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INFLUENCE OF PRIMROSE OIL AND CHOLESTEROL-3-SULFATE ON FREEZABILITY, ULTRA-STRUCTURE CHANGES AND IN VITRO FERTILIZING POTENTIAL OF RAM SPERMATOZOA

(With 4 Tables and 11 Figures)

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تأثير زيت البريمروز والكوليسترول – ٣سلفات على قابلية حيامن الكباش للتجميد والتغيرات في التركيب الدقيق ومعدل الإخصاب المعملي.

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من المعروف أن حيامن الكباش حساسة جداً لعملية التجميد حيث يتعرض الكثير منها لفقد قدرته الاخصابية أثناء عملية التجميد. ولقد وجد أن نسبة لكوليسترول إلى الأحماض الدهنية المكونة للغشاء البلازمي لحيامن الكباش تلعب دوراً حيوياً في المحافظة على سلامة الغشاء البلازمي للحيامن وكذلك المحافظة على قدرته الإخصابية. ولذلك تهدف هذه الدراسة إلى استبيان تأثير إضافة زيت البريمروز والكوليسترول بتركيزات مختلفة إلى المنى المخفف قبل التجميد على قابلية حيامن الكباش للتجميد وكذلك دراسة التغيرات في التركيب الدقيق والتغيرات الكيميائية للحيامن وقدرتها الإخصابية معمليا بعد التجميد. تم تجميع عينات المنى من ٦ كباش تبلغ من العمر ٣ سنوات حيث خففت في مخفف الترييس المضاف إليه زيت البريمروز والكوليسترول بتركيزات مختلفة وبعد تبريد وتجميد المنى بالنظام الفرنسي تم تقييم عينات المنى من حيث النسبة المئوية للحركة بعد التخفيف، بعد الإسالة وكذلك نسبة تشوهات القلنسوة وتقييم حيوية المنى بعد التجميد. أيضاً تمت دراسة التغيرات في التركيب الدقيق للحيامن بعد التجميد باستخدام الميكروسكوب الإلكتروني النافذ والتغيرات الكيميائية للحيامن بعد التجميد وتحديد القدرة الإخصابية لحيامن الكباش المجمدة باستخدام تكنولوجيا الإخصاب المعملي. وأظهرت نتائج هذه الدراسة أن إضافة ٢,٥ ملليمول زيت البريمروز إلى مخفف المنى للكباش أدى إلى زيادة معنوية كبيرة للحركة بعد الإسالة وحيوية المنى وانخفاض كبير في نسبة تشوهات القلنسوة بعد التجميد (٦٢,٥٠% ، ١٨٠,٠٠ و١٦,٢٥%

على التوالي) بالمقارنة بإضافة ٦ ملليجرام/مللي كوليسترول (٦,٢٥% ، ١٠,٦٢ و٣٦,٧٥ % على التوالي). كما أوضحت نتائج هذه الدراسة أن إضافة ٢,٥ ملليمول زيت البريمروز إلى مخفف المنى حافظ على سلامة الغشاء البلازمي للحيامن وأغشية القلنسوة وكذلك سلامة الميتوكوندريا بعد التجميد في حين أن إضافة ٦ ملليجرام/مللي كوليسترول كان له تأثير سيئ جداً على تلك التراكيب للحيامن. ومن خلال تجارب الإخصاب المعملي تبين أن هناك زيادة معنوية كبيرة في معدل الإخصاب ومعدل النمو إلى الطور التوتي وطور البلاستوسيت عند إخصاب بويضات الأغنام بالمنى مجمد و معالج بـ ٢,٥ ملليمول زيت البريمروز (٤٨,٤٥ ، ٢٧,٤٧ ، ١٦,٦٨% على التوالي) مقارنة بالمنى المعالج بـ ٦ ملليجرام/ مللي كوليسترول (١٣,٤١ ، ٢,٢٧ و ٠,٠٠ % على التوالي). ومن خلال نتائج هذه الدراسة يمكن استنتاج أن إضافة ٢,٥ ملليمول زيت البريمروز إلى مخفف المنى للكباش أدى إلى تحسين قابلية حيامن الكباش للتجميد وكذلك تحسين قدرتها الإخصابية من خلال المحافظة على سلامة التراكيب الدقيقة والمحتوى الكيميائي للحيامن بينما أن إضافة ٦ ملليجرام/مللي كوليسترول إلى مخفف المنى أدى إلى انخفاض قابلية الحيامن للتجميد وتقليل قدرتها الإخصابية من خلال تأثيره الضار على التراكيب الدقيقة والمحتوى الكيميائي لحيامن الكباش.

SUMMARY

Ram spermatozoa are most susceptible to damage during cryopreservation. Cholesterol and phospholipids are known to affect membrane stability and fertilizing potential of ram spermatozoa. The objective of the current study was to investigate the effect of primrose oil and cholesterol addition to the freezing extender on the freezability, ultrastructure, biochemical changes and in vitro fertilizing potential of ram spermatozoa. Semen was collected from 6 rams of three years old, pooled together and extended in Tris-based extender supplemented with primrose oil and cholesterol in different concentrations. Extended semen was cooled and freezed in liquid nitrogen. Sperm motility after dilution and post-thawing, acrosomal integrity and viability index were assessed subjectively. Some biochemical changes of frozen semen were determined. Representative samples from the treated ram semen were subjected to transmission electron microscopy study. Frozen-thawed ram semen treated with primrose oil and cholesterol was used to evaluate their in vitro fertilizing potential. Addition of 2.5 mM primrose oil to the semen extender significantly improved the post-thawing motility, viability index and acrosomal integrity (62.50%, 180.00 and 16.25% versus 6.25%,10.62 and 36.75%, respectively with 6 mg/ml cholesterol - 3-sulfate). Also, primrose oil addition minimized the biochemical changes and ultrastructure damage of ram spermatozoa and enhanced the in vitro fertilization rate and the morula and blastocyst stages development (48.45,27.47 and 16.68% versus 13.41,2.27 and 0.00%,

respectively with 6 mg/ml cholesterol -3-sulfate). The results regarded to 6 mg/ml cholesterol-3-sulfate supplemented ram semen extender were 13.41, 2.27 and 0.00%, respectively. In conclusion, the present results provide a novel evidence that the addition of primrose oil to the extended ram semen protects the semen from cryoinjury, improves post-thawing semen function and in vitro fertilizing potential, whereas, cholesterol-3-sulfate has a detrimental effect on the ram semen freezability and in vitro fertilizing potential.

