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INFLUENCE OF SOME FATTY ACIDS AND CHOLESTEROL ADDITION TO SEMEN EXTENDER ON FREEZABILITY AND IN VITRO FERTILIZING POTENTIALS OF RAM SPERMATOZOA

(With 6 Tables)

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تأثير إضافة بعض الأحماض الدهنية والكوليسترول لممددات المنى على قابلية حيامن الكباش للتجميد ومعدل الإخصاب المعملي

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تبين أن الأحماض الدهنية والكوليسترول الموجودة في الغشاء المحيط بحيامن الكباش تلعب دورا حيويا في تحديد قدرتها الإخصابية ولكن هذه الأحماض الدهنية تتغير نسبتها بشكل كبير أثناء تجميد حيامن الكباش مما يؤثر على قدرتها الإخصابية ولذلك تهدف هذه الدراسة إلى محاولة تحسين معدلات تجميد حيامن الكباش وكذلك قدرتها الإخصابية وذلك بإضافة بعض الأحماض الدهنية والكوليسترول بتركيزات مختلفة إلى السائل المنوي المخفف للكباش قبل التجميد. تم تجميع عينات سائل منوي من ستة كباش بتراوح عمرها من ٢ إلى ٣ سنة حيث خففت في مخفف الترس المضاف إليه تركيزات مختلفة من الأحماض الدهنية والكوليسترول. وبعد تبريد وتجميد السائل المنوي بالنظام الفرنسي تم تقييم عينات السائل المنوي من حيث النسبة المئوية للحركة بعد التخفيف، بعد الإسالة، تشوهات القلنسوة وكذلك حيوية السائل المنوي بعد التجميد كما تم استخدام السائل المنوي المعالج بتركيزات مختلفة من الأحماض الدهنية والكوليسترول لدراسة قدرتها الإخصابية وذلك باستخدام تكنولوجيا الإخصاب المعملي. ولقد أسفرت النتائج عن أن إضافة ١ مللي جرام لكل ملي من حامض البالماتيك أو ٥ مللي جرام لكل مللي من حامض اللينوليك أدت إلى زيادة معنوية في النسبة المئوية للحركة بعد الإسالة (٦٤,٠٠ و ٥٦,٠٠% على التوالي) ، حيوية السائل المنوي بعد التجميد (١٨٨,٠٠ و ١٥٤,٠٠ على التوالي) كما أدى إلى انخفاض معنوي في النسبة المئوية لتشوهات القلنسوة (١٥,٠٠ و ١٨,٠٠ % على التوالي) وكذلك أدى إلى زيادة معدل الإخصاب المعملي (٥٣,٨٤ % و ٥١,٧٢ % على التوالي) ومعدل النمو إلى طور البلاستوسيست (١٨,٧٥ و ١٦,٦٦% على التوالي). وكان على النقيض من ذلك الكوليسترول حيث وجد أن إضافته إلى السائل المنوي المخفف للكباش حتى ولو بتركيز منخفض أدى إلى انحدار معنوي في النسبة المئوية للحركة بعد الإسالة (٤٠,٠٠ %) وحيوية

السائل المنوي بعد التجميد (٩٦,٠٠) كما أدى إلى زيادة معنوية في النسبة المئوية لتشوهات القلنسوة (٢٧,٠٠ %) وكذلك انخفاض حاد في النمو إلى طور البلاستوسيست (٣,٧٧ %). ولذلك توصي هذه الدراسة بإضافة حامض البالماتيك اوحامض اللينوليك لممددات السائل المنوي للكباش لما لها من أهمية كبرى في تحسين قابلية حيامن الكباش للتجميد وكذا قدرتها الإخصابية.

