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# Influence of Egg Yolk Substitution with Nano-L-α phosphatidylcholine on Sperm Kinetics, Lipid Peroxidation, and Antioxidant Activity of Cryopreserved Buffalo Bull Spermatozoa

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## ABSTRACT

This study aimed to examine how varying concentrations (0.5%, 1.0%, 2.0%, 3.0%, and 4.0%) of nano-L-α phosphatidylcholine (nano-L-α-PC) affected the quality of post-thawed buffalo bull semen compared to the same concentrations of L-α-phosphatidylcholine (L-α-PC), while 20% egg yolk (EY) was used as a control. Ejaculates (n = 72) were collected from fertile buffalo bulls (n = 12) for 6 weeks. The ejaculates were pooled and divided into 11 groups (3 aliquots per group) extended with 20% EY (Group 1 without supplement), 0.5%, 1.0%, 2.0%, 3.0%, and 4.0% nano-L-α-PC (Groups 2–6), and 0.5%, 1.0%, 2.0%, 3.0%, and 4.0% L-α-PC (Groups 7–11) in tris buffer. After cryopreservation procedures, semen samples were thawed at 37 °C and then evaluated for sperm kinematics, acrosomes, plasma membranes, and DNA integrities. The seminal plasma was analyzed for malondialdehyde (MDA), superoxide dismutase (SOD), glutathione reduced (GSH), and catalase (CAT). Results demonstrated that extended semen samples containing 1.0-3.0% nano-L-α-PC and 1.0-4.0% L-α-PC showed high total motility compared to control, whereas at 0.5–4.0% of nano-L-α-PC and L-α-PC showed high progressive motility. Significant (P< 0.05) wobble levels were seen at 0.5, 1.0, and 3.0% nano-L-α-PC, and distance average path and velocity average path at 2.0% L-α-PC. Acrosome and plasma membrane integrities were markedly elevated (P< 0.0001) at 2.0% nano-L-α-PC and 4.0% L-α-PC. High DNA integrity metrics were noticed at 0.5–1.0% nano-L-α-PC, as well as 3.0% and 4% L-α-PC. Low MDA and high (SOD, GSH, and CAT) levels were observed at 2.0% nano-L-α-PC and 4.0% L-α-PC. In conclusion, the optimum concentration of nano-L-α-PC that improved semen quality was 2.0%, which is roughly equivalent to the effect of 4.0% L-α-PC.

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## INTRODUCTION

Cryopreserved semen is the main cornerstone in semen processing and artificial insemination in animals **(Ros-Santaella and Pintus, 2021)**. However, the cryopreservation process causes thermal, osmotic, and mechanical disturbances in sperm quality and fertility **(Singh *et al.*, 2018)**. Buffalo spermatozoa plasma membrane is characterized by high levels of polyunsaturated phospholipids and low cholesterol levels **(Ivanova *et al.*, 2019).** Such composition makes sperm cells more vulnerable to sub-lethal damage as well as oxidative stress during the freezing-thawing process **(Kumaresan *et al.*, 2006)**. As such, injury caused by the cryopreservation process can be

minimized by improving cryoprotectants in semen extenders **(Holt, 2000)**. This can be achieved by implementing non-penetrating cryoprotectants like egg yolk (EY) and soybean lecithin (SL) or penetrating cryoprotectants like glycerol, dimethyl sulfoxide, and ethylene glycol in buffalo semen extenders **(Almadaly *et al*., 2019; Rawash *et al*., 2020)**.

Egg yolk has the ability to protect spermatozoa against cold shock owing to its contents of phospholipids **(Toker *et al*., 2016)**. Nevertheless, there is a high risk of microbial contamination, which can lead to endotoxin production **(Althouse, 2008),** as well as the presence of steroid hormones that can suppress sperm metabolism and respiration and reduce sperm motility **(Lipar *et al*., 1999; Ugur *et al*., 2019)**. Concurrently, this could decrease fertility and increase the danger of disease transmission **(Beccaglia *et al*., 2009)**. Accordingly, many efforts have been made to optimize the extender by replacing egg yolk with a cryoprotectant of plant origin to get the superlative quality of frozen-thawed semen.

Soybean lecithin (SL) shares egg yolk with the active cryoprotectant components phosphatidylcholine (PC) and phospholipids. **(Oke *et al*., 2010)**. However, soybean lecithin can protect the integrity of the phospholipid membrane during the cryopreservation process, with the advantage of a low contamination risk caused by the use of cryoprotectants from animal sources such as egg yolk and milk **(Nadri *et al*., 2019)**. Recently, several studies have explored the positive effect of soybean lecithin (SL) on semen cryopreservation in buffalo bulls **(Akhter *et al*., 2010)**, bulls **(Gonzales *et al*., 2003)**, rams **(Forouzanfar *et al*., 2010),** and bucks **(Roof *et al*., 2012)**.

Substitution of egg yolk with cryoprotectants from plant origin in nanoparticle form is considered a new preferred strategy for sperm cryopreservation **(Hashem and Gonzalez-Bulnes, 2020)**.