Key words: *Primrose oil, cholesterol-3-sulfate, ram spermatozoa, biochemical assay, semen*

INTRODUCTION

One characteristic feature of the plasma membrane of mammalian sperm cells is the asymmetric transbilayer distribution of lipid (Nolan *et. al.*, 1995 and Muller *et al.*, 1998). The sperm plasma membrane transbilayer rearrangement of lipids and destabilization of the lipid phase play an important role in the fertilization process of spermatozoa (Nolan and Hammerstedt, 1997 and Muller *et al.*, 1999). However, premature destabilization may impaire the viability and the fertilizing potential of sperm cells. Ram spermatozoa are most susceptible to damage during freezing between the temperature – 10°C and – 25°C (Byrne *et al.*, 2000). Cryopreservation exerts osmotic, mechanical and chemical stresses on sperm cells (Drokin *et al.*, 1999). Damage of sperm cells by cryopreservation has been associated with alterations of the plasma membrane lipid phase. Changes in the lipid composition of the sperm cell membranes are responsible for the rise in 'membrane fluidity' upon cryotreatment (Hinkovska-Galcheva *et al.*, 1988 and Buhr *et al.*, 1994). These changes are also thought to contribute to the loss of mammalian spermatozoa motility and fertility after freezing (Holt and North, 1984 and 1986).

Cholesterol and phospholipids are known to affect membrane stability and appear to influence the fertilizing potential of spermatozoa (Grippo *et al.*, 1994). Cholesterol is mostly directed to the cell surface and, when in excess, it severely alters the fluidity of the lipid bilayer and plasma membrane properties (Liscum and Underwood, 1995 and Kellner–Weibel *et al.*, 1998). Inclusion of linoleic-olic acids to the semen diluent had a beneficial effect on preserving the sperm motility and viability (Perez-Pe *et al.*, 2001).

Primrose oil is a pure natural oil extracted from the seeds of the biennial plant. It is a unique supplement of linoleic and gamma-linolenic acid (GLA). Prostaglandins are derived from the membrane phospholipid stores of arachidonic acid (C 20:4), which are synthesized from dietary linoleic acid (C 18:2) (Filley *et al.*, 1999). The addition of PGE to the fertilization medium increased the cleavage rate (Gurevich *et al.*, 1993). Therefore, the current study was designed to investigate the effect of primrose oil and cholesterol-3-sulfate addition to the Tris-based extender on the sperm freezability, ultrastructure, biochemical changes and in vitro fertilizing potential of the ram spermatozoa.

MATERIALS and METHODS

Semen collection and extender:

Semen samples were collected twice weekly from six matured, clinically health Barki ram aged 3 years. Semen samples of at least 70% initial motility and 3 x 10^9 sperm cells /ml were used. Immediately after collection, samples were split into 7 portions and diluted at 1:19 at 30 °C with Tris-based extender (Evans and Maxwell, 1986), containing primrose oil (0.5,2.5 and 5 mM), cholesterol-3-sulfate (1,3 and 6 mg/ml) or extender as a control without any addition .

Processing of ram semen:

Immediately after dilution, the extended semen was cooled to 5 °C throughout 60 minutes in a cold handling cabinet. The cooled semen was loaded into 0.25 ml French straws (IMV, L'Aigle, France) arranged horizontally on a cooled racks, then lowered into liquid nitrogen vapor inside foam box according to Khalifa (2001), then the straws were immersed and stored in liquid nitrogen.

Evaluation of semen freezability:

After 24 hour, frozen semen samples were thawed in water bath at 40 °C for 30 seconds. Sperm motility was subjectively assessed immediately after dilution, thawing and after 1, 2 and 3 hours post-thawing. The post-thawing viability indices were recorded according to Milovanov (1962).

Preparation of semen samples for transmission electron microscopy (TEM):

Semen samples were prepared for TEM as described by (Kakar and Anand, 1984). Ram semen samples treated with 6 mg/ml cholesterol-3-sulfate and 2.5mM primrose oil revealed the worst and the best results on evaluating the post-thawing motility and viability index

were selected for TEM study. Post-thawed semen samples (one ml of each semen sample) were fixed in a primary fixative consisted of 5% glutraldehyde and 5% formaldehyde (1:1) in 0.1 M phosphate buffer (pH, 7.4) for 1 hour at 4 °C. The sperm suspension was centrifuged at 3000 rpm for 5 minutes to remove the glutraldehyde-formaldehyde mixture. The supernatant was discarded and the pellets were resuspended and washed twice with 0.1 M phosphate buffer (pH, 7.4) and then post-fixed by 1 % osmium tetraoxide in 0.1 M phosphate buffer (pH, 7.4) for 1 hour at 4 °C. Briefly, after routine dehydration with a graded series of ethanol, the sperm pellets were infiltrated successively with absolute propylene-oxide and Spurr's epoxy resin, then with absolute Spurr's epoxy resin. Finally, the sperm pellets were embedded in the beam capsules with fresh Spurr's epoxy resin. Ultrathin sections were cut out with a diamond knife and the grids were contrasted with uranyl acetate and lead citrate (Reynolds, 1963). The sections were examined under transmission electron microscope (Jeol 100S, at VACSERA- Electron Microscopy Unit).

