Effect Of The Addition Of Natural And Lyophilized Hen's Egg Yolk, Egg Yolk Plasma And LDL To Semen Extender On The Freezability And DNA Integrity Of Arab Stallion Spermatozoa

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ABSTRACT

The present study aimed to investigate the cryoprotective capacities of natural and lyophilized forms of whole egg yolk, egg plasma and low-density lipoprotein (LDL) on Arab stallion spermatozoa. Semen samples were collected from 5 Arabian stallions and diluted with INRA-82 extender containing natural egg yolk (15%), lyophilized egg yolk (15%), natural egg plasma (5, 7.5, 10 and 15%), lypholized egg plasma (10%), natural LDL (5, 7.5, 10 and 15%) and lypholized LDL (7.5%). Semen aliquots were processesed for cryopreservation and sperm pos-thaw motility, membrane, acrosome, mitochondria and DNA integrities were recorded. The results showed that, 10% egg plasma and 7.5% LDL were superior to other tested concentrations. Natural forms of LDL and egg plasma improved quality of frozen-thawed stallion spermatozoa in terms of pos-thaw motility, viability index, membrane, acrosome, mitochondria and DNA integrities. Using the lyophilized forms of LDL and egg plasma in stallion semen extender resulted in the worst semen quality endpoints, while, using the lyophilized egg yolk resulted in satisfactory results. In conclusion, the addition of the natural forms of LDL and egg plasma as well as lyophilized egg yolk to semen extender could be good alternatives to the whole egg yolk as they improves stallion sperm quality after cryopreservation in terms of motility, integrities of mitochondria, membrane and acrosome as well as reduction of DNA damage.

Key words: Stallion-semen-cryopreservation-LDL-egg plasma-lyophilized-egg yolk

INTRODUCTION

Cryopreservation and subsequent thawing places several stressors on sperm cells; the most detrimental of these include ice crystal formation, osmotic changes, and temperature shock *(1)*. Sperm of different species tolerate the cryopreservation process differently,for example, stallion sperm do not tolerate the freezing process as well as bovine or human sperm *(2)*. Therefore, optimization of cryopreservation protocols for stallion sperm is an active field of research.

Hen egg yolk has been routinely used with success in freezing extenders for semen of many domestic animals including horses *(3)* in a concentrations ranging from 2% to 22% *(4-7)*. In recent years, arguments concerning the presence of cryoprotective antagonists in egg yolk had reinforced interest in the use of only the LDL extracted from egg yolk in the extenders, rather than complete egg yolk. Egg yolk contains substances that impede respiration of spermatozoa which may lead to decrease their motility *(8-11)*. In addition, egg yolk increases the risk of microbial contamination *(12)* which may lead to endotoxin production that can reduce the potential fertilizing capacity of spermatozoa, and increase the risk of disease transmission through the transportation of egg yolk-based extenders in the international exchange of stored semen *(13)*.

In semen extenders, LDL had been used to replace egg yolk because of its better sperm

preservation property *(8, 14)*. Simply adding clarified egg yolk plasma also has been shown to improve stallion sperm freezability *(15)*. It was hypothesized that the use of powdered egg yolk compared to natural egg yolk in an extender for the cryopreservation of ram *(16)* and Zebu bull *(17)* semen, may improve post-thaw semen quality.

Egg yolk is normally used as a protective agent to freeze semen of stallions. However, addition of egg yolk in extenders is not without disadvantages and the demand to find cryoprotective alternatives is strong. So, the objective of this study was to test the cryoprotective capacities of natural and lyophilized forms of whole egg yolk, egg plasma and LDL.

MATERIALS AND METHODS

Extraction of egg plasma from egg yolk

Extraction of LDL was done according to the method of ***Pillet et al. (15)***. Hen's eggs were thoroughly cleaned and rinsed with distilled water. The egg shells were cracked then the yolk and albumen were separated. The viteline membrane was perforated, the egg yolk was aspirated, diluted 1:2 (v:v) in 0.17 M sodium chloride solution, homogenized with a magnetic stirrer for 1 h at 4°C. The homogenate was then centrifuged (10000 Xg for 45min at 4°C). The supernatant was carefully collected with a glass pipette to avoid any contamination by the pellet (granules) then transferred into a new centrifuge tube for a second centrifugation, under the same conditions mentioned above, in order to completely remove granules. Following the second centrifugation the supernatant was considered as egg yolk plasma fraction with a satisfactory degree of purity *(18)*. One portion of natural egg plasma was designated to the lyophilization process.

