the addition of osmoregulators like sucrose, is critical for minimizing sperm damage and preserving fertility during cryopreservation.

Table (1): Effect of glycerol level with and without sucrose on sperm motility, fertility, and hatchability %

Items	Experimental groups					Sig.
	Control	8 % GLY	11 % GLY	8 % GLY+ 5 % sucrose	11 % GLY+ 5 % sucrose	
Number of eggs	30	62	59	57	53	
Number of fertile eggs	23	14	14	13	15	
Number of hatched chicks	21	0	0	4	5	
Motility%	95 ^{a2}	20^{b}	20^{b}	10 ^{c}	9 ^{c}	***
Fertility%	76.7 ^{a}	22.6^{b}	23.7^{b}	22.8^{b}	28.3^{b}	*
Scientific hatchability%	91.3 ^{a}	0 ^{c}	0 ^{c}	30.7 ^{b}	33.3 ^{b}	**
Commercial hatchability %	70.0 ^{a}	0 ^{c}	0 ^{c}	9 ^{b}	13.5 ^{b}	**

$^{a\text{-}b \text{ and } c}$ means in the same row having different letters are significantly at $P \leq 0.05$.
* Significant at ($P \leq 0.05$); ** Significant at ($P \leq 0.01$); *** Significant at ($P \leq 0.001$)

Table (2): Effect of dimethylacetamide (DMA) levels with and without sucrose on sperm motility, fertility, and hatchability %

	Control	5 % DMA	7% DMA	5 % DMA+ 5 % sucrose	7% DMA+ 5 % sucrose	Sig.
Number of eggs	29	56	61	84	55	
Number of fertile eggs	21	4	19	15	10	
Number of hatched chicks	10	0	0	4	0	
Motility%	95^{a2}	40^{b}	48^{b}	35^{b}	40^{b}	***
Fertility%	72.4^{a}	7.1 ^{d}	31.14^{b}	17.9 ^{c}	18.2 ^{c}	*
Scientific hatchability%	47.6 ^{a}	0 ^{c}	0 ^{c}	26.6 ^{b}	0 ^{c}	**
Commercial hatchability %	34.5 ^{a}	0 ^{c}	0 ^{c}	4.76 ^{b}	0 ^{c}	**

$^{a\text{-}b \text{ and } c}$ means in the same row having different letters are significantly at $P \leq 0.05$.
* Significant at ($P \leq 0.05$); ** Significant at ($P \leq 0.01$); *** Significant at ($P \leq 0.001$)