Biochemical assay of frozen ram semen:

The assays for alanine transaminase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were carried out according to (Upreti *et al.*, 1996). Total cholesterol and high density lipoprotein (HDL) of frozen-thawed ram semen samples were determined by colorimetric method (Specord 40, analytikjene CEAG, Germany) according to Watson (1960) and Cohn *et al.* (1988).

Evaluation of in vitro fertilizing potential of the treated semen:

Frozen semen samples treated with primrose oil and cholesterol-3-sulfate were used for assessing of their fertilizing potential in vitro.

In vitro maturation:

Oocytes were collected, selected and matured in vitro in TCM-199 medium supplemented with 10 µg/ml LH, 5 µg/ml FSH and 1 µg/ml estradiol-17 β at 39 °C for 24 hour in a humidified atmosphere of 5%CO_2 in air according to O'Brien *et al.* (1996).

In vitro sperm preparation:

Three straws of frozen ram semen from each treatment were thawed in a water bath at 37 °C for 30 seconds. Immediately after thawing, the most motile ram spermatozoa was separated by swim up method in S-TALP medium containing 6 mg/ml BSA, for 1 hour (Parrish *et al.*, 1988). The uppermost layer of spermatozoa was selected and washed twice by centrifugation at 2000 rpm for 10 minutes. Sperm pellet was resuspended in F-TALP medium containing 10 µg/ml heparin

for sperm capacitation. After appropriate dilution, 2 ml of sperm suspension was added per fertilization drop at a final concentration of 2.5×10^5. Gametes were co-incubated in fertilization drops under silicon oil for 18 hour at 39 °C in 5%CO_2 in a humidified air. After sperm-oocyte incubation, some of oocytes were fixed in acetic acid-ethanol (1:3) and stained with 1% aceto-orcein stain to assess the fertilization rate. Fertilization was considered to be occurred when male pronucleus or enlarged sperm head or even sperm tail was present in the oocyte ooplasm.

In vitro culture:

Groups of the inseminated oocytes were then freed from cumulus cells and the attached spermatozoa by gentle pipetting and cultured in TCM-199 medium containing 25mM HEPES. The proportion of cleaved oocytes was recorded 48 hour after insemination and those developed to the morula and blastocyst stages were recorded 5- to 7-days post-insemination (Winter berger-Torres and Sevellec, 1987).

Statistical analysis:

The statistical analysis was performed using the Costat Computer Program, version 3.03 copyright (1986) Cottort Software, all data were subjected to analysis of variance (ANOVA). A probability of less than 0.05 ($P<0.05$) was considered statistically significant.

RESULTS

Data regarding the influence of different primrose oil and cholesterol-3-sulfate concentrations on the sperm function in the cryopreserved ram semen are illustrated in Table 1. Supplementation of Tris-based extender with 0.5 or 2.5 mM primrose oil increased significantly ($P< 0.001$) the post-thawing sperm motility compared to the control .Additionally, primrose oil at concentrations of 0.5 and 2.5 mM enhanced significantly ($P<0.001$) the post-thawing sperm motility as compared with 3 mg/ml and 6 mg/ml cholesterol-3-sulfate. Likewise, supplementation of Tris-based extender with 0.5 and 2.5 mM primrose oil significantly increased ($P<0.001$) the viability indices compared to the control and significantly increased ($P<0.001$) compared to 3 and 6 mg/ml cholesterol-3-sulfate. Furthermore, the present results revealed that the percentage of acrosomal defect was significantly decreased ($P< 0.001$) with 0.5 and 2.5mM primrose oil treated semen compared to 3 and 6 mg/ml cholesterol-3-sulfate .

Table1: Influence of different primrose oil and cholesterol-3-sulfate concentrations on post-thawing motility, viability indices and acrosomal defect of ram spermatozoa. (Mean±SE).

Treatments	Concentration /ml	Motility		Viability index	Acrosomal defect
		After dilution	Post-thawing		
Control	extender alone	a 80.00±2.04	b 40.00±4.08	b 120.00±6.12	c 17.25±2.29
Primrose Oil	0.5 mM	a 82.50±3.23	a 62.50±2.50	a 176.25±10.68	c 13.75±2.21
	2.5 mM	a 86.25±2.39	a 62.50±4.79	a 180.00±10.55	c 16.25±1.86
	5 mM	a 81.25±3.15	b 37.50±4.80	bc 111.25±10.68	c 20.50±3.52
cholesterol-3-sulfate	1 mg/ml	a 82.50±1.44	bc 30.00±4.08	c 86.25±8.26	b 29.50±1.19
	3 mg/ml	a 82.50+1.44	c 25.00±2.89	d 55.00±2.04	b 32.25±2.89
	6 mg/ml	a 80.00+2.04	d 6.25±1.25	e 10.62±1.88	a 36.75±0.48
Significance		NS	P<0.001	P<0.001	P<0.001

Values are from 4 trials for each treatment
Values with different letters in the same column were significantly different (P<0.001)

Ultrastructure findings:

The ultrastructure study, by transmission electron microscopy (TEM) of ram semen samples treated with 2.5 mM primrose oil before freezing, revealed enhancement in the freezability of the spermatozoa and reduced the effect of the freezing and thawing. Examination by TEM illustrated that the plasma membrane and acrosomal membranes were intact in the majority of sperm cells. The plasma membrane appeared as thick electron-dense material surrounding the whole head region of sperms with loose attachment at the acrosome and the nucleus content was homogenous in the electron- density (Fig. 1). The outer and inner acrosomal membranes and the subacrosomal space were evident (Fig. 2). The mitochondria were slightly vacuolated and were more closely attached to the underlying dense fibers (Fig. 3).

Examination of ram semen samples treated with 6 mg/ml cholesterol-3-sulfate by TEM showed severe damage in the plasma membrane along the sperm cell, acrosomal membranes, and the

mitochondrial helix. Plasma membrane of sperm cells was completely lost at the head region in the majority of spermatozoa (Fig. 4). Many electron-dense materials in the space between the plasma membrane and the outer acrosomal membrane could be observed at the head region (may be destructed materials of the outer acrosomal membrane) (Fig. 5). Also, the plasma membrane at the mid-piece region of the tail was detached from the underlying mitochondrial helix and evaginated at some areas and contained electron-dense materials (may be destructed mitochondrion) (Fig. 6) and it appeared thin and with less electron-density at the end-piece (Fig. 7).