SUMMARY

Cryopreservation of ram semen disrupts the membrane integrity of spermatozoa and reduces the motility and the fertilizing potential. The transbilayer dynamics of lipids in the plasma membrane of mammalian sperm cells is crucial for the fertilization process. The aim of the present study is to develop a treatment supporting the membrane integrity of ram spermatzoa during cryopreservation. Semen samples were collected from six rams aged 2-3 years, pooled together and extended in Tris based extender supplemented with different fatty acids and cholesterol concentrations. Extended semen was cooled and freezed. Percentages of motility after dilution, post – thawing and acrosomal defects as well as viability index were subjectively assessed. Frozen – thawed ram semen treated with low or high concentrations of fatty acids and cholesterol were used to assess their in vitro fertilizing potential. Addition of 1 mg/ml palmitic acid or 5mg /ml linoleic acid significantly increased ($P<0.001$) post-thawing sperm motility (64.00 and 56.00%, respectively), viability indices (188.00 and 154.00, respectively), decreased acrosomal defect (15.00 and 18.00 %, respectively), enhanced significantly ($P<0.05$) in vitro fertilization rate (53.84 and 51.72 %, respectively) and in vitro blastocyst production (18.75 and 16.66%, respectively). On the other hand, addition of cholesterol to the diluted ram semen, even in low concentration, resulted in a drastic decrease in the post - thawing motility (40.00 %), viability index (96.00), increased acrosomal defect (27.00%) and inhibited severely in vitro blastocyst production (3.77%). In conclusion, the current results illustrated that, the addition of 1mg/ml palmitic or 5mg/ml linoleic acid to ram semen extender, protected ram sperm plasma membrane against cryoinjury, improved semen freezability and in vitro fertilizing potential. By contrast, cholesterol addition to the diluted ram semen drastically inhibited ram semen freezability and in vitro fertilizing potential.

Key words: *Fatty acids, cholesterol, semen, fertilizing potentials, ram, spermatozoa.*

INTRODUCTION

The plasma membranes of mammalian spermatozoa contrast sharply with those of mammalian somatic cells in their composition and biophysical properties (Wolf *et al.*, 1990 and Cerolini *et al.*, 2001). Sperm plasma membranes have unusually high levels of ether-linked lipids or of highly unsaturated fatty acyl groups (White, 1993).The movement and distribution of phospholipids between the exoplasmic and cytoplasmic leaflet of the plasma membrane of ram spermatozoa is a highly dynamic, ATP- dependent process (Muller *et al.*, 1997).The transbilayer dynamics of lipid in the plasma membrane of ram spermatozoa is crucial for the fertilization process (Nolan and Hammerstedt, 1997 and Muller *et al.*, 1999) .

Cryopreservation has been associated with a dramatic alteration of the plasma membrane lipid content and phospholipids release (Darin-Bennett *et al.*, 1973 and Oliw *et al.*, 1993). These changes of the lipid composition of sperm membranes have been responsible for the increase in the plasma membrane fluidity upon cryotreatment (Hinkovska-Galcheva *et al.*, 1989; Buhr *et al.*, 1994). The susceptibility of ram spermatozoa to damage during cryopreservation is linked with a high ratio of unsaturated: saturated fatty acids in the phospholipids of the plasma membrane and low cholesterol content (White, 1993).

Cholesterol phospholipids ratio in the sperm membrane may in fact play a mechanistic role in membrane stability and appear to influence the fertilizing potential of ram spermatozoa (Langlais and Roberts, 1985 and Grippo *et al.*, 1994). Recently, there is a strong evidence that, the addition of phospholipids to diluted ram semen may provide immediate protection against cryopreservation effects and improve sperm motility. However, the physiological role of membrane phospholipids and cholesterol in maintaining the fecundity of ram spermatozoa during cryopreservation remains to be elucidated. Therefore the present study was designed to investigate the effects of some fatty acids and cholesterol addition to ram semen extender on their freezability and fertilizing potential in vitro.

MATERIALS and METHODS

Animals:

Six mature, clinically health Barki rams, aged 2-3 years were used. They were kept at the experimental farm of Animal Reproduction Research Institute (ARRI) Giza, Egypt.

Semen collection and extender:

Semen samples were collected twice weekly from rams using artificial vagina that was adjusted to a proper condition. Semen samples of at least 70% initial motility and 3×10^9 sperm cells / ml were used. Immediately after collection, samples were split into 16 portions and diluted at 1:19 ratio at 30°C with Tris based extender (Evans and Maxwell 1986), containing different fatty acid concentrations as follow; extender alone (control); palmitic acid (1,2 and 4mg / ml); linoleic acid (5,10 and 20 mg/ml); arachidonic acid (1, 5 and 10 mg /ml) ; lecithin (5, 10, 20 mg/ml) and cholesterol (2,5 and 10 mg/ml).

Processing of ram semen:

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

Evaluation of semen freezability:

After 24 hour, frozen ram semen samples were thawed in water bath at 40°C for 30 second. Sperm motility was subjectively assessed immediately after dilution, post- thawing and after 1,2and 3 hours of thawing. The post-thawing viability indices were recorded according to Milovanov (1962). The percentage of abnormal acrosome was recorded after thawing in smears stained by Fast green FCF according to Wells and Awa (1970).

Evaluation of fertilizing potential of the treated semen in vitro:

Frozen semen samples treated with low or high concentrations of fatty acid and cholesterol were used for evaluation of their fertilizing ability in vitro.