Nanotechnology reveals new prospects for establishing innovative approaches for sperm handling and improving the outcomes of artificial insemination technology **(Feugang *et al*., 2019).** Nanoparticles can augment semen quality and freezability and shield spermatozoa from cryo-injury **(Khalil *et al*., 2019)**. Nano-soya lecithin is hypothesized to have a higher solubilizing capacity and smaller particle sizes, resulting in a super protective effect of spermatozoa and lessening cold shock damage during freezing-thawing processes of buck **(Nadri *et al*., 2019)** and rooster **(Sun *et al*., 2021)** semen. To our knowledge, no accessible research employed nano-L-α-phosphatidylcholine (nano-L-α-PC) in buffalo bull sperm cryopreservation. Therefore, this study aimed to explore the effects of nano-L-α-phosphatidylcholine (nano-L-α-PC) as a cryoprotectant on the quality and antioxidant properties of frozen-thawed buffalo bull semen in comparison to L-α-phosphatidylcholine (L-α-PC) and egg yolk.

## MATERIALS AND METHODS

This investigation was carried out following the values outlined and approved by the Faculty of Veterinary Medicine ethics committee, Benha University, Egypt (BUFVTM 04–02-23).

### 1. Nanoparticles preparation

The dried thin film of soybean lecithin (L-α-Phosphatidylcholine (product number: KJ512547)) purchased from Karlsruhe, Germany, was hydrated with the addition of 10 mL of sterile phosphate-buffered solution (×PBS) (pH = 7.4) and then vortexed by an XH-D Vortex mixer (Wincom Company, Ltd., China) for 1 hour to obtain a milky aqueous suspension. The coarse emulsion was homogenized by sonication (S 4000 Misonix sonicator, QSONICA, Newtown, CA, USA) at 60% amplitude in an ice bath to prevent heating for 30 min (5-sec sonication and 10-sec rest intermittently to allow cooling of the sample) **(Mousavi *et al*., 2019)**. The mean diameter of the particle sizes was characterized by a zeta analyzer (Microtrac MRB's NANOTRAC Wave II/Q/Zeta) manufactured by Verder Scientific. Ltd. (UK), which is based on dynamic light scattering (DLS).

### 2. Animals' selection and semen collection

Twelve sexually mature, proven fertile Egyptian buffalo bulls aged 5–6 years were used for semen collection (n = 72 ejaculates) during the period from January to March 2023, where one ejaculate was collected per bull for 6 weeks using a bovine artificial vagina (AV) (Neustadt/Aisch, Müller, Nürnberg, Germany). Bulls were healthy and reared on the farm of the Animal Reproduction Research Institute (ARRI) under optimum nourishment, lighting, and housing systems. Before semen collection, each bull was exposed to teasing by false mounting to gain a high-quality semen sample **(Şahin *et al*., 2020)**.

### 3. Semen extension, freezing and thawing

A pilot study was conducted before the experiments, trying different doses from 1 to 8% to determine the suitable range of doses adopted. We transported the semen samples to the laboratory immediately after collection to select the highest quality ejaculates before semen processing. Ejaculates possessing > 70% sperm motility, viability greater than 75%, and a concentration > $800\times10^6$ sperm/mL were pooled. Semen samples were primarily diluted at 30 °C with tris-buffer: tris (375 mM), citric acid (124 mM), glucose (41.6 mM), and glycerol (7%) (Sigma-Aldrich) in 100 mL of deionized water (pH 6.8) **(Shokry *et al*.,**

**2024)**. The pooled semen samples were equally divided into 33 aliquots (n = 3 groups) and diluted with 20% Tris-Egg yolk (EY) extender (1st group), 0.5, 1.0%, 2.0%, 3.0%, and 4.0% nano-L-α-phosphatidylcholine (nano-L-α-PC; 2nd-6th group), and 0.5, 1.0%, 2.0%, 3.0% and 4.0% L-α-phosphatidylcholine (L-α-PC; 7th-11th group) in Tris-buffer to obtain ($80 \times 10^6$ sperm/mL). After that, the extended semen samples were slowly cooled to 5 °C in a cold cabinet (IMV Co., France) within 2 hrs. The cooled semen samples were packed into 0.25 mL labeled polyvinyl straws (IMV Co., France) ($20 \times 10^6$ sperm/straw) for identification and equilibrated at 5 °C for 4 hrs. On a cooled rack the straws were ordered horizontally 6 cm above liquid nitrogen vapors inside a foam box for 15 min. **(Khalifa, 2001)**. Finally, the straws were plunged into LN2 tanks at -196 °C and stored at least for 24 hours until analysis.

## 4. Evaluation of sperm quality

### 4.1. Assessment of sperm kinematics

Sperm kinematics (Wobble (WOB), distance path average (DAP), velocity average path (VAP), straightness (STR), and distance curved line (DCL), distance straight line (DSL), straight liner velocity (SLV), curved liner velocity (CLV), linearity (LIN), and beat cross frequency (BCF) were assessed by means of a computer-assisted sperm analysis system (CASA; CEROS II, version 1.10; Hamilton Thorn Beverly). Directly after thawing in warm water (37 °C for 45 Sec), approximately 7 µL of frozen-thawed semen was mounted on a clean, pre-warmed CASA slide with 20 µm depth, and 200 sperms were evaluated for motion characteristics (**Naz *et al*., 2018)**.

### 4.2. Assessment of acrosomal membrane integrity (AMI)

The acrosome integrity of buffalo bull spermatozoa was evaluated after thawing by using a silver nitrate stain, according to **Chinoy *et al.*, (1992)**. Semen films were spread over glass slides and dried out at room temperature, then fixed with 70% ethyl alcohol for 2 min and 95% ethyl alcohol for another 2 min. In an incubator at 65 °C and complete humidity, the preparations were stained with silver nitrate for 2 hours. The chemical reaction was stopped after the color of the preparation turned into gold. Immediately, the slides were washed with distilled water many times. The slides were then dried out at room temperature. A total of 200 spermatozoa were evaluated under a light microscope (100 × magnification) for acrosomal membrane integrity.