The outer acrosomal membrane was segmented (Fig. 4). Also, the acrosome was swollen as indicated by increasing the distance between the outer and inner acrosomal membranes (Fig. 4). The mitochondria at the neck and mid-piece regions of the tail are found to have vacuolated matrix (marked electron translucent spaces) and some mitochondria appeared swollen (Fig. 6) or completely destructed (only ghosts of mitochondria) (Fig. 8). However, the damage of mitochondria was more evident in the neck region of the tail (Fig. 8).The dense fibers of the axonemal complex at the neck and mid-piece regions of the tail were indistinct (Fig. 7 and Fig. 8).

Biochemical assay:

The data regarding the effect of primrose oil and cholesterol on the enzyme activities of cryopreserved ram semen are presented in Table 2.

The AST and ALP enzymes leakage during freezing was significantly decreased ($P<0.001$) with 2.5 mM primrose oil supplemented ram semen extender (84.25 ± 3.49 U/L, 3164.00±16.72 U/L, respectively). In contrast, addition of 6 mg/ml cholesterol-3-sulfate to the extended ram semen drastically increased the AST and ALP enzymes leakage during freezing (141.25 ±4.27 and 5248.00 ±102.94 U/L, respectively). Likewise, the results presented in Table 2, revealed that addition of 2.5 mM of primrose oil to the extended semen, significantly decreased the HDL and cholesterol (20.60±1.25 mg/dl and 55.61 ± 6.03 mg/dl, respectively) compared to 6 mg/ml cholesterol-3-sulfate (37.02±2.30 mg/dl and 113.12 ± 2.23 mg/dl, respectively).

Table 2: Influence of different primrose oil and cholesterol-3-sulfate concentrations on post-freezing enzymes and lipids activity of ram semen (Means± S E.)

Treatments	Concentrations /ml	ALT* (U/ml)	AST** (U/ml)	ALP*** (U/ml)	HDL**** mg/dl	Cholesterol mg/dl
Control	Extender alone	b 189.25±2.17	a 136.25±4.27	c 3408.75±33.32	b 25.77±1.64	a 104.03±4.98
Primrose oil	1 mM	b 186.00±4.55	b 105.00±7.18	de 3198±26.97	c 21.77±0.39	b 86.59±2.09
	2.5 mM	b 183.25±3.49	c 84.25±3.49	e 3164.00±16.72	c 20.60±1.25	c 55.61±6.03
	5 mM	b 189.50±2.10	b 103.75±10.45	cd 3340.25±25.43	b 28.29±1.43	b 87.05±1.89
Cholesterol -3-sulfate	1 mg/ml	b 189.50±2.10	a 127.50±3.23	cd 3334.25±50.54	b 26.83±0.61	a 105.85±4.63
	3 mg/ml	b 187.50±3.07	a 136.25±4.27	b 3595.00±50.54	a 28.58±0.63	a 108.69±2.32
	6 mg/ml	a 200.00±4.56	a 141.25±4.27	a 5248.00±102.94	a 37.02±2.30	a 113.12±2.23
Significance		P<0.001	P<0.001	P<0.001	P<0.001	P<0.001

Values are from 4 trials for each treatment.
Values with different letters in the same column were significantly different (P<0.001)
* ALT: alanine transaminase ** AST: Aspritate aminotransferase enzyme
*** ALP: Alkaline phosphatase enzyme **** HDL: high density lipoprotein

In vitro fertilizing potential :

The results presented in Table 3 and Fig. 9 showed that the treatment with 2.5 mM primrose oil significantly increased (P<0.001) the penetration and fertilization rates (68.37±1.97 and 48.45 ±3.56%, respectively) as compared with 3 mg/ml cholesterol-3-sulfate (54.01± 4.63 and 21.32 ±7.78%, respectively) and 6 mg/ml cholesterol-3-sulfate (42.44 ±5.48 and 13.41 ± 4.79%, respectively). Furthermore, 2.5 mM primrose oil treated frozen ram semen significantly enhanced (P<0.01) the cleavage rate, morula and blastocyst development (Table 4 and, Figs. 10 and 11) (43.35 ±3.14 , 27.47 ±3.61 and 16.68 ±2.59%, respectively) as compared with 3 mg/ml cholesterol-3- sulfate treated semen (17.40 ± 2.56,5.07 ±2.93 and 0.00%, respectively) and with 6 mg/ml cholesterol-3-sulfate (12.02 ± 1.85 , 2.27 ± 1.33 and 0.00%, respectively). Additionally, 2.5 mg/ml primrose oil treated semen improved the embryo developmental rates as compared with the control.

Table 3: Influence of different primrose oil and cholesterol-3-sulfate concentrations on in vitro fertilization dynamics of sheep oocytes (Mean ± SE).

Treatment	Concentrations /ml	No.of oocytes	Penetration rate	Fertilization rate
Control	Extender alone	62	65.98±5.87 ab	33.82±2.76 bc
Primrose oil	0.5 mM	65	63.75±3.99 ab	43.75±3.99 ab
Primrose oil	2.5 mM	54	68.37±1.97 a	48.45±3.56 a
Primrose oil	5 mM	60	65.33±2.09 ab	37.08 ±1.72 ab
Cholesterol	1 mg/ml	52	60.83±3.94 ab	43.16±2.09 bc
Cholesterol	3 mg/ml	55	54.01±4.63 bc	21.32±7.78 cd
Cholesterol	6 mg/ml	62	42.44±5.48 c	13.41+4.79 d
Significance			P<0.01 **	P<0.001 ***

Values are from 4 trials for each treatment
Values with different letters in the same column were significantly different (P<0.001)

Table 4: Influence of different primrose oil and cholesterol-3-sulfate concentrations on in vitro sheep embryonic development (Mean ± SE).