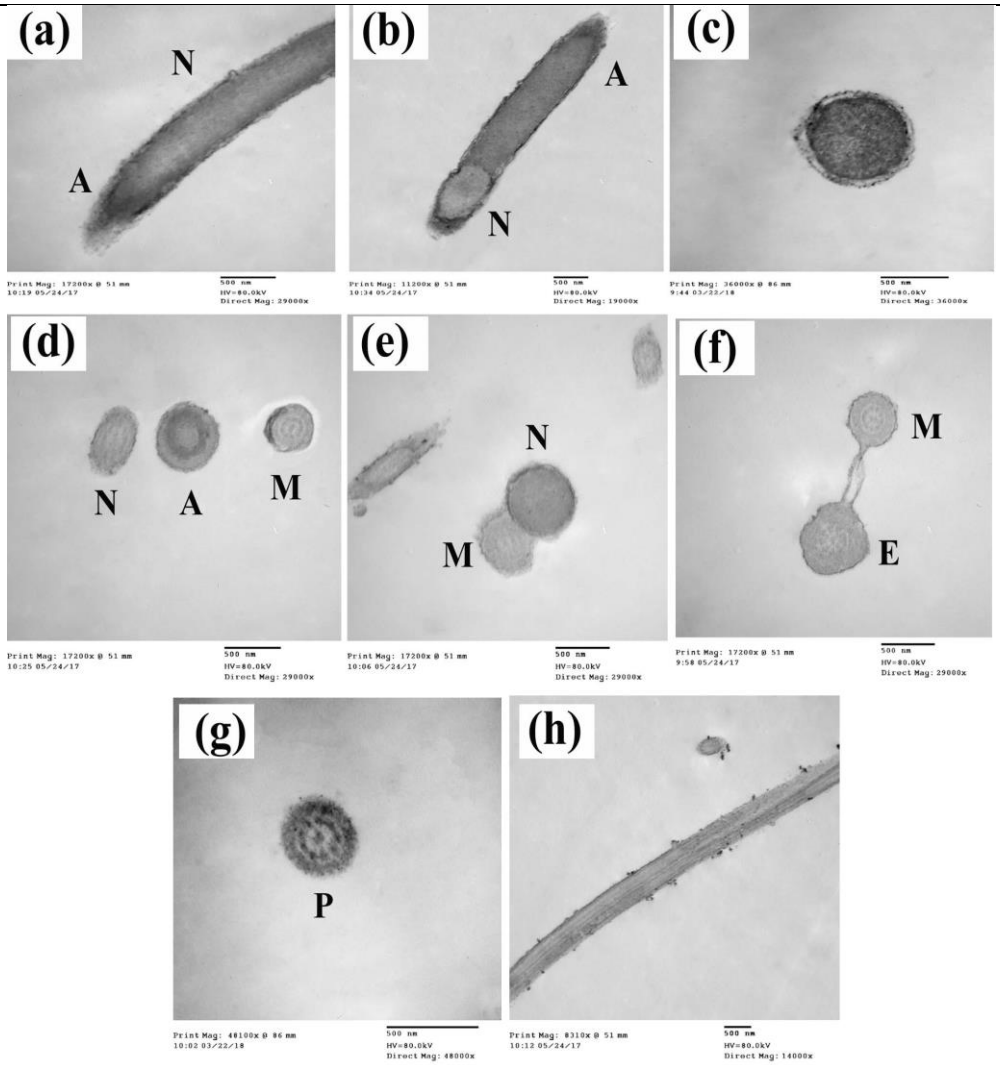

Figure (1): Transmission electron micrographs (TEM) of fresh spermatozoa. micrograph (a and b): longitudinal sections of the sperm head x (17200 and 11200, respictivly) ,(c) a cross section of nucleus (x 36000) , (d) cross sections in nucleus, acrosome and midpiece (x 17200) , (e) cross sections in nucleus and midpiece (x 17200) ,(f) cross sections in midpiece and endpiece (x 17200) , (g) a cross section in principal piece (x 17200) , (h) a longitudinal section of tail (x 8310).
-* A: Acrosome, M: midpiece, N: nucleus, E: endpiece, P:Principle piece.

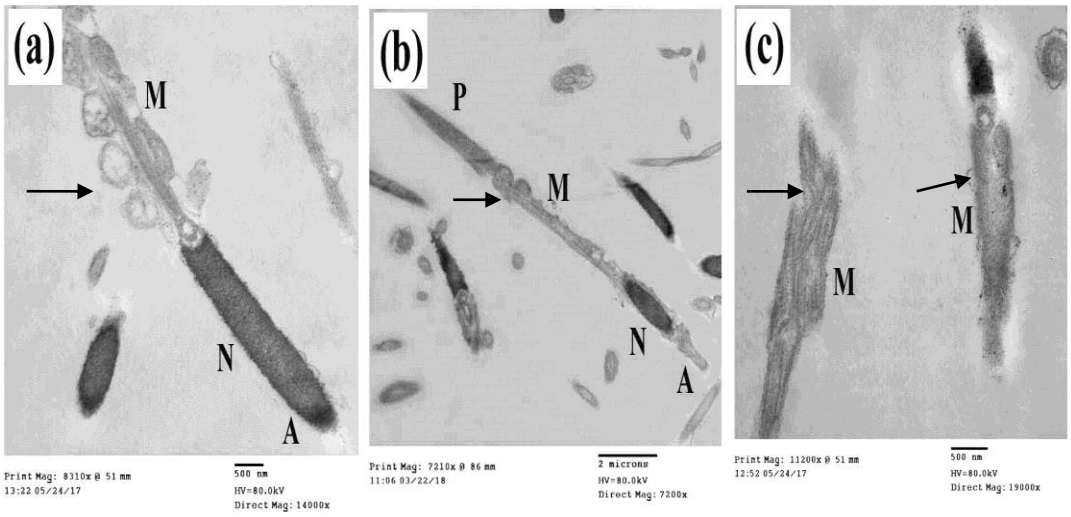

Figure (2): Transmission electron micrographs (TEM) of spermatozoa frozen using 5 % DMA.
Micrograph (a and b) show longitudinal sections in acrosome, nucleus, midpiece and principal piece, mitochondria were abnormally swollen and the midpiece area lacks plasma membrane (→)(x 8310) and x 7210, respictivly), (c) two longitudinal sections shows absence of plasma membrane around the midpiece (→) (x 11200).
-* A: Acrosome, M: midpiece, N: nucleus.