### 4.3. Assessment of plasma membrane integrity (PMI)

Plasma membrane integrity of buffalo bull spermatozoa was evaluated by incubating 50 µL of frozen-thawed semen sample with 500µL hypo-osmotic swelling solution (HOS, consisting of 0.735 g sodium citrate and 1.35 g fructose in 100 mL of distilled water, osmolality, 190 mOsmol/kg) for 30 min **(Revell and Mrode, 1994)**. On a pre-warmed, non-greasy glass slide, a drop of incubated semen was placed and covered with a cover slip. A total of 200 spermatozoa were evaluated under a phase contrast microscope (Olympus Bx40, Japan) at (400 × magnification) for intact plasma membrane (coiling in the tail region) and damaged plasma membrane (no coiling in the tail region).

### 4.4. Assessment of sperm DNA injury

In a warm water bath (37 °C), six straws per treatment were thawed and centrifuged at 1500 rpm for 15 min, and the sediment was collected to evaluate the integrity of DNA by the COMET assay **Boe-Hansen *et al.*, (2005)**. 500 µL of agarose (1%) at its normal melting point were placed on a frosted slide at 45 °C and covered with a cover slip. Following electrophoresis, the slide settled onto a tray that had been pre-cooled. Subsequently, they were dried at 5 °C overnight, hydrated again with distilled water for ten min, dyed with acridine orange (stain 1%), and viewed under a fluorescence microscope (Olympus, Japan) at 400× magnifying power. An image analysis system (TriTek Comet-Score, shareware version 1.5) was used to evaluate DNA fragmentation based on DNA damage percentage, tail length, tail DNA percentage, and tail moment **(Fraser, 2004)**. The images were categorized depending on the strength of the comet's fluorescence light. Sperm damaged DNA, forming a 'comet' pattern, while intact sperm were without comets.

### 4.5. Biochemical assessment of antioxidant

For each treatment, five straws were thawed at 37 °C, pooled, and centrifuged at 1500 rpm for 15 min, and then the supernatant was collected for the spectrophotometric analysis of malondialdehyde (MDA) level, superoxide dismutase (SOD), glutathione reduced (GSH), and catalase (CAT) enzymatic activity according to **Ognjanović *et al., (*2008); Partyka *et al*., (2012)**; **Campos-Shimada *et al*., (2020)** and **Bendou *et al*., (2021),** respectively, using diagnostic kits (MDA 25 29, SD 25 21, GR 25 11, and CA 25 17) purchased from Bio Diagnostic (Cairo, Egypt) at wavelength 534 nm, 560 nm, 510 nm, and 405 nm, respectively.

## 5. Statistical analysis

According to **Ho (2013)**, the data were checked for normal distribution using the Shapiro-Wilk test. Data were presented as mean ± SEM and analyzed with One-Way ANOVA and Tukey's HSD post-hoc test using IBM® SPSS® Statistics (Version 25). A statistical test resulted in a significant difference at $P< 0.05$.

## RESULTS

### 1. Estimation of L-α-phosphatidylcholine nanoparticles size and charge

The mean particle size of nano-L-α-phosphatidylcholine used in the current research measured by zeta analyzer based on dynamic light scattering is displayed in **Fig.1**, which represents the mean particle size (28.09 nm). The zeta potential was 19.6 mV and negatively charged.

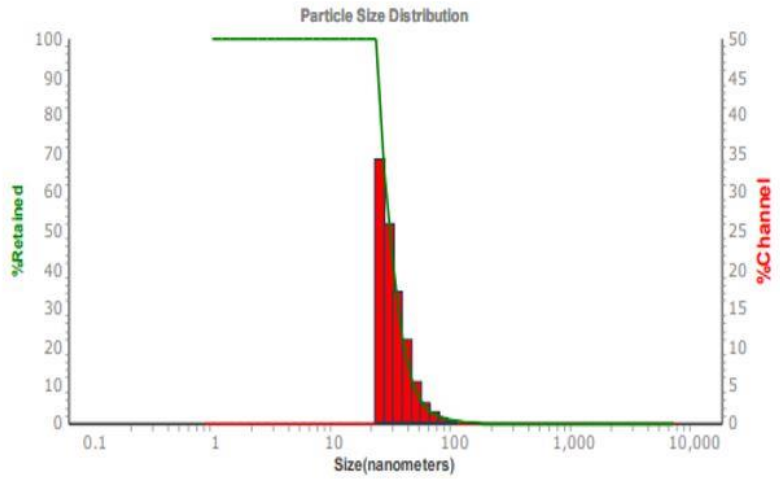


**Fig. 1:** Zeta particle size analysis of nano L-α-phosphatidylcholine based on dynamic light scattering.

### 2. Effect of nano-L-α-phosphatidylcholine and L-α-phosphatidylcholine on spermatozoa kinematics, and acrosomal, and plasma membrane integrities of frozen-thawed buffalo semen