Treatment	Concentrations /ml	No. of oocytes	Cleavage rate	Morula	Blastocyst
Control	Extender	42	31.25±3.15 b	8.75±5.91 bc	2.08±2.08 bc
Primrose oil	0.5 mM	46	34.23±4.76 ab	13.26±2.76 bc	6.92±2.37 b
Primrose oil	2.5 mM	48	43.35±3.14 a	27.47±3.61 a	16.68±2.59 a
Primrose oil	5 mM	42	30.89±3.21 b	14.91±3.14 b	4.28±2.54 bc
Cholesterol	1 mg/ml	45	29.16±6.29 b	8.33±2.89 bc	2.50±1.45 bc
Cholesterol	3 mg/ml	45	17.40±2.56 c	5.07±2.93 bc	0.00±0.00 c
Cholesterol	6 mg/ml	49	12.02±1.85 c	2.27±1.33 c	0.00±0.00 c
Significance			P<0.001	P<0.001	P<0.001

Values are from 4 trials for each treatment
Values with different letters in the same column were significantly different (P<0.001

DISCUSSION

The transbilayer dynamics of lipids in the plasma membrane of mammalian sperm cells is crucial for the fertilization process (Muller *et al.*, 1999). Recently, comprehensive overviews on the lipid dynamics in the plasma membrane of mammalian sperm cell and its possible physiological relevance have been given (Martinez and Morros, 1996 and Nolan and Hammerstedt *et al.*, 1997). The present findings clearly show that cryo- preservation of ram semen resulted in a significant decrease in the post-thawing sperm motility, viability and the in vitro fertilizing potential. These results are consistent with those of Bhosrekar

et al. (1991) and Muller *et al.* (1999) who found that, cryotreatment exerted osmotic, mechanical and chemical stresses on sperm cell that impaired dramatically the integrity of the plasma membrane. Additionally, Muller *et al.* (1999) indicated that, the damage of sperm cell by cryopreservation has been associated with alteration of the plasma membrane lipid phase. Any untimed destabilization of the lipid phase may abolish irreversibly the fertilizing potential of sperm cells (Watson, 1995). The present results reveal that, the membrane constituents (phospholipids and cholesterol) have an important role in preserving the fertilizing ability of spermatozoa .

The current results emphasize that, addition of 2.5 mg/ml primrose oil to the diluted ram semen, had an immediate beneficial effect on sperm freezability, in vitro fertilizing potential and significantly minimized the ultrastructure damage and biochemical changes that occur to many spermatozoa during cryopreservation. Gurevich *et al.* (1993) and Kolev and Dimov (1998) attributed the improvement of sperm freezability and fertilizing potential of primrose oil treated ram semen to the presence of gamma linolenic acid which is the precursor of prostaglandin E. Addition of prostaglandin to semen extender increased the life span of the spermatozoa and the cleavage rate of the inseminated oocytes. On the contrary, addition of 6 mg/ml cholesterol-3-sulfate to the extended semen, drastically inhibit ram semen freezability, in vitro fertilizing potential and lead to a pronounced damage in the sperm cell ultrastructure and increased the biochemical changes. These results are consistent with Grippo *et al.* (1994), Zeng and Terada (2000) and Perez-Pe *et al.* (2001). Cholesterol, when in excess, severely alters the fluidity of the lipid bilayer and plasma membrane properties while removal of cholesterol from the cell, is of great importance for cell function and survival (Johson *et al.* 1991; Liscum and Underwood, 1995 and Kellner-Weibet *et al.* 1998). Additionally, Watson (1979) suggested that, cholesterol have an important role in the sperm cells stability. Moreover, Mazur (1985) found that, by increasing membrane fluidity and permeability, the amount of intracellular ice formation, which is lethal to the cell on cryopreservation, will be decreased.

Transmission electron microscopy findings reveal a great damage in the plasma membrane, acrosomal membranes as well as in the mitochondrial helix of ram spermatozoa after freezing in cholesterol enriched extender in respect to primrose oil enriched extender. These results are in accordance with those reported by (Krongenaes *et al.*

(1994). Hammerstedt *et al.* (1990) and Watson (1995) related the drastic decrease in spermatozoal freezability and fertilizing potential to the effect of cholesterol-3-sulfate treated ram semen on the integrity of the plasma membrane. Moreover, Windsor (1997) found that mitochondrial respiration plays an important role in the fertilizing potential of ram spermatozoa and thus, mitochondrial injury during freezing is likely to be implicated in poor fertility of frozen ram semen. However, TEM examination of sperm cells treated with primrose oil show slight mitochondrial damage and intact plasma membrane in the majority of sperm cells. Therefore, the improvement in the sperm function and fertilizing potential of primrose oil treated frozen ram semen may be attributed to the decreased damage of plasma membrane, acrosomal membranes and mitochondrial helix during freezing. The observed disturbance in the plasma membrane integrity by TEM examination, lead to cytoplasmic enzymes leakage, which in high concentrations can be toxic to the spermatozoa. These results are in agreement with (White, 1993; Ollero *et al.* 1996 and Drokin *et al.*, 1999).

The present study show that, most of ALP and AST enzymes were leaked during freezing with cholesterol treated ram semen in respect to primrose oil treated semen. These results are consistent with those reported by (Krebs, 1972, Tang and Hoskins, 1975 and Dhami and Kodagali 1988 and 1990) who found that, AST and ALP enzymes leakage could be used as a marker for optimization of freezability and fertilizing ability of cryopreserved ram semen.

In conclusion, the present study provides a novel evidence that, the addition of primrose oil to ram semen diluents improved the ram semen freezability and in vitro fertilizing potential presumably by protecting the mitochondria, plasma membrane and acrosomal membranes from damage during cryopreservation. Meanwhile, cholesterol-3-sulfate treatment resulted in a pronounced reduction in sperm function and severe damage in sperm cells ultrastructures.