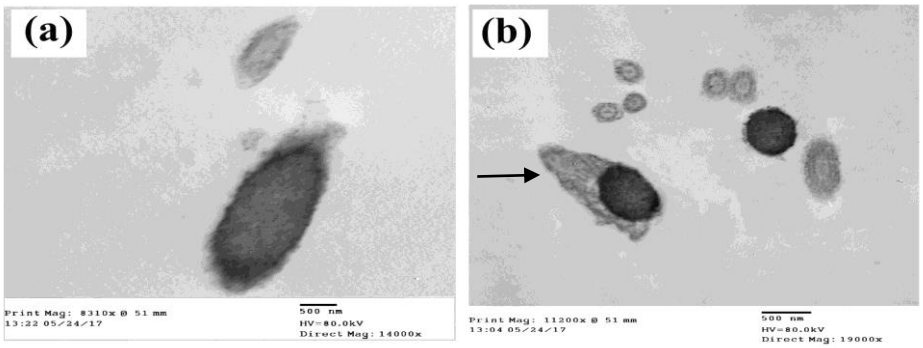

Figure (3): Transmission electron micrographs (TEM) of spermatozoa frozen using 5 % DMA.
Micrograph (a) shows a cross section in a nucleus with an interrupted nuclear membrane (x14000), (b) cross sections of two nuclei, one exhibiting cytoplasm leakage (→) (x11200).

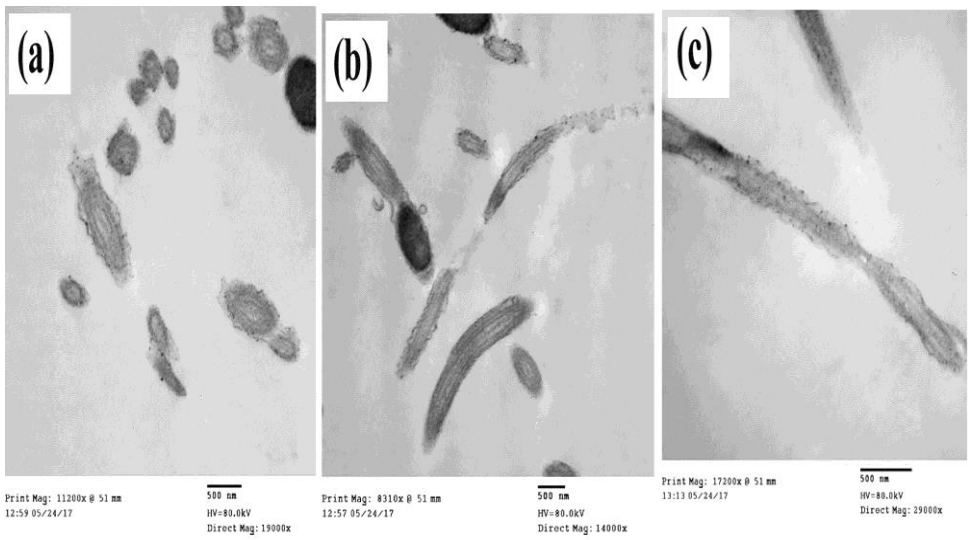

Figure (4): Transmission electron micrographs (TEM) of spermatozoa frozen using 5 % DMA.
Micrograph (a) cross sections in tails with ruffled plasma membrane (x11200), (b) longitudinal sections of principal pieces with a completely broken tail (x3810), (c) a longitudinal section in partially torn tail (x17200).