The data in **Table (1)** showed that adding 1-2% nanoparticles L-α-PC as or1-4% L-α-PC to the extender increased total motility compared to egg yolk. At doses of 0.5 to 4.0% nano-L-α-PC and L-α-PC, progressive motility was higher than the egg yolk. Kinematics characteristics showed that 0.5%, 1.0%, and 3.0% nano-L-α-PC improved WOB, while at 2.0% L-α-PC increased DAP and APV compared to egg yolk. Furthermore, 2.0% nano-L-α-PC and 4.0% L-α-PC considerably ($P< 0.0001$) improved post-thawing acrosomal and plasma membrane integrities compared to egg yolk **(Table 2).**

**Table 1:** Effect of nano-L-α-phosphatidylcholine and L-α- phosphatidylcholine on spermatozoa kinematics of frozen-thawed buffalo semen.

| Treatment | Conc. | Total motility(%) | Progressive motility (%) | WOB (%) | DAP (µm) | VAP (µm/s) | STR (%) | DCL (µm) |
|---|---|---|---|---|---|---|---|---|
| Egg yolk | 0 | 46.06 ± 2.08$^{c}$ | 28.13 ± 2.42$^{c}$ | 0.62 ± 0.03$^{c}$ | 15.33 ± 0.41$^{b}$ | 34.94 ± 1.66$^{b}$ | 0.72 ± 0.00 | 25.04 ± 1.46 |
| Nano-L-α-phosphatidyl choline | 0.5 | 60.59 6.38$^{abc}$ | 41.61 ± 0.97$^{b}$ | 0.72 ± 0.02$^{ab}$ | 16.07 ± 0.44$^{ab}$ | 36.46 ± 1.72$^{b}$ | 0.71 ± 0.03 | 22.35 ± 0.37 |
| | 1.0 | 72.06 ± 4.56$^{ab}$ | 55.81 ± 1.28$^{a}$ | 0.70 ±0.01$^{abc}$ | 15.36 ± 0.27$^{b}$ | 35.10 ± 0.47$^{b}$ | 0.71 ± 0.03 | 21.96 ± 0.71 |
| | 2.0 | 68.61 ± 2.4$^{ab}$ | 50.08 ± 2.93$^{a}$ | 0.67 ± 0.01$^{bc}$ | 15.36 ± 0.93$^{b}$ | 36.72 ± 2.24$^{b}$ | 0.67 ± 0.01 | 22.74 ± 1.09 |
| | 3.0 | 63.55 ±4.13$^{abc}$ | 40.71 ± 1.45$^{b}$ | 0.75 ± 0.03$^{a}$ | 14.83 ± 0.29$^{b}$ | 34.38 ± 0.82$^{b}$ | 0.68 ± 0.01 | 21.89 ± 0.32 |
| | 4.0 | 54.55 ± 3.30$^{bc}$ | 35.70 ± 0.34$^{b}$ | 0.62 ± 0.02$^{c}$ | 15.07 ± 1.30$^{b}$ | 38.49 ± 3.34$^{b}$ | 0.64 ± 0.02 | 24.44 ± 2.64 |
| L-α-phosphatid ylcholine | 0.5 | 59.16 ± .42$^{abc}$ | 35.82 ± 0.90$^{b}$ | 0.68 ± 0.01$^{bc}$ | 15.98 ± 0.61$^{ab}$ | 37.22 ± 0.42$^{b}$ | 0.69 ± 0.01 | 23.66 ± 1.24 |
| | 1.0 | 66.55 ± 4.35$^{ab}$ | 39.27 ± 0.86$^{b}$ | 0.69 ± 0.01$^{bc}$ | 15.29 ± 0.12$^{b}$ | 36.63 ± 1.35$^{b}$ | 0.69 ± 0.01 | 22.34 ± 0.43 |
| | 2.0 | 68.50 ± 4.03$^{ab}$ | 41.22 ± 1.09$^{b}$ | 0.69 ± 0.01$^{bc}$ | 18.44 ± 0.98$^{a}$ | 42.06 ± 2.55$^{a}$ | 0.67 ± 0.01 | 26.66 ± 1.19 |
| | 3.0 | 71.63 ± 3.51$^{ab}$ | 50.97 ± 0.36$^{a}$ | 0.63 ± 0.01$^{c}$ | 16.42 ± 0.42$^{ab}$ | 37.02 ± 0.55$^{b}$ | 0.70 ± 0.01 | 26.03 ± 0.37 |
| | 4.0 | 75.49 ± 2.72$^{a}$ | 54.98 ± 0.88$^{a}$ | 0.66 ± 0.02$^{bc}$ | 15.90 ± 0.16$^{ab}$ | 35.38 ± 0.64$^{b}$ | 0.70 ± 0.03 | 24.39 ± 0.84 |
| P value | | 0.0001 | 0.0001 | 0.0001 | 0.02 | 0.03 | 0.29 | 0.06 |

Data were expressed as (Mean ± SEM, n = 6 straws/replicate). WOB, DAP, VAP, STR, and DCL referred to wobble, distance path average, velocity average path, straightness, and distance curved line, respectively. Data with different superscript letters within the same column were significantly different.