Extraction of LDL from egg yolk

Extraction of LDL was done according to the method of ***Moussa et al. (8)***. Egg yolk plasma was prepared as mentioned above. Ammonium sulphate solution (40%) was added drop-twice (20 to 30min) to the cooled egg plasma (4°C; *19)*. The mixture was maintained at 4 °C, with continuous stirring, for another hour. The mixture was then centrifuged, under the same conditions mentioned above, and the supernatant dialyzed in cellophane membrane against MilliQ water over night, followed by centrifugation of the dialysis membrane content. The upper floating part containing the LDL was carefully withdrawn from the centrifuge tube, avoiding contamination by the fluid portion located at the bottom of the tube. One portion of natural LDL was designated to the lyophilization process.

Preparation of extenders

Modified INRA-82 extender *(20)* was prepared by mixing equal amounts of ultra-heat treated skim milk and glucose–saline solution (25 g\L glucose monohydrate, 1.5 g\L lactose monohydrate, 1.5 g\L raffinose pentahydrate, 0.4 g\L potassium citrate monohydrate, 0.3 g\L sodium citrate dihydrate, 4.76 g HEPES, 0.035 g SDS, pH 7.0, 500 mg\L penicillin, 500 mg\L gentamycin). Natural egg yolk (15%), lyophilized egg yolk (15%), natural egg plasma (5, 7.5, 10 and 15%), lypholized egg plasma (10%), natural LDL (5, 7.5, 10 and 15%) and lypholized LDL (7.5%) were added separately to the freezing extender. Before adding them to the extender, lyophilized forms of egg yolk, egg plasma and LDL were re-suspended in 0.1 M phosphate buffer pH 7.0 plus Tween 20 (1.0 %).

Animals and semen collection

On a once weekly collection schedule, four ejaculates per stallion were obtained from 5 Arabian stallions (A total of 20 ejaculates), aged 6-11 years, and individually housed at Al-Zahraa horse stud, Cairo, Egypt. The collection was done in the early morning; a mare in estrus was used as a mount animal. Semen was collected using Colorado model artificial vagina with an inline filter to separate the gel fraction.

Semen processing

Immediately following collection, the gel-free portion of the ejaculate was evaluated for volume and progressive motility, and concentration was determined with a hemocytometer. Only ejaculates with at least 60% progressively motile sperm and 250 x 10^6 sperm cells /ml were used for freezing. The semen was extended 1:1 (semen:extender) in INRA-82 extender that had been warmed to 38°C. The diluted samples were placed into 15-mL tubes and centrifuged for 10 minutes at 400 Xg. ***(21)***. At least 95% of the supernatant was removed ***(22)*** and each pellet was diluted with INRA-82 (containing 5% glycerol and 15% egg yolk or its substitutes according to the experimental design) to a final sperm concentration of 100x 10^6 motile sperm/ml. Each aliquot was cooled slowly to 5 °C over one hour under aerobic conditions, and then incubated at 5 °C for 30 min ***(23)***. The extended semen was drawn into 0.5-mL straws, sealed with a sealing powder and placed 4 cm above liquid nitrogen in the vapor phase in foam box for 10 min before being plunged into the liquid phase ***(24)***. The straws were then stored in goblets in canisters and kept immersed in liquid nitrogen. For thawing, two straws per treatment were warmed in a water bath at 38 °C for 30 sec. Individual motility was recorded just after thawing, 1, 2 and 3 hours post-thawing. The post-thawing viability indices were estimated according to ***Milovanov (25)***. Also, acrosomal integrity was estimated using fast green stain ***(26)***

Hypo-osmotic swelling (HOS): The procedure described by ***Nie and Wenzel (27)*** was used to determine the percentage of HOS positive cells in each sample. A 100 µl aliquot of each semen sample was mixed in 1.0 ml of a pre-warmed 100 mOsm sucrose solution (1.712g sucrose dissolved in 50 ml of sterile de-ionized water). The mixture was incubated at 37°C for 60 minutes in a 1.5 ml micro-centrifuge tube. Following incubation, a small drop of sample was placed on a microscope slide and cover-slipped for examination by using phase contrast microscopy (400X) to evaluate 100 spermatozoa for evidence of swelling and curling changes.

MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay

The MTT assay was performed according to the method of ***Mosmann (28)***. For each fresh or frozen-thawed sample, six wells of the 96-well microplate were used. The 100 µl of semen sample plus 10 µl of MTT stock solution (5 mg MTT/ml of PBS) was placed in each well. The rates of MTT reduction were determined using an ELISA reader at a wavelength of 550 nm. The optical density of frozen-thawed semen samples was measured 2 times (immediately and after 1 h of incubation at 37 °C). MTT reduction rates (optical density) for each semen sample was calculated by concurring the difference between the first and second reading of the ELISA reader. The viability of spermatozoa (Y) could be calculated by knowing the MTT reduction rate (X) by using a regression equation; Y= 251.65X-18.751 ***(29)***.