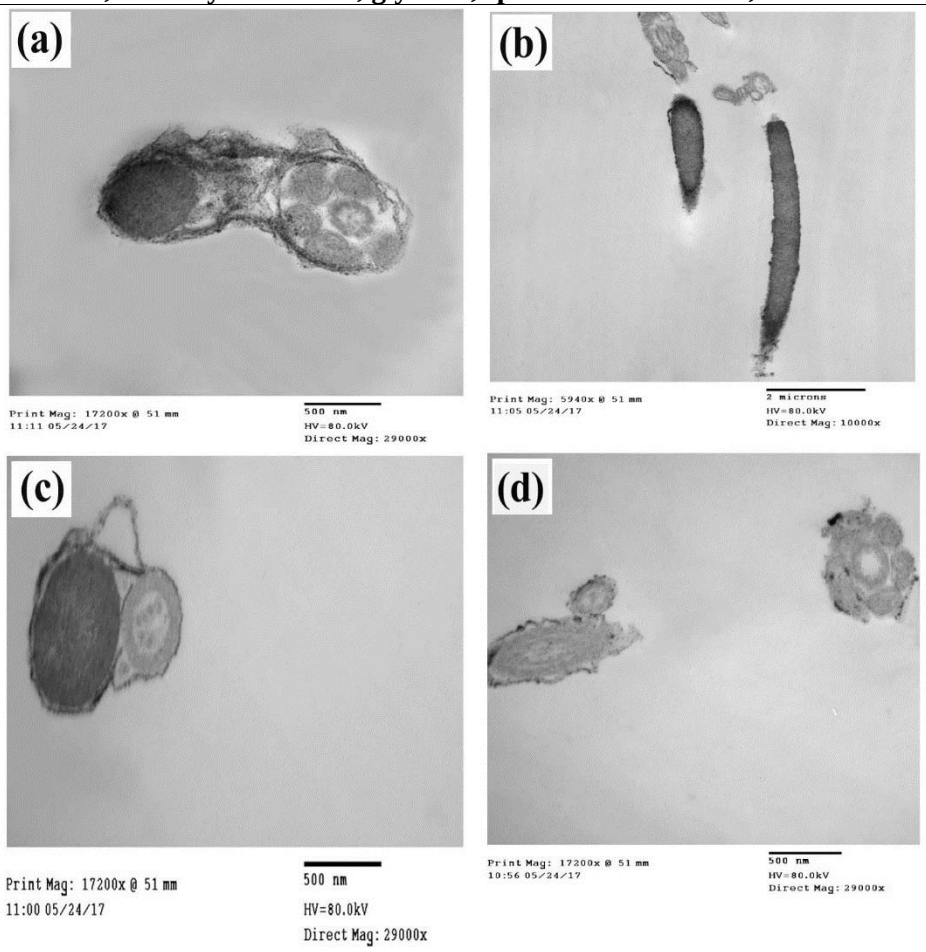

Figure (5): Transmission electron micrographs (TEM) of spermatozoa frozen using 5 % DMA with 5 % sucrose.
Micrograph (a) shows a cross section in a midpiece with swollen mitochondria and distended nucleus (x 17200), (b) longitudinal sections of sperm heads with a breakage at the region between nucleus and distorted midpiece (x 5940), (c) a cross section in bent sperm with intact plasma membrane (x 17200), (d) a cross section in principal piece with ruffled plasma membrane (the left section) and a midpiece containing swollen mitochondria with ruptured plasmalemma (the right section) (x 17200).

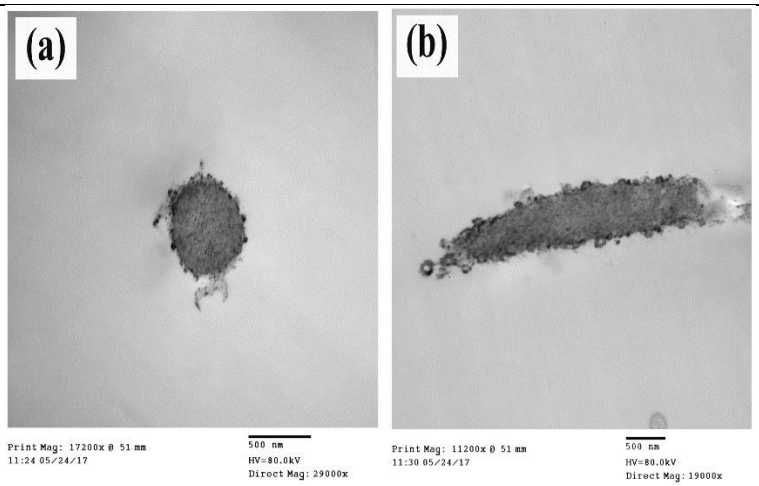

Figure (6): Transmission electron micrographs (TEM) of spermatozoa frozen using 5 % DMA with 5 % sucrose.
Micrographs a and b show cross and longitudinal sections in nucleus, The nucleus material was dense, but with granulated chromatin (picture (a) x17200 and picture (b) x11200, respictivly).