In vitro oocyte maturation:

Cumulus – oocyte complexes (COCs) were obtained by slicing of sheep ovaries and collecting groups of 20 COCs with homogenous ooplasm surrounded by compact multi layers of cumulus cells were selected for culture in 50- µl drops of TCM – 199 medium supplemented with10 µg / ml luteinizing hormone, 5µg / ml follicle stimulating hormone and 1µg / ml estradiol-17β, under sterile mineral oil at 39 °C for 24 hour in an atmosphere of 5% CO_2 in air (O'Brien *et al.*, 1996).

In vitro preparation of the sperm

Straws of frozen ram semen from each treatment were thawed in a water bath at 37 °C for 30 sec. 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 resupended in F-TALP media containing 10-µg/ml heparin. After appropriate dilution, 2µl of sperm suspension was added per fertilization drop at a final concentration of 2.5×10^5 sperm cell / ml. Gametes were co-incubated in fertilization drops under silicon oil for 18 hours at 39 °C in 5% CO_2 in humidified air. After oocyte – sperm incubation, some of oocyte were fixed in acetic acid - ethanol (1:3) and stained with 1 % aceto- orcein stain to assess fertilization at 400x magnification. Fertilization was defined by the presence of either the sperm tail associated with the male pronucleus or the presence of 2 pronuclei. Groups of the inseminated oocyte were then freed from cumulus cell and attached spermatozoa by gentle pipetting and cultured in TCM-199 medium with HEPES modification. The proportion of cleaved oocytes was recorded 48h after insemination and those developed to the morula and blastocyst stages were recorded at 5- to 7- days post insemination (Wintenberger - Torres and Sevellec, 1987).

Statistical analysis:

By using the Costat Computer Program, version 3.03 copyright (1986) cottort software, all data were subjected to analysis of variance (ANOVA). Treated means were compared by the least significant difference test (LSD) at 1% and 5% level at probability. In vitro fertilization rate and embryos development were analyzed using Chi-square analysis (X^2). The Pearson correlation test was used to calculate the correlation between semen parameters measured and in vitro fertilization rate and in vitro embryo production.

RESULTS

Data regarding the effects of different fatty acids and cholesterol concentrations on the motility of cryopreserved ram semen are outlined in Table 1. The percentage of motility immediately after dilution was nearly similar among different treatments. However, the percentage of the post- thawing sperm motility was significantly increased ($P < 0.001$) with 1 mg/ml palmitic acid and 5 mg/ml linoleic acid (64.00±2.44 and 56.00±2.44%, respectively) compared with control, cholesterol and

arachidonic acid at all concentrations .On the other hand, the percentage of post- thawing sperm motility was significantly decreased with the addition of high cholesterol concentration to the Tris based extender (7.00±1.22%). Additionally, the highest percentage of sperm motility reduction was recorded with the high cholesterol concentration (91.32±1.56%), while the lowest percentage of sperm motility reduction was recorded with inclusion of low concentration of both palmitic acid and linoleic acid (21.90 ± 2.68 and 32.50 ±2.96%, respectively).

Table 1: Effect of different concentrations of fatty acids and cholesterol on post-thawing motility of ram spermatozoa (Means±SE).

Treatment	Concentration / ml	Motility after Dilution (%)	Post - thawing motility (%)	Reduction of sperm motility (%)
Control		83.00 ± 1.22^{a}	38.00 ± 1.99^{def}	54.11 ± 2.68^{cde}
Palmitic Acid	1 mg	82.00 ± 1.22^{a}	64.00 ± 2.44^{b}	21.90 ± 2.68^{g}
	2 mg	86.00 ± 2.44^{a}	56.00 ± 5.87^{ab}	35.27 ± 5.36^{fg}
	4 mg	85.00 ± 2.73^{a}	38.00 ± 3.73^{def}	55.52 ± 3.36^{bcde}
Linoleic Acid	5 mg	83.00 ± 1.22^{a}	56.00 ± 2.44^{ab}	32.50 ± 2.96^{fg}
	10 mg	82.00 ± 1.99^{a}	52.00 ± 4.89^{abc}	36.68 ± 5.50^{fg}
	20 mg	85.00 ±1.57^{a}	56.00 ± 2.44^{ab}	33.88 ± 3.76^{fg}
Lecithin	5 mg	82.00 ± 1.22^{a}	48.00 ± 7.34^{bcd}	41.91 ± 8.04^{ef}
	10 mg	85.00 ± 1.57^{a}	48.00 ± 6.63^{bcd}	43.59 ± 7.60^{ef}
	20 mg	83.00 ± 1.22^{a}	47.00 ± 6.63^{bcd}	43.38 ± 7.60^{ef}
Arachidonic Acid	1 mg	85.00 ± 1.57^{a}	38.00 ± 1.99^{def}	55.09 ± 2.68^{bcde}
	5 mg	81.00 ± 3.31^{a}	32.00 ± 3.73^{ef}	60.07 ± 4.47^{bcd}
	10 mg	86.00 ± 1.86^{a}	26.00 ± 2.44^{f}	69.75 ± 2.68^{b}
Cholesterol	2 mg	84.00 ± 1.86^{a}	40.00 ± 3.16^{cdef}	52.41 ± 3.35^{de}
	5 mg	81.00 ± 0.99^{a}	26.00 ± 2.44^{f}	67.94 ± 2.90^{bc}
	10 mg	81.00 ± 0.99^{a}	7.00 ± 1.22^{g}	91.32 ± 1.56^{a}
Over all mean		83.37 ± 0.45	42 ± 1.83	49.71 ± 2.17
Significance		Ns (F=0.97)	(F= 12.05) P< 0.0001	(F= 13.17) P < 0.0001