**Table 2:** Effect of nano-L-α-phosphatidylcholine and L-α-phosphatidylcholine on spermatozoa kinematics, and acrosomal, and plasma membrane integrities of frozen-thawed buffalo semen.

| Treatment | Concentration | DSL (µm) | SLV (µm/s) | CLV (µm/s) | LIN (%) | BCF (Hz) | Intact acrosome (%) | HOST (%) |
|---|---|---|---|---|---|---|---|---|
| **Egg yolk** | **0** | **11.73 ± 0.44** | **26.80 ± 1.19** | **57.19 ± 3.57** | **0.47 ± 0.01** | **20.73 ± 1.89** | **74.33 ± 1.38$^{c}$** | **37.5 ± 1.54$^{d}$** |
| **Nano-L-α-phosphatidyl-choline** | **0.5** | **11.53 ± 0.87** | **26.33 ± 2.52** | **50.41 ± 1.56** | **0.46 ± 0.01** | **20.04 ± 0.11** | **77.00 ± 1.71$^{bc}$** | **46.67 ± 1.31$^{cd}$** |
| | **1.0** | **10.89 ± 0.30** | **25.05 ± 1.43** | **50.12 ± 0.25** | **0.49 ± 0.03** | **20.72 ± 0.45** | **79.33 ± 2.03$^{abc}$** | **45.33 ± 2.72$^{cd}$** |
| | **2.0** | **10.30 ± 0.70** | **24.71 ± 1.68** | **54.15 ± 2.68** | **0.45 ± 0.01** | **20.21 ± 1.67** | **85.00 ± 1.90$^{ab}$** | **61.33 ± 3.39$^{ab}$** |
| | **3.0** | **10.12 ± 0.11** | **23.37 ± 0.30** | **50.76 ± 0.98** | **0.46 ± 0.00** | **20.30 ± 0.59** | **76.17 ± 1.19$^{c}$** | **43.67 ± 2.99$^{cd}$** |
| | **4.0** | **10.83 ± 0.52** | **24.80 ± 1.82** | **51.09 ± 0.20** | **0.44 ± 0.03** | **20.62 ± 1.15** | **76.17 ± 2.50$^{c}$** | **38.83 ± 2.81$^{cd}$** |
| **L-α-phosphatidyl choline** | **0.5** | **11.14 ± 0.29** | **25.90 ± 0.25** | **54.66 ± 1.43** | **0.47 ± 0.01** | **20.75 ± 1.46** | **78.17 ± 1.28$^{bc}$** | **45.67 ± 3.08$^{cd}$** |
| | **1.0** | **10.53 ± 0.21** | **25.55 ± 1.26** | **53.14 ± 2.14** | **0.48 ± 0.00** | **17.61 ± 2.08** | **79.33 ± 1.71$^{abc}$** | **41.67 ± 3.03$^{cd}$** |
| | **2.0** | **12.42 ± 0.67** | **27.73 ± 1.50** | **59.17 ± 2.56** | **0.46 ± 0.01** | **19.69 ± 0.79** | **79.83 ± 1.89$^{abc}$** | **50.33 ± 3.19$^{bc}$** |
| | **3.0** | **11.50 ± 0.41** | **26.00 ± 0.84** | **58.70 ± 1.55** | **0.44 ± 0.01** | **21.20 ± 1.30** | **82.67 ± 2.12$^{abc}$** | **50.00 ± 3.58$^{bcd}$** |
| | **4.0** | **11.26 ± 0.53** | **25.11 ± 1.47** | **54.39 ± 2.45** | **0.45 ± 0.01** | **22.01 ± 0.38** | **87.50 ± 1.23$^{a}$** | **64.67 ± 1.99$^{a}$** |
| **P value** | | **0.08** | **0.75** | **0.08** | **0.21** | **0.54** | **0.0001** | 0.0001 |

Data were expressed as (Mean ± SEM, n = 6 straws/replicate). DSL, SLV, CLV, LIN, BCF, HOS referred to distance straight line, straight liner velocity, curved liner velocity, linearity, beat cross frequency, and hypo-osmotic swelling test, respectively. Data with different superscript letters within the same column were significantly different.

### 3. Effect of nano-L-α-phosphatidylcholine and L-α-phosphatidylcholine on DNA integrity of frozen-thawed buffalo spermatozoa

The result of the comet assay of frozen-thawed buffalo semen demonstrated that 1.0% nano-L-α-PC reduced the damage in DNA, tail length, and tail moment compared to the egg yolk. Nano-L-α-PC reduced tail moment at concentrations (0.5, 1.0, 3.0, and 4.0%). Supplementation with L-α-PC markedly reduced DNA damage, tail length, and tail moment ($P< 0.0001$) at concentrations of 3.0% and 4.0%, as well as DNA in the tail at concentrations of 2.0% and 3.0% compared to egg yolk **(Table 3).**

**Table 3:** Effect of nano-L-α-phosphatidylcholine and L-α-phosphatidylcholine on DNA integrity of frozen-thawed buffalo spermatozoa.