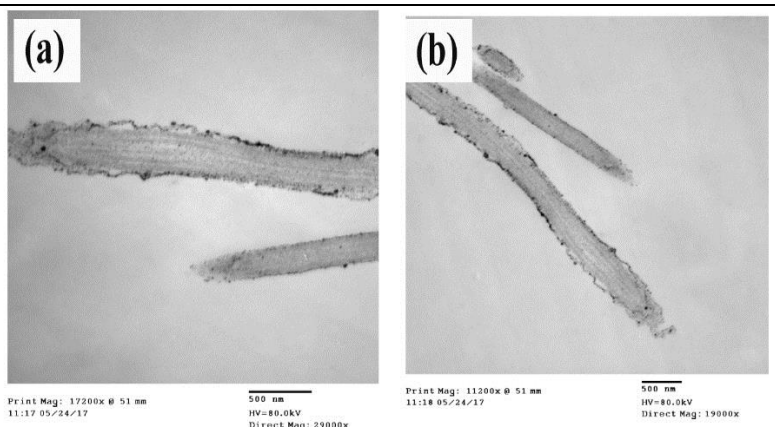

Figure (7): Transmission electron micrographs (TEM) of spermatozoa frozen using 5 % DMA with 5 % sucrose.
Picture (a and b) shows longitudinal sections of ruffled plasma membrane (x17200 and x11200, respectively).

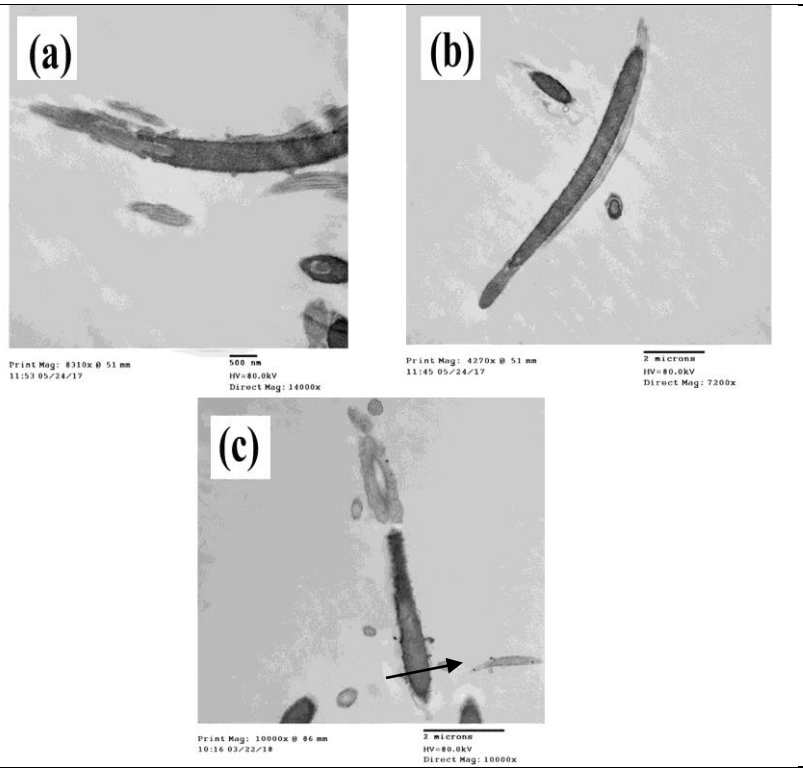

Figure (8): Transmission electron micrographs (TEM) of spermatozoa frozen using 8 % GLY.
Picture (a) a partial tear in acrosome region (x8310), (b) a bent spermatozoon and a cross section of a nucleus with a distended plasmalemma (x4270), (c) a longitudinal section in a spermatozoon with a fraction between nucleus and the midpiece (→) with swollen mitochondria (x10000).