Comet (Single cell gel electrophoresis assay): The alkaline comet assay for spermatozoa was carried out according to ***Hughes et al. (30)***. Fully frosted glass slides were covered with 100 µl of 0.5% normal melting point agarose (Sigma), a coverslip was added and the agarose was allowed to solidify. The coverslips were removed and 1x10^5 sperm cells in 50 µl PBS (7.2 pH) were mixed with 50 µl of 1.2% low melting point agarose and used to form the second layer. The slides with coverslips removed, were then placed in lysis buffer for 1 h (2.5 M NaCl, 100 mM Na EDTA, 10 mM Tris, 1% Triton X at a pH of 10). The slides were incubated at 37°C in 100 µl/ml of proteinase K in lysis buffer overnight. After draining the proteinase K solution from the slides, they were placed in a horizontal electrophoresis unit filled with freshly prepared alkaline electrophoresis solution containing 300 mM NaOH and 1 mM EDTA for 20 min to allow the DNA to denature. Electrophoresis was performed at room temperature, at 25 V (0.714 V/cm) and 300 mA, obtained by adjusting the buffer level, for 10 min. Then the slides were washed with a neutralizing solution

of 0.4 M Tris at pH 7 to remove alkali and detergents. After neutralization, the slides were each stained with 50 µl of 20 µg/ml ethidium bromide and mounted with a coverslip. A total of 200 sperm cells were examined under fluorescent microscope (400X). The intensity of the stain in the comet tail region is presumed to be related to the DNA content, and DNA damage is estimated from measurements of the percent DNA in tail, tail length and tail moment, using an image analysis system (Comet-Score program). Spermatozoa with fragmented DNA (damaged, Fig. 1) display increased migration of the DNA from the nucleus towards the anode, while spermatozoa with non-fragmented DNA (undamaged) do not form a "comet" ***(31)***.

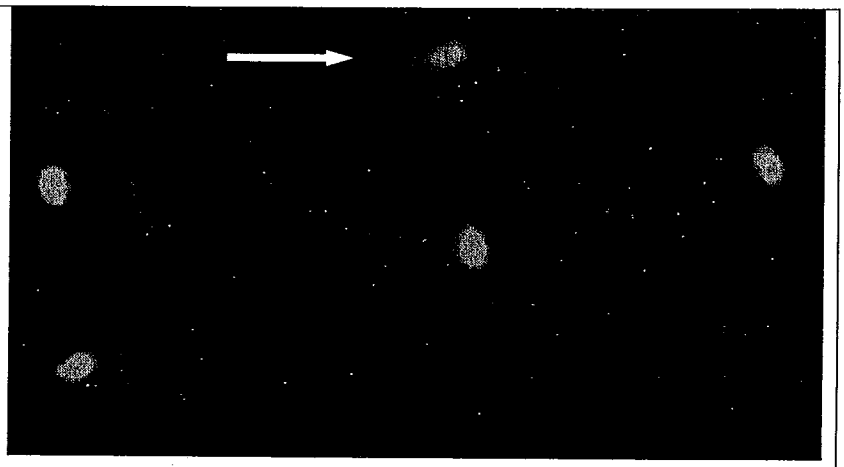

Fig. 1. Comet picture of stallion spermatozoa, frozen in extender containing lyophilized egg yolk, with (arrow) or without DNA fragmentation.

Statistical analysis

Two way analysis of variance and Duncan's multiple range tests were done for the obtained data after transformation of percentages to their corresponding arcsin values ***(32)***. Data were analyzed using the 1986-version of Costat (version 3.03 copyright software), and $P \leq 0.05$ was considered as statistically significant.

RESULTS

Data regarding the effects of adding different concentrations of natural hen egg plasma to semen extender on post-thaw characteristics and DNA integrity of Arab stallion frozen-thawed spermatozoa were presented in table 1. The post-thaw total and progressive motility, viability index, sperm membrane and acrosome integrities were highest ($P \leq 0.05$) when 10% egg plasma was included in semen freezing extender, intermediate ($P \leq 0.05$) when 7.5% egg plasma was used, and lowest ($P \leq 0.05$) in extenders containing 5% and 15% egg plasma. The percentage of spermatozoa with non fragmented DNA and the percentage of DNA in the head of comet were highest ($P \leq 0.05$) when 10% egg plasma was included in semen freezing extender, intermediate ($P \leq 0.05$) when 7.5% egg plasma was used, and lowest ($P \leq 0.05$) in extenders containing 5% and 15% egg plasma. On the other hand, the percentage of DNA in the tail of comet, tail length and Olive tail moment were lowest ($P \leq 0.05$) when 10% egg plasma was included in semen freezing extender, intermediate ($P \leq 0.05$) when 7.5% egg plasma was used, and highest ($P \leq 0.05$) in extenders containing 5% and 15% egg plasma.