| Treatment | Concentration | DNA damage (%) | Tail length (µm) | DNA in tail (%) | Tail moment |
|---|---|---|---|---|---|
| **Egg yolk** | 0 | **19.32 ± 0.66$^{a}$** | **16.62 ± 0.92$^{a}$** | **12.40 ± 1.40$^{a}$** | **2.08 ± 0.17$^{a}$** |
| | 0.5 | **12.91 ± 0.81$^{bcd}$** | **12.99 ± 0.53$^{abc}$** | **8.98 ± 0.43$^{a}$** | **1.16 ± 0.07$^{b}$** |
| **Nano-L-α-** | 1.0 | **10.05 ± 0.44$^{cd}$** | **6.90 ± 0.49$^{d}$** | **10.72 ± 1.41$^{a}$** | **0.74 ± 0.10$^{b}$** |
| **phosphatidyl** | 2.0 | **16.12 ± 1.17$^{abc}$** | **13.56 ± 0.77$^{ab}$** | **10.25 ± 0.70$^{a}$** | **1.36 ± 0.03$^{ab}$** |
| **choline** | 3.0 | **15.42 ± 0.50$^{abc}$** | **12.89 ± 0.25$^{abc}$** | **9.33 ± 1.25$^{a}$** | **1.21 ± 0.18$^{b}$** |
| | 4.0 | **18.25 ± 0.63$^{ab}$** | **11.80 ± 0.47$^{ab}$** | **10.23 ± 0.84$^{a}$** | **1.18 ± 0.05$^{b}$** |
| | 0.5 | **14.08 ± 1.78$^{abcd}$** | **14.08 ± 0.57$^{ab}$** | **10.93 ± 1.32$^{a}$** | **1.36 ± 0.06$^{ab}$** |
| **L-α-** | 1.0 | **11.51 ± 0.25$^{cd}$** | **14.14 ± 0.08$^{ab}$** | **9.20 ± 0.69$^{a}$** | **1.35 ± 0.09$^{ab}$** |
| **phosphatidyl** | 2.0 | **11.88 ± 0.57$^{cd}$** | **13.19 ± 0.65$^{ab}$** | **8.02 ± 0.44$^{b}$** | **1.06 ± 0.09$^{b}$** |
| **choline** | 3.0 | **11.45 ± 0.76$^{cd}$** | **10.37 ± 0.34$^{bcd}$** | **7.96 ± 0.35$^{b}$** | **0.82 ± 0.03$^{b}$** |
| | 4.0 | **8.97 ± 0.82$^{cd}$** | **8.28 ± 0.72$^{cd}$** | **9.01 ± 0.66$^{a}$** | **0.78 ± 0.10$^{b}$** |
| **P value** | | **0.0001** | **0.0001** | **0.04** | **0.0001** |

Data was expressed as (Mean ± SEM, n = 6 straws/replicate). Data with different superscript letters within the same column were significantly different.

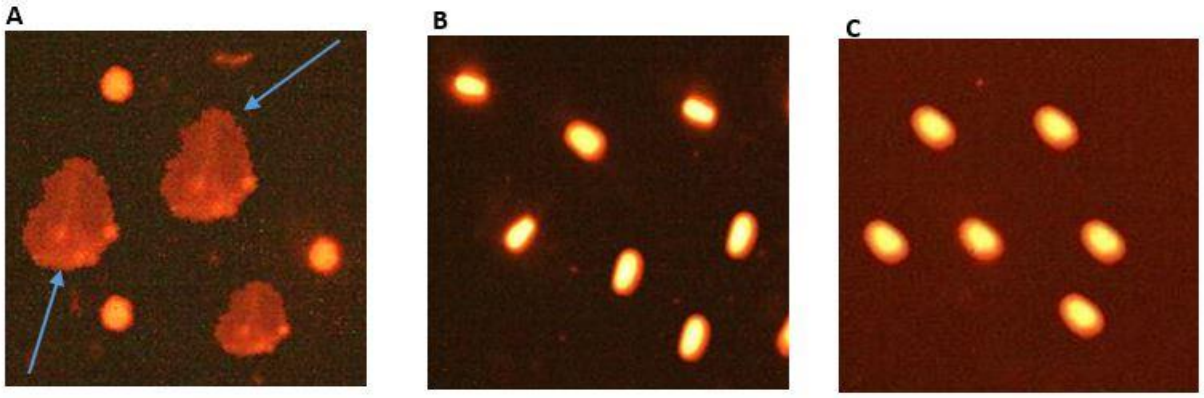


**Fig. 2:** Representative microscopic images of comet assay of frozen-thawed buffalo bull semen extended with Tri's egg yolk (A), 1% nano-L-α-phosphatidylcholine-Tris (B), and 4% L-α-phosphatidylcholine-Tris (C) extenders. The blue arrows indicated spermatozoa with damaged DNA (comet).

**4. Effect of nano-L-α-phosphatidylcholine and L-α-phosphatidylcholine on oxidative stress biomarkers in frozen-thawed buffalo semen.**

Supplementation with nano-L-α-PC at concentrations of 0.5, 2.0%, and L-α-PC at 4.0% significantly (P< 0.0001) lowered MDA levels. Supplementing nano-L-α-PC at 2.0-4.0% and L-α-PC at 1.0 and 4.0% boosted SOD activity markedly (P< 0.0001). Implementing a buffalo bull semen extender with 2.0% nano-L-α-PC and 4.0% L-α-PC led to increased GSH and CAT activities **(Table 4).**

**Table 4**: Effect of different concentrations of nano-L-α phosphatidylcholine and L-α phosphatidylcholine on oxidative stress biomarkers in frozen-thawed buffalo semen.