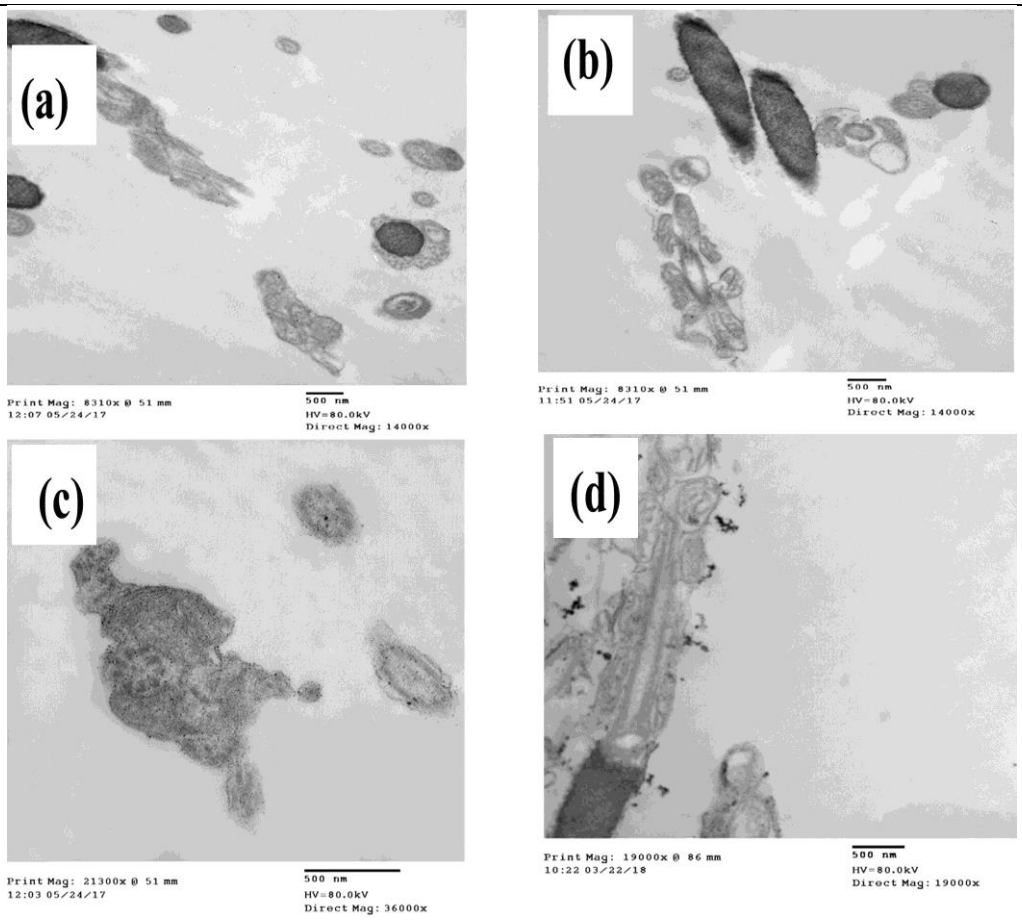

Figure (9): Transmission electron micrographs (TEM) of spermatozoa frozen using 8 % GLY.
Microghraph (a and b): cross sections in nucleus, midpiece and end piece of spermatozoa. The midpiece was characterized by the absences of plasmalemma, swollen and displaced mitochondria. The nucleus with distended plasmalemma. Micrograph C shows a number of irregular agglutinated distended endpieces. Micrograph (D) shows a longitudinal section of damaged midpiece.

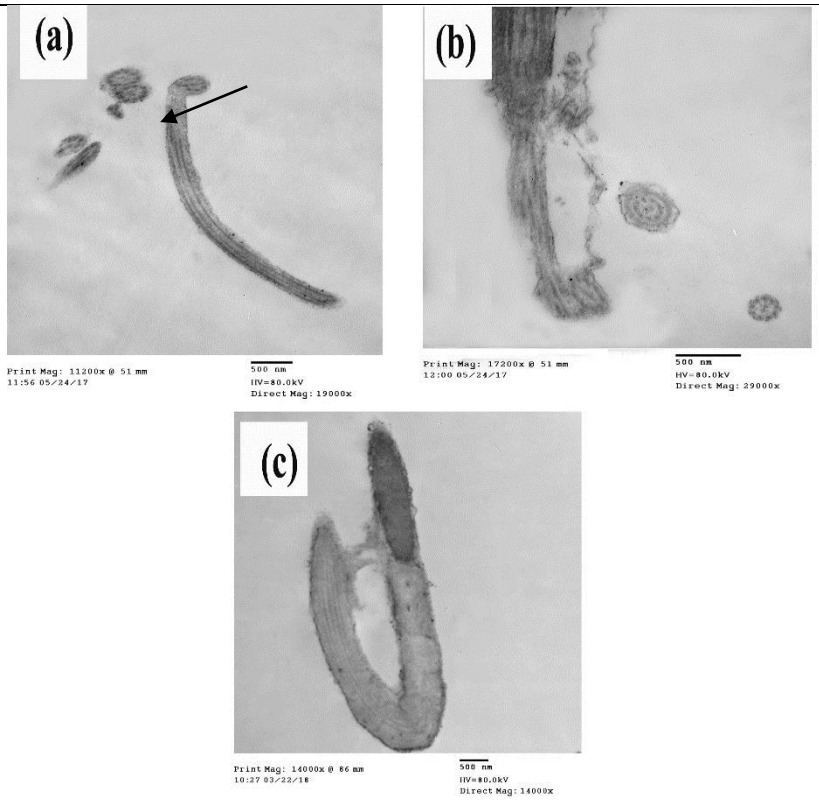

Figure (10): Transmission electron micrographs (TEM) of spermatozoa frozen using 8 % GLY.
Picture (a) a cross section of the tail with ruffle and distended plasmalemma, furthermore a longitudinal section in bent spermatozoon (→) with intact tail (x 11200), (b) a longitudinal section of damaged tail with ruppted plasma membrane (x 17200), (c) a longitudinal section of a bent spermatozoon with a coiled tail (x 14000).