Table 1. Effects of adding different concentrations of natural hen egg plasma to semen extender on post-thaw characteristics and DNA integrity of Arab stallion frozen-thawed spermatozoa.

Parameters (No = 20)*		Concentration of egg plasma			
		5.0%	7.5%	10.0%	15%
Post-thaw total motility (%)	0h	20.00 ± 1.12^{d}	45.00 ± 1.77^{b}	52.63 ± 0.76^{a}	30.00 ± 1.12^{c}
	1h	20.00 ± 0.79^{b}	40.50 ± 1.00^{a}	45.25 ± 1.43^{a}	22.00 ± 0.61^{b}
	2h	15.00 ± 1.12^{c}	32.50 ± 1.25^{b}	40.00 ± 1.26^{a}	17.50 ± 1.25^{c}
	3h	10.00 ± 1.12^{b}	25.00 ± 1.77^{a}	30.00 ± 1.40^{a}	12.50 ± 0.56^{b}
Post-thaw progressive motility (%)	0h	15.00 ± 1.12^{d}	37.50 ± 1.25^{b}	42.38 ± 0.56^{a}	26.00 ± 0.93^{c}
	1h	12.50 ± 1.12^{d}	30.00 ± 1.12^{b}	37.63 ± 0.76^{a}	20.00 ± 0.56^{c}
	2h	10.00 ± 0.79^{c}	25.00 ± 1.12^{a}	30.00 ± 1.26^{a}	17.50 ± 0.56^{b}
	3h	7.50 ± 0.56^{b}	20.00 ± 1.12^{a}	22.13 ± 0.83^{a}	12.50 ± 1.26^{b}
Viability index		55.00 ± 2.90^{c}	120.50 ± 4.61^{b}	141.56 ± 2.46^{a}	67.00 ± 2.86^{c}
Swollen spermatozoa (HOS +ve %)		27.20 ± 0.99^{b}	36.00 ± 1.01^{ab}	39.53 ± 1.54^{a}	30.20 ± 0.89^{b}
Normal acrosomes (%)		26.20 ± 1.21^{c}	33.20 ± 0.64^{ab}	36.48 ± 0.72^{a}	31.00 ± 1.01^{bc}
Comet Assay: Sperm with non-fragmented DNA (%)		86.00 ± 2.20^{b}	93.80 ± 0.84^{ab}	95.85 ± 0.97^{a}	92.80 ± 0.93^{ab}
Comet Assay: DNA in head of comet (%)		91.00 ± 0.71^{d}	96.88 ± 0.16^{b}	98.35 ± 0.08^{a}	94.78 ± 0.35^{c}
Comet Assay: DNA in tail of comet (%)		9.00 ± 0.71^{a}	3.12 ± 0.16^{c}	1.65 ± 0.08^{d}	5.22 ± 0.35^{b}
Comet Assay: Tail length (pixel)		15.08 ± 0.54^{a}	10.30 ± 0.53^{bc}	8.47 ± 0.33^{c}	12.18 ± 0.49^{b}
Comet Assay: Olive tail moment		0.87 ± 0.01^{a}	0.60 ± 0.01^{c}	0.54 ± 0.17^{d}	0.70 ± 0.02^{b}

Means with different alphabetical superscripts within row are significantly different at $P \leq 0.05$.
* No= number of ejaculates.

Data regarding the effects of adding different concentrations of natural hen LDL to semen extender on post-thaw characteristics and DNA integrity of Arab stallion frozen-thawed spermatozoa were presented in table 2. The post-thaw total and progressive motility, viability index and acrosome integrities were highest ($P \leq 0.05$) when 7.5% LDL was included in semen freezing extender, intermediate ($P \leq 0.05$) when 10% LDL was used, and lowest ($P \leq 0.05$) in extenders containing 5% and 15% LDL.

The inclusion of 7.5% and 10% natural LDL in stallion semen extender resulted in significantly ($P \leq 0.05$) higher percentage of spermatozoa with intact membranes (HOS +ve) as compared to inclusion of 5% and 15% LDL.