Values are from 5 trials for each treatment.
Values with different letters in the same column are significantly different at least (P<0.001).

The data presented in Table 2, showed that, supplementation of Tris- based extender with 1 mg / ml palmitic acid significantly increased (P< 0.001) viability index (188.00 ±14.45) as compared to control (125.00 ± 3.87) and all other fatty acid concentrations. Whereas, the lowest viability index was recorded with 10 mg / ml cholesterol (13.5 ± 0.60). Likewise, the results presented in Table 2, showed that low

concentration of palmitic acid, linoleic acid and lecithin supplemented Tris- based extender significantly decreased (P<0.001) the percentage of acrosomal defect (15.00 ± 1.64, 18.00± 1.64 and 18.4 ± 2.67 %, respectively). On the other hand, the highest percentage of acrosomal defect was recorded with high concentration of cholesterol, arachidonic acid and control (33.6 ± 1.06, 33.20 ± 0.91 and 30.00 ± 5.93 %, respectively).

Table 2: Effect of different concentrations of fatty acids and cholesterol on the viability indices and acrosomal defect of ram spermatozoa Means±SE).

Treatment	Concentration / ml	Viability indices	Acrosomal defect (%)
Control		125.00 ± 3.87 ^{cde}	30.00 ± 5.93 ^{ab}
Palmitic Acid	1 mg	188.00 ± 14.45 ^{a}	15.00 ± 1.64 ^{e}
	2 mg	163.00 ± 16.01 ^{ab}	18.60 ± 2.20 ^{cde}
	4 mg	129.00 ± 9.31 ^{cd}	20.40 ± 4.35 ^{cde}
Linoleic Acid	5 mg	154.00 ± 11.22 ^{bc}	18.00 ± 1.64 ^{de}
	10 mg	144.00 ± 12.87 ^{bc}	18.40 ± 0.39 ^{cde}
	20 mg	149.00 ± 8.85 ^{bc}	21.00 ± 2.60 ^{cde}
Lecithin	5 mg	150.00 ± 15.57 ^{bc}	18.40 ± 2.67 ^{cde}
	10 mg	111.00 ± 9.13 ^{de}	20.40 ± 1.74 ^{cde}
	20 mg	144.00 ± 14.08 ^{bc}	24.80 ± 3.48 ^{bcd}
Arachidonic Acid	1 mg	111.00 ± 5.99 ^{de}	21.00 ± 1.89 ^{cde}
	5 mg	94.00 ± 1.86 ^{e}	24.60 ± 2.20 ^{bcd}
	10 mg	62.00 ± 8.74 ^{f}	33.20 ± 0.91 ^{a}
Cholesterol	2 mg	96.00 ± 2.44 ^{e}	27.00 ± 1.81 ^{abc}
	5 mg	55.00 ± 2.73 ^{f}	31.20 ± 2.59 ^{ab}
	10 mg	13.5 ± 0.60 ^{g}	33.60 ± 1.06 ^{a}
Over all mean		118.03 ± 5.42	23.47 ± .0. 87
significance		(F= 20.78) P< 0.001	(F= 4.88) P< 0.001

Values are from 5 trials for each treatment.
Values with different letters in the same column are significantly different at least (P<0.001).