| Treatment | Concentration | MDA (nmol/mL) | SOD (U/mL) | GSH (U/L) | CAT (U/L) |
|---|---|---|---|---|---|
| Egg yolk | 0 | 12.94 ±1.20$^{a}$ | 17.44 ± 0.91$^{d}$ | 3.11 ± 0.20$^{c}$ | 1.85 ±0.08$^{b}$ |
| Nano-L-α- | 0.5 | 8.43 ± 0.30$^{bc}$ | 21.41 ± 2.18$^{cd}$ | 3.61 ± 0.32$^{c}$ | 2.35 ±0.39$^{b}$ |
| phosphatidyl | 1.0 | 10.82 ±1.33$^{abc}$ | 24.50 ± 1.45$^{bcd}$ | 4.77 ± 0.49$^{bc}$ | 1.91 ±0.15$^{b}$ |
| choline | 2.0 | 7.32 ± 0.78$^{c}$ | 30.85 ± 2.08$^{ab}$ | 6.97 ± 0.60$^{ab}$ | 4.61 ±0.27$^{a}$ |
| | 3.0 | 11.62 ± 0.94$^{ab}$ | 25.75 ± 1.51$^{abc}$ | 4.32 ± 0.41$^{c}$ | 2.32 ±0.44$^{b}$ |
| | 4.0 | 10.31 ± .00$^{abc}$ | 25.87 ± 1.59$^{abc}$ | 4.82 ± 0.40$^{bc}$ | 2.76 ±0.40$^{b}$ |
| L-α- | 0.5 | 10.71 ±0.27$^{abc}$ | 21.69 ±1.20$^{cd}$ | 4.15 ± 0.72$^{c}$ | 2.29 ±0.24$^{b}$ |
| phosphatidyl | 1.0 | 11.09 ±0.43$^{abc}$ | 26.69 ±1.13$^{abc}$ | 3.80 ± 0.10$^{c}$ | 2.15 ± .18$^{b}$ |
| choline | 2.0 | 10.33 ±0.76$^{abc}$ | 23.15 ±1.26$^{cd}$ | 3.81 ± 0.34$^{c}$ | 2.25 ±0.20$^{b}$ |
| | 3.0 | 10.16 ±0.37$^{abc}$ | 22.60 ± 2.00$^{cd}$ | 4.71 ± 0.26$^{bc}$ | 2.78 ±0.15$^{b}$ |
| | 4.0 | 8.21 ± 0.61$^{bc}$ | 32.0 ± 1.41$^{a}$ | 7.32 ± 0.80$^{a}$ | 4.48 ±0.18$^{a}$ |
| P value | | 0.0001 | 0.0001 | 0.0001 | 0.0001 |

Data was expressed as (Mean ± SEM, n = 5 straws/replicate). Data with different superscript letters within the same column showed significant differences.

## DISCUSSION

Cryopreservation of semen reduces sperm quality due to physical or chemical injury **(Wang *et al*., 2020)**. In the majority of animal species, egg yolk is the most commonly employed cryoprotectant **(Gamal *et al*., 2016)**. Egg yolk shields spermatozoa from temperature shock, but it faces various criticisms, mostly due to variable composition, high-density lipoproteins (HDLs), egg yolk granules, and hygienic problems, i.e., the potential of bacterial contamination **(Ansari *et al*., 2010)**. Phosphatidylcholine (PC) is a main component of soy lecithin, a plant-derived cryoprotectant with the capacity to overcome the aforementioned limitations of egg yolk **(Sun *et al*., 2021)**.

According to our study, adding 2% nano-L-α-phosphatidyl choline (nano-L-α-PC) or 4.0% L-α-phosphatidylcholine (L-α-PC) improved sperm quality comparable to egg yolk (EY). **Nadri *et al*., (2019)** showed that fortification of 2.0% soybean lecithin nanoparticles into buck extenders improved sperm quality, which is also consistent with our findings. However, previous sperm cryopreservation studies in other species found that the best concentration ranged from 1.0% in rams **(Forouzanfar *et al*., 2010)**, 1.5% in goats **(Taşdemir *et al*., 2013)**, 0.8% in dogs **(Kmenta *et al*., 2011)**, and 6.0% in boars **(Wojtusik *et al*., 2018)**. These species differences related to variations in the composition of sperm plasma membrane and seminal plasma proteins, particularly in bovine **(Manjunath *et al*., 2002)**, may accuse the variant amount of phosphatidylcholine necessarily incorporated in the extender **(Bencharif *et al*., 2008)**.

The computer-assisted sperm analysis (CASA) is a comprehensive and effective technique used to

evaluate sperm quality in many mammalian' species **(Zakošek Pipan *et al*., 2020)**. Sperm total motility and progressive motility are the key elements that determine the fertility potential of spermatozoa **(Sun *et al.*, 2021)**. Our study revealed that, for both nano-L-α-PC and L-α-PC, the best concentration that enhanced total and progressive motility was 1% nano-L-α-PC and 4% L-α-PC when compared with egg yolk, same as **Sun *et al*. (2021)** results in rooster and **Nowier and Saad (2022)** in bovine, respectively, when compared with 20% egg yolk extender. Semen extended with 0.5, 1.0, or 3.0% nano-L-α-PC showed higher WOB, and these data are consistent with data reported by **Nadri *et al*. (2019).** In extenders containing 2.0% L-α-PC, the values of DAP and APV, which have a good association with sperm progression, were higher. This could be because phosphatidylcholine promotes ATP generation **(Adami *et al*., 2020)** and reduces oxidative stress **(Motlagh *et al*., 2014)**, accordingly improving the motion characteristics. However, **Mafolo *et al*., (2020)** found that ram semen extended with the EY extender had greater sperm motility. This is perhaps due to high dead sperms and reactive oxygen species release after semen cryopreservation **(Peris-Frau *et al*., 2020),** which increases in buffalo-bulls due to low temperature intolerance associated with high levels of polyunsaturated phospholipids and low cholesterol contents **(Ivanova *et al.*, 2019).**