The percentage of spermatozoa with non fragmented DNA and the percentage of DNA in the head of comet were highest ($P \leq 0.05$) when 7.5% LDL was included in semen freezing extender, intermediate ($P \leq 0.05$) when 10% LDL was used, and lowest ($P \leq 0.05$) in extenders containing 5% and 15% LDL. On the other hand, the percentage of DNA in the tail of comet, tail length and Olive tail moment were lowest ($P \leq 0.05$) when 7.5% LDL was included in semen freezing extender, intermediate ($P \leq 0.05$) when 10.0% egg plasma was used, and highest ($P \leq 0.05$) in extenders containing 5% and 15% LDL.

As presented in table 3, inclusion of natural LDL in stallion semen extender resulted in the highest ($P \leq 0.05$) frozen-thawed semen quality in terms of post-thaw total and progressive motility as well as sperm membrane and acrosome integrities. Addition of natural egg plasma to the extender resulted in the second highest frozen-thawed semen quality. The use of lyophilized forms of egg plasma and LDL in semen extender gave the worst ($P \leq 0.05$) post-thaw motility, sperm membrane and acrosome integrities.

Table 2. Effects of adding different concentrations of natural hen LDL to semen extender on post-thaw characteristics and DNA integrity of Arab stallion frozen-thawed spermatozoa.

Parametrs (No = 20)*		Concentration of LDL			
		5.0%	7.5%	10.0%	15%
Post-thaw total motility (%)	0h	30.00 ± 1.12^{c}	55.00 ± 1.66^{a}	50.00 ± 1.12^{ab}	40.00 ± 1.12^{b}
	1h	25.00 ± 1.12^{d}	47.63 ± 0.67^{a}	42.50 ± 0.55^{b}	35.00 ± 1.12^{c}
	2h	20.00 ± 1.77^{c}	39.75 ± 2.00^{a}	35.00 ± 1.77^{ab}	27.50 ± 1.25^{bc}
	3h	15.00 ± 1.12^{c}	32.63 ± 0.76^{a}	30.00 ± 1.77^{ab}	25.00 ± 0.38^{b}
Post-thaw progressive motility (%)	0h	25.00 ± 1.12^{c}	45.25 ± 1.12^{a}	40.00 ± 1.77^{ab}	35.00 ± 1.77^{b}
	1h	20.00 ± 1.77^{c}	40.25 ± 1.28^{a}	35.00 ± 1.77^{ab}	27.50 ± 0.55^{bc}
	2h	15.00 ± 1.12^{b}	30.8 ± 1.08^{a}	27.50 ± 0.55^{a}	20.00 ± 1.77^{b}
	3h	10.00 ± 1.12^{b}	25.00 ± 1.26^{a}	22.50 ± 0.55^{a}	15.00 ± 1.12^{b}
Viability index		75.00 ± 3.21^{d}	147.50 ± 3.05^{a}	132.50 ± 4.11^{b}	107.50 ± 2.50^{c}
Swollen spermatozoa (HOS +ve %)		32.26 ± 0.84^{b}	42.30 ± 0.83^{a}	40.98 ± 0.95^{a}	35.48 ± 0.93^{b}
Normal acrosomes (%)		29.14 ± 0.67^{c}	38.58 ± 0.93^{a}	36.26 ± 0.51^{ab}	33.48 ± 0.51^{bc}
Comet Assay: Sperm with non-fragmented DNA (%)		89.68 ± 1.24^{c}	95.60 ± 1.20^{a}	93.62 ± 0.77^{ab}	91.06 ± 1.03^{bc}
Comet Assay: DNA in head of comet (%)		93.58 ± 0.29^{c}	98.52 ± 0.02^{a}	96.78 ± 0.17^{ab}	95.68 ± 0.17^{b}
Comet Assay: DNA in tail of comet (%)		6.42 ± 0.29^{a}	1.48 ± 0.02^{c}	3.22 ± 0.17^{b}	4.32 ± 0.17^{ab}
Comet Assay: Tail length (pixel)		15.72 ± 0.19^{a}	10.53 ± 0.18^{c}	12.14 ± 0.21^{bc}	14.20 ± 0.40^{ab}
Comet Assay: Olive tail moment		0.88 ± 0.02^{a}	0.25 ± 0.01^{d}	0.57 ± 0.02^{c}	0.76 ± 0.01^{b}

Means with different alphabetical superscripts within row are significantly different at $P \leq 0.05$
* No= number of ejaculates.

Concerning the viability indices of stallion spermatozoa, inclusion of natural forms of whole egg yolk, egg plasma and LDL as well as lyophilized egg yolk resulted in satisfactory viability indices (ranged from 132.75 to 147.50). The inclusion of lyophilized forms of egg plasma and LDL gave unsatisfactory viability indices (ranged from 10.00 to 22.50). The sperm mitochondrial activity, in terms of MTT reduction rate and viability according to MTT reduction, was highest ($P \leq 0.05$) when the natural forms of egg plasma and LDL were included in semen freezing extender, intermediate ($P \leq 0.05$) when the natural and lyophilized forms of egg yolk are used, and lowest ($P \leq 0.05$) in extenders containing the lyophilized forms of egg plasma and LDL.