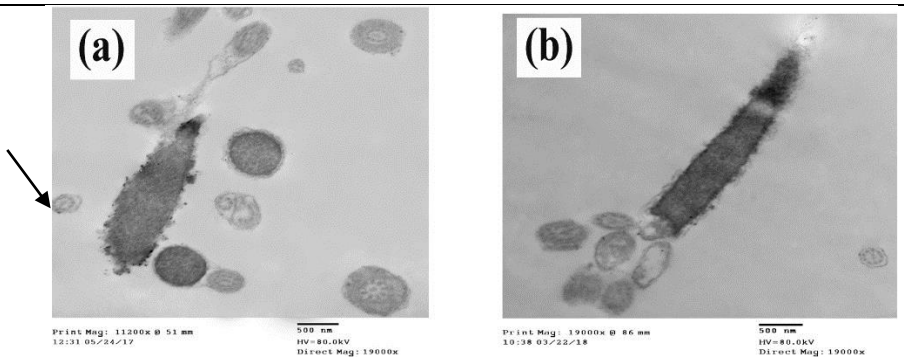

Figure (11). Transmission electron micrographs (TEM) of spermatozoa frozen using 8 % GLY with 5 % sucrose.
Picture (a): an irregular nucleus and the nuclear material was granulated (→) (x11200), (b) the midpiece without plasma membrane, swollen and separated mitochondria, the acrosome without any deformity and the nuclear material was dense, homogeneous but had little granulated chromatin.

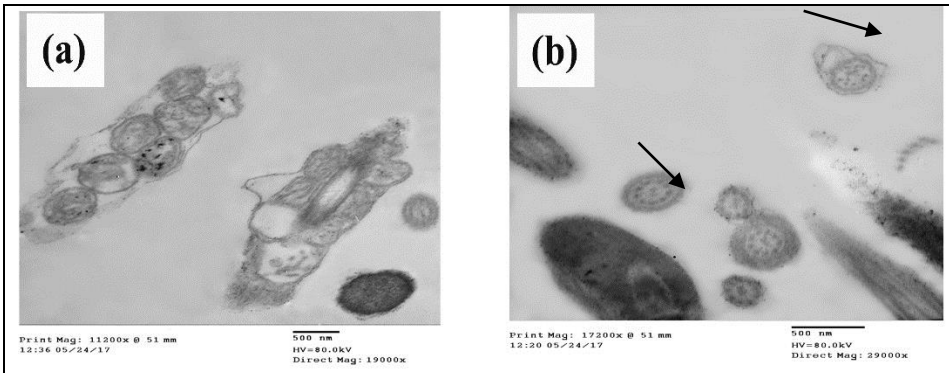

Figure (12): Transmission electron micrographs (TEM) of spermatozoa frozen using 8 % GLY with 5 % sucrose.

Micrograph (a) longitudinal sections of two midpiece with distended plasmalemma and swollen mitochondria and a cross section of a nucleus with a dense nuclear material (x 19000), (b) cross sections of intact and inflated principal piece and nucleus (→) (x 17200).

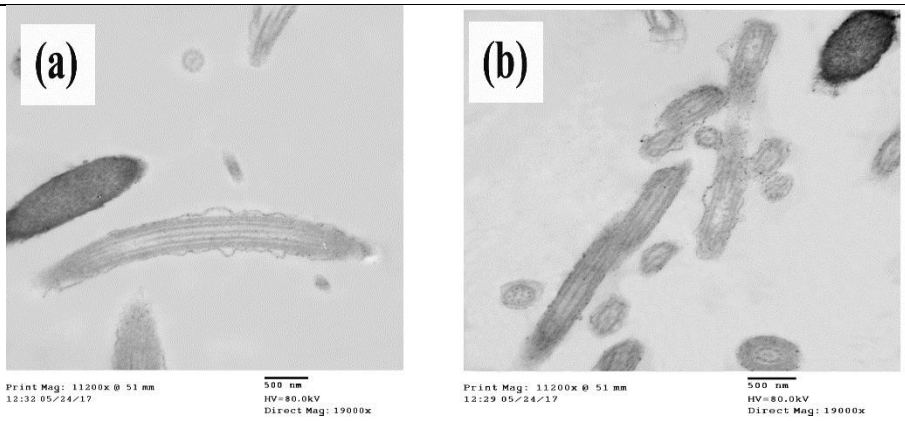

Figure (13): Transmission electron micrographs (TEM) of spermatozoa frozen using 8 % GLY with 5 % sucrose.
Micrograph (a and b): longitudinal sections in tails with puffy and continuous plasma membranes (x 11200).