Semen quality is linked to both acrosome and plasma membrane stability. Acrosomal integrity impacts sperm penetration and fusion and affects the fertility of frozen semen **(Castiglione Morelli *et al*., 2021)**. Based on our results, 2.0% nano-L-α-PC and 4.0% L-α-PC are highly effective in protecting acrosomes from freezing-thawing damage as compared with EY. In the meantime, **Nadri *et al*. (2019)** in caprine and **Nowier and Saad (2022)** in bovine studies reported similar results. The protective effect of the PC is excreted through prevention of the premature acrosomal reaction **(Simpson *et al*., 1986)**, especially in its nanoform. On the other hand, the previous study showed that bull sperm in EY had better acrosomal integrity compared to SL-based extenders **(Celeghini *et al*., 2008)**. Nevertheless, **Singh *et al*. (2018)** and **Mafolo *et al*., (2020)** observed no significant difference in acrosome integrity between the PC liposome and the EY extender in buffalo bull and ram, respectively.

Plasma membrane integrity is critical in regulating sperm activity directly associated with fertility, which is altered by the chemical content or temperature variations of the extender **(Najafi *et al*., 2017)**. In the present study, nano-L-α-PC and L-α-PC significantly ameliorated plasma membrane damage as compared with EY. These results are similar to those of **Akhter *et al*. (2011)** in buffalo bull and **Nadri *et al*. (2019)** in goat**.** It was thought that nano-L-α-PC preserves the stability of plasma membranes by reducing the loss of phospholipids, changing the membrane's composition, or modifying permeability to water and cryoprotective substances **(Nadri *et al*., 2019)**. **Mafolo *et al*., (2020)** recorded the failure of the phosphatidylcholine liposome to protect the sperm membrane. On the other side, **Masoudi *et al*., (2016)** found no significant difference in membrane integrity between the PC of SL and EY after cryopreservation of ram semen.

Spermatozoa DNA integrity is crucial in the development of embryos reliant on the existence of normal DNA **(Chelucci *et al*., 2015)**. Our study recorded an obvious improvement in most parameters of DNA integrity when semen extender was supplemented with nano L-α-PC at doses of 0.5 and 1.0% and L-α-PC, particularly at doses of 3.0% and 4.0%. These results are similar to those of buck **(Chelucci *et al*., 2015)** with a concentration of 2.5% and stallion **(Aboelmaaty *et al*., 2023)** at a dose of 1.0%. The successful maintenance of DNA integrity might be attributed to lower lipid peroxide generation and better membrane integrity **(Aboelmaaty *et al*., 2023).** Another study **(Kadirvel *et al*., 2009)** reported no effect of the SL-based extender on buffalo bull sperm chromatin. However, certain studies in Simmental bulls **(Celeghini *et al*., 2008)** and in cattle bulls **(Waterhouse *et al*., 2010)** suggested that the extender can affect DNA integrity during cryopreservation.

Spermatozoa are highly susceptible to oxidative stress, which can permanently damage their lipids, proteins, and DNA **(Abdel-Khalek *et al.*, 2022).** A study in ram verified that the semen cryopreservation creates significant levels of reactive oxygen species, which may compromise sperm shape and function **(Masoudi *et al*., 2016)**. The current data showed a marked decrease in MDA level and increased SOD, GSH and CAT activities in 2.0% nano-L-α-PC and 4.0% L-α-PC groups compared to the egg yolk-Tris group. This is consistent with the findings in Ram (Motlagh ***et al*., 2014)** and Rooster **(Sun *et al*., 2021). Sun *et al*., (2021)** showed that phosphatidylcholine of soybean lecithin has antioxidant properties, which improve sperm viability and motility as well as reduce reactive oxygen species production after freezing and thawing, besides it alleviates the efflux of phospholipids or cholesterol, therefore decreasing the intracellular ice crystal formation and prevent cryo-damage of spermatozoa.

## CONCLUSIONS

Our outcomes demonstrated that supplementation of cryo-diluent media with 2% nano-

L-α-PC or 4% L-α-PC provided excellent cryoprotective and antioxidant properties for buffalo bull spermatozoa. Meaningfully, 2% nano-L-α-PC or 4% L-α-PC enhanced post-thawing motility, acrosomal integrity, plasma membrane intactness, and DNA integrity, which was accomplished by lowering lipid peroxidation and high antioxidant activity.

## Author contribution

All authors contributed to the conception and design of the study. Material preparation, data collection and analysis were performed by [Asmaa Salah Ghania], [Alaa Elsayed Abdel-Ghaffar], [Gamal Abdel-raheem Mohamed Sosa], [Magdy Ramadan Badr], [Mohsen Abd Elhafeez Agag], [ Mohamed Mahmoud M. Kandiel], [Abdelaziz Mostafa Sakr], and [Laila Esmaail Kortam]. The first draft of the manuscript was written by [Asmaa Salah Ghania], and [Mohamed Mahmoud M. Kandiel] and all authors read and approved the final manuscript.

## Data availability

All data used in this study is included in this published article.

## Declarations Ethical approval

This experiment was approved and carried out following the ethical guidelines of the Animal Care and Use Committee of the Faculty of Veterinary Medicine, Benha University, Egypt.

## Informed consent

Not applicable as our study did not involve humans.

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None

## Declaration of interest

All authors declared that there is no conflict of interest among them concerning this manuscript.

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