Table 3. Effects of adding natural and lyophilized forms of egg yolk, egg plasma and LDL to semen extender on post-thaw characteristics and DNA integrity of Arab stallion frozen-thawed spermatozoa.

Parametrs (No = 20)*		Egg yolk (15%)		Egg plasma (10%)		LDL (7.5%)	
		Natural	lyophilized	Natural	lyophilized	Natural	lyophilized
Post-thaw total motility (%)	0h	49.75±1.35^{b}	45.00±1.40^{c}	52.63±0.76^{ab}	15.00±0.85^{d}	55.00±1.66^{a}	10.00±1.15^{e}
	1h	42.88±1.11^{bc}	39.75±1.56^{c}	45.25±1.43^{ab}	10.00 ±1.26^{d}	47.63±0.67^{a}	5.00±1.09^{e}
	2h	35.00±1.40^{b}	32.63±0.91^{b}	40.00±1.26^{a}	5.00±0.89^{c}	39.75±2.00^{a}	0.00±0.00^{d}
	3h	30.00±1.40^{ab}	27.38±0.91^{b}	30.00±1.40^{ab}	0.00±0.00^{c}	32.63±0.76^{a}	0.00±0.00^{b}
Post-thaw progressive motility (%)	0h	40.00±1.36^{bc}	37.33±1.28^{c}	42.38±0.56^{ab}	10.00±1.15^{d}	45.25±1.12^{a}	7.63±0.45^{d}
	1h	35.00±1.26^{b}	29.75±1.28^{c}	37.63±0.76^{ab}	7.63±0.76^{d}	40.25±1.28^{a}	2.38±0.56^{e}
	2h	29.75±1.06^{a}	24.75±1.06^{b}	30.00±1.26^{a}	3.38±0.51^{c}	30.8±1.08^{a}	0.00±0.00^{d}
	3h	22.88±0.84^{b}	20.00±1.09^{c}	22.13±0.83^{b}	0.00±0.00^{d}	25.00±1.26^{a}	0.00±0.00^{d}
Viability index		132.75±3.14^{b}	122.25±1.96^{c}	141.56±2.46^{a}	22.50±1.89^{d}	147.50±3.05^{a}	10.00±1.23^{e}
Swollen spermatozoa (HOS +ve %)		37.10±1.00^{b}	36.20±1.05^{b}	39.53±1.54^{ab}	27.05±0.81^{c}	42.30±0.83^{a}	24.20±0.85^{c}
Normal acrosomes (%)		34.25±0.81^{b}	30.75±1.68^{c}	36.48±0.72^{ab}	28.28±0.44^{c}	38.58±0.93^{a}	30.15±0.92^{c}
Mitochondrial	MTT reduction rate	0.30±0.01^{b}	0.29±0.02^{b}	0.33±0.01^{a}	0.19±0.01^{c}	0.35±0.02^{a}	0.13±0.00^{d}
	Viability acc. to MTT reduction	56.74±1.78^{b}	54.22±1.12^{b}	64.29±1.78^{a}	29.06±0.89^{c}	69.33±1.78^{a}	13.96±0.89^{d}
Comet Assay	Sperm with non-fragmented DNA (%)	96.00±1.23^{a}	87.90±1.69^{b}	95.85±0.97^{a}	88.85±1.41^{b}	95.60±1.20^{a}	84.58±1.13^{c}
	DNA in head of comet (%)	98.39±0.05^{a}	96.05±0.28^{b}	98.35±0.08^{a}	96.15±0.03^{b}	98.52±0.02^{a}	95.10±0.05^{c}
	DNA in tail of comet (%)	1.61±0.05^{c}	3.95±0.28^{b}	1.65±0.08^{c}	3.85±0.03^{b}	1.48±0.02^{c}	4.90±0.05^{a}
	Tail length (pixel)	5.53±0.15^{f}	12.72±0.30^{b}	8.47±0.33^{e}	11.42±0.29^{c}	10.53±0.18^{d}	16.53±0.31^{a}
	Olive tail moment	0.53±0.08^{b}	0.58±0.01^{b}	0.54±0.17^{b}	0.55±0.01^{b}	0.25±0.01^{c}	0.99±0.02^{a}

Means with different alphabetical superscripts within row are significantly different at $P \leq 0.05$
* No= number of ejaculates.