REFERENCES

Bhosrekar, M.R; Mokashi,S.; Purohit, J.R.; Gokhale, S.B. and Mangurkar, B.R. (1991): Studies on the effect of deep freezing and seasons on the leakage of aspartate amino transferase into extra cellular medium and sperm morphology of murrah buffalo bulls. Anim. Reprod. Sci.; 26: 219-226.

Buhr, M.M.; Curtis, E.F. and Somnapan Kakuda, N. (1994): Composition and behavior of head membrane lipids of fresh and cryopreserved boar sperm. Cryobiology; 31: 224-238.

Byrne, G.P.; Lonergan, P.; Wade, M.; Duffy, P.; Donovan, A.; Hanrahan, J.P. and Boland, M.P. (2000): Effect of freezing rate of ram spermatozoa on subsequent fertility in vivo and in vitro. Anim. Reprod. Sci.; 62 (4): 265-275.

Cohn, J.S.; McNamara, J.R. and Schaefer, E.J. (1988): Lipoprotein cholesterol concentrations in plasma of human subjects as measured in the fed and fasted states. Clin.Chem.; 34: 2456.

Costat Computer Program (1986): Version 3.03 copyright software.

*Dhami, A. J. and Kodagali, S.B.(1988):*Leakage of phosphatases from buffalo spermatozoa during freezing: effect of bulls, seasons, extenders and freezing stages. Indian J. Anim. Reprod.; 9: 25–31.

Dhami, A.J. and Kodagali, S.B. (1990): Freezability, enzyme leakage and fertility of buffalo spermatozoa in relation to the quality of semen ejaculates and extenders. Theriogenology, 34: 853–863.

Drokin, S.I.; Vaisberg, T.N.; Kopeika, E.F.; Miteva, K.D. and Pironcheva, G.L. (1999): Effect of cryopreservation on lipids and some physiological features of spermatozoa from rams pastured in highlands and in valleys. Cytobios.;100 (393):27-6.

Evans, G. and Maxwell, W.M.C. (1986): Salamon's Artificial Insemination of Sheep and Goats. Butterworths, Sydney.

Filley, S.J.; Turner, H.A. and Stormshari, F. (1999): Prostaglandin F_2 concentrations, fatty acid profiles, and fertility in lipid-infused post partum beef heifers.Biol. Reprod.; 61: 1317-1323.

Grippo, A.A.; Anderson, S.H.; Capman, D.A.; Henault, M.A. and Killian, G.J. (1994): Cholesterol, phospholipid and phospholipase activity of ampullary and isthmic fluid from the bovine oviduct. J. Reprod. Fertil.; 102: 87–93.

Gurevich, M.; Harel - Markowitz, E.; Marcus, S.; Shore, L.S. and Shemesh, M. (1993): Prostaglandin production by the oocyte cumlus complex around the time of fertilization and the effect of prostaglandin E on the development of the early bovine embryo. Reprod. Fertil. Dev.; 5 (3): 281–283.

Hammerstedt, R.H.; Graham, J.K. and Nolan, J.P. (1990): Cryopresevation of mammalian sperm: What we ask them to survive. J. Andr.:1173–88.

Hinkovska-Galcheva, V.; Peva, D.; Momchilowa-Pnkova, A.; Petkova, D. and Koumanov, K.(1988): Phosphatidylcholine and phosphatidylethanolamine derivativas, membrane fluidity and changes in the lipolytic activity of ram spermatozoa plasma membranes. Int. J. Biochem.; 20: 867–871.

Holt, W.V. and North, R.D. (1984): Partially irreversible cold-induced lipid phase transitions in mammalian sperm plasma membrane domains: freeze-fracture study. J. Exp. Zool.; 230: 473-483.

Holt, W.V. and North, R.D. (1986): Thermotropic phase transition in the plasma membrane of ram spermatozoa. J. Reprod. Fertil.; 78: 447-457.

Johson W.J.; Mahlberg, F.H.; Rothblat, G.H. and Phillips, M.C. (1991): Cholesterol transport between cells and high density lipoproteins. Biochem. Biophys. Acta, 1085: 273–298.

Kakar, S.S. and Anand, S.R. (1984): A transmission electron microscopic study of fresh and frozen buffalo spermatozoa .Indian j. Exper .Biol .; 22: 11.

Kellner-Weibel, G.; Jerome, W.G.; Small, D.M.; Warner, G.J.; Stoltenborg, J.K.; Kearney, M.A.; Corjay, M.H.; Phillips, M.C. and Rothblat, G.H. (1998): Effect of intracellular cholesterol accumulation on macrophage viability: a model for foam cell death. Arterioscler. Thromb. Vasc. Boil.; 18: 423–431.

Khalifa, T.A.A. (2001): Effect of some antioxidants on viability of preserved buffalo and ram semen. Ph. D.Thesis, Theriogenology, Fac. Vet. Med. Cairo University.

Kolev, S.I. and Dimov, K. (1998): Influence of synthetic PGF_2 alpha and oxytocin on life time of ram's spermatozo with and without sperm plasma at 46 °C. Bulgarian J. Agr. Sci., 4:6, 831–835.

Krebs, E.G. (1972): Protein kinases. In: B.L. Horecker and E.R. stadman (Eds.), Regulation. Vol.5, Academic press, New York, pp.99-133.

Krogenaes, A.; Andersen Berg, K.; Hafne, A.L. and Engeland, E. (1994): Membrane alterations in bull spermatozoa after freezing and thawing and after in vitro fertilization. Acta., Vet., Scand.; 35 (1): 17-26.

Liscum, L. and Underwood, K.W. (1995): Interacelluar cholesterol transport and compartmentation. J. Biol. Chem.; 270: 15443-15446.

Martinez, P. and Morros, A. (1996): Membrane lipid dynamics during human sperm capacitation. Frontiers Bioscience, 1: 103-117.