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الملخص العربي

تحسين تقنيات الحفظ بالتجميد للحيوانات المنوية لديوك الدندراوي: تأثيرات المواد الواقية من أضرار التجميد والسكروز على الحركة، والخصوبة، والتركيب الدقيق للحيوانات المنوية

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يعد الحفظ بالتجميد أمرًا محوريًا للحفاظ على التنوع الجيني ومنع انقراض الأنواع. لذا تهدف هذه الدراسة إلى تحسين تقنيات الحفظ بالتجميد للحيوانات المنوية لديوك الدندراوي من خلال تجربتين.

في التجربة الأولى استخدمت فيها المستويات المثلى لاثنين من المواد الواقية من أضرار التجميد، مع أو بدون اضافة السكروز، في محاولتين تجريبيتين. تم تجميع السائل المنوي من ٢٠ ديك و تم تخفيفه وتقسيمه إلى خمس معاملات. في المحاولة التجريبية الاولى، بقيت قسامة واحدة طازجة (مجموعة مقارنة)، في حين تم تجميد القسامات الاخرى في ماصات التجميد باستخدام الجلسرين (GLY) بتركيزات ٨٪ و١١٪، مع أو بدون سكروز ٥٪. في المحاولة التجريبية الثانية، تم استخدام معاملة واحدة بدون اضافات كمجموعة مقارنة، بينما تم تجميد القسامات الأخرى في كريات باستخدام مادة مخفف تجميد يحتوي على ٥% أو ٧% ثنائي ميثيل أسيتاميد (DMA)، مع أو بدون سكروز ٥٪. تم تلقيح الدجاجات الدندراوي (العدد = ١٠ لكل مجموعة) بحيوانات منوية طازجة (مجموعة مقارنة) أو مجمدة ومذابة، وتم تكرار كل محاولة ستة مرات.

في التجربة الثانية، تم تقسيم السائل المنوي المجمع أيضا إلى خمسة معاملات. كانت المعاملة الأولى بمثابة الكنترول (مجموعة مقارنة)، في حين تم تجميد المعاملتين ٢ و٣ في كريات باستخدام مخفف التجميد يحتوي على ٥% من DMA، مع وبدون ٥% سكروز، على التوالي. تم تجميد القسامتين ٤ و٥ في ماصات التجميد باستخدام مخفف التجميد يحتوي على ٨% GLY، مع وبدون ٥% سكروز، على التوالي. تم استخدام المجهر الإلكتروني للإرسال (TEM) لتقييم الحيوانات المنوية من حيث الضرر بالتبريد.

لم يؤثر مستوى GLY بشكل كبير على الحركة أو الخصوبة أو الفقس، لكن السكروز حسّن بشكل ملحوظ نسبة الفقس مع استخدام ٨٪ و ١١٪ GLY. لم يؤثر DMA بشكل كبير على الحركة، على الرغم من أن ٧% DMA أظهر أعلى خصوبة. يعزز السكروز قابلية الفقس مع ٥% DMA ولكن ليس مع ٧% DMA. كشف تحليل TEM عن مستويات أعلى من تلف الجسيم الطرفي (الأكروسوم) في الحيوانات المنوية المجمدة باستخدام GLY مقارنةً بـ DMA، مع تخفيف السكروز باستمرار لإصابات الجسيم الطرفي. ولوحظ ضرر معتدل في منطقة الذيل، مع الحفاظ على النواة والقطعة الوسطى.

في الختام، فإن اختيار طريقة التجميد وإدراج السكروز أثر بشكل كبير على نتائج تجميد الحيوانات المنوية، مما يدل على دورها الحاسم في الحفاظ على جودة الحيوانات المنوية. وكان السكروز فعالا بشكل خاص في جميع العلاجات، ولا سيما في حماية الأكروسوم.

الكلمات المفتاحية: الدجاج الدندراوي، ثنائي ميثيل الاسيتاميد، الجليسرول، التركيب الدقيق للحيوانات المنوية، السكريات الثنائية