The percentage of spermatozoa with non fragmented DNA and the percentage of DNA in the head of comet were highest ($P \leq 0.05$) when the natural forms of whole egg yolk, egg plasma and LDL were included in semen freezing extender, intermediate ($P \leq 0.05$) when the lyophilized forms of egg yolk and egg plasma are used, and lowest ($P \leq 0.05$) in the lyophilized LDL containing extender.

On the other hand, the percentage of DNA in the tail of comet, tail length and Olive tail moment were lowest ($P \leq 0.05$) when the natural forms of whole egg yolk, egg plasma and LDL were included in semen freezing extender, intermediate ($P \leq 0.05$) when the lyophilized forms of egg yolk and egg plasma are used, and highest ($P \leq 0.05$) in the lyophilized LDL containing extender.

DISCUSSION

The purpose of this study was to determine the cryoprotective effect of natural and lyophilized forms of egg yolk, egg plasma and LDL on Arab stallion spermatozoa. According to all measured parameters herein, the extenders containing 10% egg plasma and 7.5 LDL showed the optimal cryoprotective effects on frozen–thawed Arabian stallion spermatozoa. It appeared that 8% LDL was found to be more suitable for cryopreservation of bull *(8,33)*, ram *(34)* and caprine *(35)* sperm. Based on work with canine sperm, ***Bencharif et al. (9)*** demonstrated that 6% LDL gave the best post-thaw results. This difference in the optimum concentration of LDL could be due to the difference in the composition of sperm plasma membranes between different animal

species. In our study, at 5%, the egg plasma and LDL were insufficient to guarantee the protection of equine sperm during cooling and freezing processes. Whereas at 15% LDL, the osmotic pressure of the extender may not have been within the range specifically required for equine spermatozoa. It is reported by ***Moussa et al. (8)*** that with the increase in LDLs concentration above 10%, there is a decline in the osmotic pressure of extender, due to salt precipitation or the LDL aggregation effect, resulting in a decrease in spermatozoa performance after freezing ***(8,14).***

Our results revealed that post-thaw total and progressive motility of stallion spermatozoa were significantly ($P \leq 0.05$) higher in extenders containing natural LDL and natural egg plasma as compared to that contained fresh egg yolk. Similarly, ***Pillet et al. (15)*** and ***Rodgers et al. (2)*** concluded that simply adding clarified egg plasma has been shown to improve stallion sperm. Also, better post-thaw sperm motility in extender containing LDLs than egg yolk has also been reported in equine ***(36),*** buffalo bull ***(37)***, bull ***(33-38)***, boar ***(14)*** and dog semen ***(39)***. The LDL acts by adherence to the sperm cell membrane ***(38)*** and forming an interfacial film between fatty acids and water ***(40).*** The LDL would promote the entry of phospholipids and cholesterol into the cell membrane, building a complex with seminal plasma proteins, which were deleterious to sperm, making them unavailable to function in the membrane ***(38).*** In the present study, the post-thaw functional integrities of plasma membrane (HOS +ve) and acrosome of stallion spermatozoa were significantly ($P \leq 0.05$) higher in extender containing natural forms of egg plasma and LDL compared with that containing egg yolk. Similarly, higher proportion of sperm with intact membranes was observed in extender containing LDL than in the extender containing egg yolk ***(36-37).*** ***Moreno et al. (36)*** stated that LDL provided good protection of acrosome integrity, possibly via a direct action through the exchange or repair of acrosomal membrane phospholipids or possibly simply because the extender has lower progesterone content than egg yolk because of the filtering effect of the dialysis membrane. The progesterone found in egg yolk plays a role in the capacitation of spermatozoa in horses ***(41)*** which was unwanted during cryopreservation.

Mitochondria are one of most important effective organelles in sperm because they supply energy needed for sperm motility ***(42).*** In our study, the post-thaw mitochondrial activity (MTT reduction rate) of stallion spermatozoa were significantly ($P \leq 0.05$) higher in extender containing natural forms of egg plasma and LDL compared with that containing egg yolk. This finding came in accordance with the concerns that egg yolk contains substances that impede respiration of spermatozoa which may lead to decrease their motility ***(8-9,11).***

The integrity of sperm DNA was very important for the success of fertilization and the development of fetus and offspring ***(43).*** Based on our data of Comet assay, concerning the percentage of spermatozoa with intact DNA, there was no significant difference between stallion spermatozoa in extender containing natural forms of fresh egg yolk, egg plasma and LDL. Moreover, by using these extenders, the DNA was preserved in more than 95% of the spermatozoa. ***Moreno et al. (36)*** used the acridine orange test to assess DNA integrity of stallion spermatozoa and they found that there were no significant differences among extenders containing LDL or egg yolk and the DNA was preserved in 98% of the spermatozoa.