Mazur, P. (1985): Basic concepts in freezing cells. In 'Deep Freezing of Boar Semen'. Eds. *L.A.Johnson and K. Larsson.* pp.91-111. Swedish Univ. Agric. Sci., Uppsala.

Milovanov, V.K. (1962): Biology of reproduction and artificial insemination of farm animals. Monograph. Selkohz. Lit. J. and Plakatov, Moscow.

Muller, K.; Pomorski, T.; Muller, P. and Herrmann, A. (1998): Stability of transbilayer phospholipids symmetry in viable ram cell after cryotreatment . J. Cell Sci., 112: (1): 11-20.

Muller, K.; Pomorski, T.; Muller, P. and Herrmann, A. (1999): Stability of transbilayer phospholipid asymmetry in viable ram sperm cells after cryotreatment. J. Cell Sci., 112: 11-20.

Nolan, J.P.; Magargee, S.F.; Posner, R.G. and Hammerstedt, R.H. (1995): Flow cytometric analysis of transmembrane phospholipids movement in bull sperm. Biochemistry, 34: 3907-3915.

Nolan, J.P. and Hammerstedt, R.H. (1997): Regulation of membrane stability and the acrosome reaction in mammalian sperm. FASEB J.; 11: 670-682.

O'Brien, J.K.; Dwarte, D.; Ryan, J.P.; Maxwell, W.M.C. and Evans, G. (1996): Developmental capacity, energy metabolism and ultrastructure of mature oocytes from prepubertal and adult sheep .Reprod. Fert. Dev.; 8: 1029–1037.

Ollero, M.; Blanco, T.M.; Lopez-Perez, M.J. and Cebrian- Perez, J.A. (1996): Surface changes associated with ram sperm cryopreservation revealed by counter-current distribution in an aqueous two-phase system. Effect of different cryoprotectant. J. Chrom .B. Biom. Appl.; 17:680(1-2):157-164.

Parrish, J.J.; Susko-Parrish, J.; Winer, M.A. and First, N.L. (1988): Capacitation of bovine sperm by heparin. Biol. Reprod.; 38: 1171-1180.

Perez-Pe, R.; Cebrian-Perez, J.A. and Muino-Blanco, T. (2001): Semen plasma proteins prevent cold-shock membrane damage to ram spermatozoa. Theriogenology,56 (3): 425-434.

Reynolds, E.S. (1963): The use of lead citrate at high pH as an electron obaque stain in electron microscopy. J. Cell. Biol .;17:258.

Tang, F.Y. and Hoskins, D.D. (1975): Phosphoprotein phosphatase of bovine epididymal spermatozoa. Biochem .Biophys .Res. Comm., 62: 328-335.

Upreti, G.C.; Payne, S.R.; Duganzich, D.M.; Oliver, J.E. and Smith, J.F. (1996): Enzyme leakage during cryopreservation of ram spermatozoa. Anim. Reprod. Sci.; 41: 27-36.

Watson, D. (1960): Cholesterol colorimetric method. Clin. Chem. Acta, 5: 637.

Watson, P.F. (1979): The preservation of semen in mammales. In 'Oxford Reviews of Reproductive Biology', Vol. 1. Eds. *C.A.Finn.* pp.283-350. Oxford University Press, New York.

Watson, P.F. (1995): Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. Reprod. Fert. Dev.; 7: 871-891.

White, I.G. (1993): Lipids and calcium uptake of sperm in relation to cold shock and preservation: A review. Reprod. Fert. Dev.; 5 (6): 639–658.

Windsor, D.P. (1997): Mitochondrial function and ram sperm fertility. Reprod. Fert. Dev.; 9(3): 279-284.

Wintenberger–Torres, S. and Sevellec, C. (1987): 'Atlas of the Early Development of the Sheep Embryo. (Institute National de la Recherche Agronomique, Paris).

Zeng, W. and Terada, T. (2000): Freezability of boar spermatozoa is improved by exposure to 2-hydroxypropyl-betacylodextrin. Reprod. Fert. Dev. ; 12: 223-228

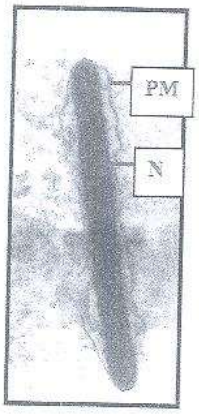

Fig. 1

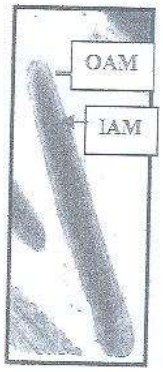

Fig. 2

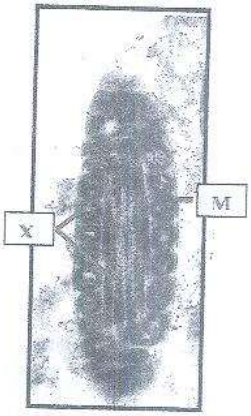

Fig. 3.

Fig. 1: Electron micrograph for a sagittal section in the sperm head from a frozen-thawed semen sample treated with primrose oil showing intact plasma membrane (PM) and the nucleus content (N) is homogenous in the electron density. (X 14 000).

Fig. 2: Electron micrograph for a sagittal section in the sperm head from a frozen-thawed semen sample treated with primrose oil illustrating intact outer (OAM) and inner acrosomal membranes (IAM). Also, the subacrosomal space is evident. (X 14 000).

Fig. 3: Electron micrograph for a transverse section in the neck region of the tail from a frozen-thawed semen sample treated with primrose oil showing mitochondria (M) with slight electron translucent spaces in the mitochondrial matrix. The mitochondrial helix is closely attached to the underlying axonemal complex. (X 28 000).

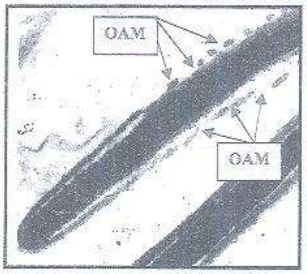

Fig. 4: Electron micrograph for a sagittal section in the sperm head from a frozen-thawed semen sample treated with cholesterol-3-sulphate illustrating complete loss of the plasma membrane, segmentation of the outer acrosomal membrane (OAM) (arrows), and swollen acrosome. (X 35 000).

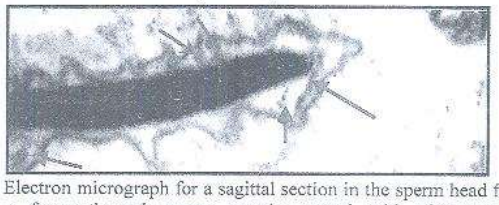

Fig. 5: Electron micrograph for a sagittal section in the sperm head from a frozen-thawed semen sample treated with cholesterol-3-sulphate showing multiple electron dense materials just underneath the plasma membrance (may be destructed material of the outer acrosomal membrane) (arrows). (X 28 000).

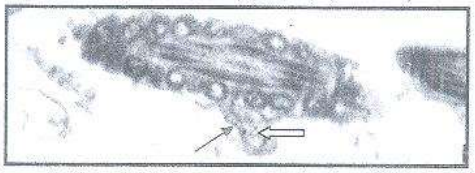

Fig. 6: Electron micrograph for a cross section in the mid-piece region of the tail from a frozen-thawed semen sample treated with cholesterol-3-sulphate illustrating severe degeneration of the mitochondria that contain electron-translucent spaces with absence of their transverse cristae. Also the plasma membrane, at some areas, appeared evaginated and detached (thin arrow) from the underlying mitochondrial helix and contained electron dense materials within the evagination (may be destructed mitochondrion) (open arrow). (X 28 000).

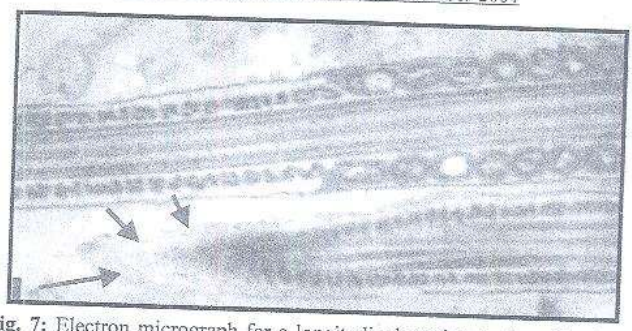

Fig. 7: Electron micrograph for a longitudinal section in the mid-piece (upper) and en-piece (lower) of the tail from a frozen-thawed semen sample treated with cholesterol-3-sulphate showing marked vacuolation of the mitochondrial matrix (marked electron translucent spaces) and the plasma membrance appeared thin and with less electron density at the end-piece (arrows). (X 35 000).

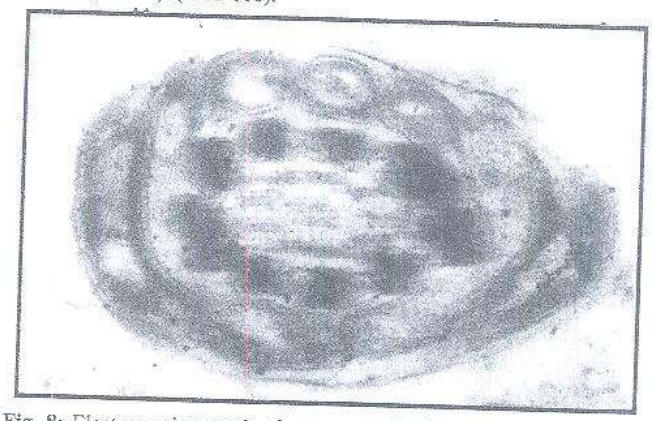

Fig. 8: Electron micrograph of a cross section in the neck region (note the presence of mitochondria in different orientation) showing severe degeneration in the mitochondria that contained electron-translucent spaces with complete absence of the transverse cristae and some mitochondria are completely disappeared. (X 40 000).

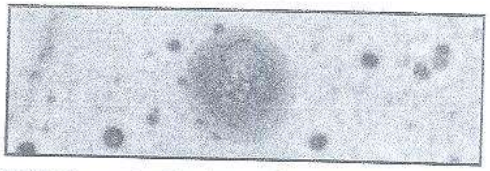

Fig. 9: Fertilized oocyte showing male pronucleus formation from matured oocyte that inseminated with primrose oil-treated frozen ram semen. (X 10).

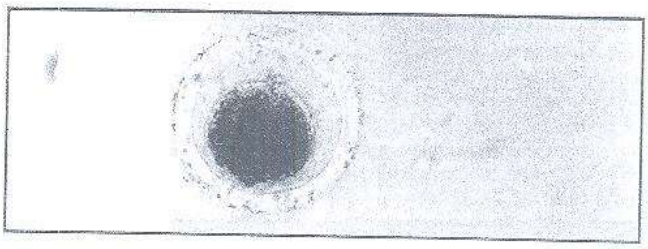

Fig. 10: Compact morula stage from matured oocyte that inseminated with primrose oil-treated frozen ram semen. (X 10).

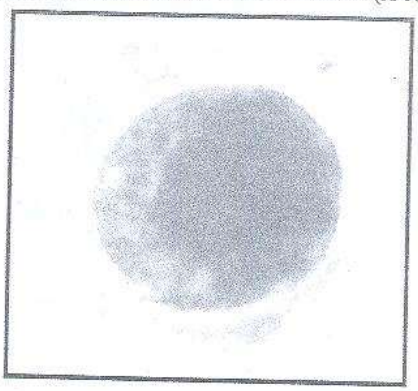

Fig. 11: Blastocyst stage from matured oocyte that inseminated with primrose oil-treated frozen ram semen. (X10).