In the current study, the post-thaw motility, acrosome and DNA integrity of stallion spermatozoa were significantly ($P \leq 0.05$) lower in extenders containing lyophilized egg yolk as compared to that contained fresh egg yolk, the sperm membrane integrity and mitochondrial activity were not significantly different between the two extenders. Concerning the studied semen parameters herein, using the lyophilized egg yolk in stallion semen extender resulted in satisfactory results. Similar satisfactory post-thaw semen quality was obtained when using powdered egg yolk in an extender for the cryopreservation of ram ***(16)*** and Zebu bull ***(17)*** semen.

Concerning all the studied semen quality endpoints in the present study, using the lyophilized forms of LDL and egg plasma in stallion semen extender resulted in the worst results. A similar preparation of lyophilized LDL was used by ***Moustacas et al. (44)*** and ***Neves et al. (45)*** to cryopreserve ram and dog spermatozoa, respectively, and lyophilized LDL was also ineffective in protecting sperm during the freezing thawing process. ***Watson (46)*** reported that the drying process could cause loss of LDL activity, due to denaturation resulting from the removal of water. Also, ***Neves et al. (45)*** stated that these low results of lyophilized LDL may be attributed to that Tween 20 may have had deleterious effects on sperm such as disnaturing proteins of the plasmatic membrane or may have provoked other injuries, which lead to disrupted membrane integrity. Although, in the present study Tween 20 was also used for reconstitution of lyophilized egg yolk without such deleterious effect on stallion spermatozoa.

In conclusion, the addition of the natural forms, but not lyophilized forms, of LDL and egg plasma as well as lyophilized egg yolk to semen extender could be good alternatives to the whole egg yolk as they improves stallion sperm quality after cryopreservation in terms of motility, integrities of mitochondria, membrane and acrosome as well as reduction of DNA damage.

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الملخص العربي

تأثير صفار بيض الدجاج ، بلازما صفار البيض و الليبوبروتين منخفضي الكثافة الطازج والمجفف على قدرة حيامن الخيول العربية على التجميد وسلامة حمضها النووى

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قسم التلقيح الاصطناعى ونقل الأجنة[١] ووحدة المناعة الدوائية[٢] و قسم الفحوص الحقلية[٣] ، معهد بحوث التناسليات الحيوانية – مركز البحوث الزراعية - الجيزة

يهدف هذا البحث إلى دراسة تأثير صفار البيض ، بلازما صفار البيض و الليبوبروتين منخفض الكثافة الطازج والمجفف على قدرة حيامن الخيول العربية على التجميد. تم تجميع السائل المنوى من خمسة خيول عربية أصيلة وتم تخفيف السائل المنوى بمخفف الأترا-٨٢ الذى يحتوى على صفار البيض الطازج (١٥%) و المجفف (١٥%) و بلازما صفار البيض الطازجة (٥ و ٧,٥ و ١٠ و ١٥%) و بلازما صفار البيض المجففة (١٠%) و الليبوبروتين منخفض الكثافة الطازج (٥ و ٧,٥ و ١٠ و ١٥%) و الليبوبروتين منخفض الكثافة المجفف (٧,٥%). تم تجميد عينات السائل المنوى بإستخدام النيتروجين السائل وتم فحص حركة الحيامن و سلامة أغشية الحيامن و القلنسوة و الميتوكوندريا و الحامض النووى. وقد أوضحت النتائج أن أفضل تركيز لبلازما صفار البيض و الليبوبروتين منخفض الكثافة الطازجين هو ١٠% و٧,٥%، على الترتيب. وقد أدت إضافة بلازما صفار البيض و الليبوبروتين منخفض الكثافة الطازجين إلى تحسين خصائص السائل المنوى المجمد بالمقارنة بصفار البيض الطازج. أدى إستخدام بلازما صفار البيض و الليبوبروتين منخفض الكثافة المجففان إلى تدهور خصائص السائل المنوى المجمد بينما كانت خصائص السائل المنوى المجمد مرضية عند إستخدام مخفف يحتوى على صفار البيض المجفف . الأستنتاج: إن إضافة بلازما صفار البيض و الليبوبروتين منخفض الكثافة الطازجين و صفار البيض المجفف لمخفف تجميد السائل المنوى للخيول يمكن أن يحل محل صفار البيض الطازج حيث أنهم قد حسنوا خصائص السائل المنوى المجمد (الحركة بعد الأسالة، سلامة أغشية الحيامن و الميتوكوندريا و القلنسوة و الحامض النووى).