The results presented in Table 3, revealed that, although the sperm penetration rates did not differ among low concentrations of different treatments and control, the rate of male pronuclei formation (MPN) was significantly increased (P< 0.05) in oocyte that was

inseminated with frozen - thawed ram semen treated with low concentrations of palmitic acid (53.84%) and linoleic acid (51.72 %) compared with control (32.61%). Moreover, the rates of development to the blastocyst, were significantly enhanced (P<0.05) in oocytes that inseminated with low concentrations of palmitic acid and linoleic acid (18.75 and 16.66 %, respectively) as compared to cholesterol and control (3.77 and 2.44 %, respectively), Table, 4.

Table 3: Influence of low concentrations of fatty acids and holesterol on in vitro fertilization of sheep oocytes.

Treatments	Concentrations / ml	No. of oocytes	Penetration rate		Fertilization rate	
			No.	(%)	No.	(%)
Control		46	29	(63.04)^{a}	15	(32.61)^{b}
Palmitic acid	1 mg	52	36	(69.23)^{a}	28	(53.84)^{a}
Linoleic acid	5 mg	58	40	(68.97)^{a}	30	(51.72)^{a}
Lecithin	5 mg	67	44	(65.67)^{a}	26	(38. 81)^{ab}
Arachidonic acid	1 mg	65	45	(69.23)^{a}	25	(38.46)^{ab}
cholesterol	2 mg	55	34	(61.82)^{a}	20	(36.36)^{ab}

Values with different letters in the same column are significantly different at least (P<0.05).

Table 4: Influence of low concentration of fatty acids and cholesterol on in vitro sheep embryonic development.

Treatments	Concentrations / ml	No. of oocytes	Cleavage rate		Morula		Blastocyst	
			No.	(%)	No.	(%)	No.	(%)
Control		41	14	(34.15)^{a}	4	(9.76)^{b}	1	(2.44)^{b}
Palmitic acid	1 mg	48	22	(45.83)^{a}	13	(27.08)^{a}	9	(18.75)^{a}
Linoleic acid	5 mg	36	15	(41.67)^{a}	10	(27.78)^{a}	6	(16.66)^{a}
Lecithin	5 mg	45	17	(37.78)^{a}	8	(17.78)^{ab}	5	(11.11)^{a}
Arachidonic acid	1 mg	49	18	(36.73)^{a}	6	(12.24)^{ab}	3	(6.12)^{a}
Cholesterol	2 mg	53	18	(33.96)^{a}	5	(9.43)^{b}	2	(3.77)^{a}

Values with different letters in the same column are significantly different at least (P<0.05).

As shown in Tables 5 and 6, insemination of sheep oocytes with frozen – thawed semen treated with high concentration of cholesterol decreased significantly (P<0.05) male pronuclei formation, cleavage rate, morula and blastocysts development (26.92, 21.43, 4.76 and 0.00 %, respectively) compared to high concentrations of palmitic acid and linoleic acid. Additionally, there was a non-significant difference among other treated groups regarding the in vitro fertilization rate or the embryo developmental rates.

Table 5: Influence of high concentrations of fatty acids and cholesterol on in vitro fertilization rate of sheep oocytes.

Treatments	Concentrations / ml	No. of oocytes	Penetration rate		Fertilization rate	
			No.	(%)	No.	(%)
Control		55	35	(63.64)^a	21	(38.18)^a
Palmitic acid	4 mg	48	31	(64.58)^a	23	(47.92)^a
Linoleic acid	20 mg	62	37	(59.67)^a	25	(40.32)^a
Lecithin	20 mg	56	32	(57.14)^a	21	(37.50)^a
Arachidonic acid	10 mg	42	23	(54.76)^a	17	(40.48)^a
Cholesterol	10 mg	52	22	(42.31)^b	14	(26.92)^b

Values with different letters in the same column are significantly different at least ($P<0.05$).

Table 6: Influence of high concentration of fatty acids and cholesterol on in vitro sheep embryonic development.

Treatments	Concentrations / ml	No. of oocytes	Cleavage rate		Morula		Blastocyst	
			No.	(%)	No.	(%)	No.	(%)
Control		48	17	(35.42)^a	4	(8.33)^b	2	(4.17)^a
Palmitic acid	4 mg	39	17	(43.59)^a	8	(20.51)^a	4	(10.26)^a
Linoleic acid	20 mg	51	22	(43.14)^a	8	(15.69)^{ab}	4	(7.84)^a
Lecithin	20 mg	44	15	(34.09)^a	5	(11.36)^{ab}	2	(4.55)^a
Arachidonic acid	10 mg	36	13	(36.11)^a	4	(11.11)^{ab}	2	(5.56)^a
Cholesterol	10 mg	42	9	(21.43)^b	2	(4.76)^b	0	(0.00)^b

Values with different letters in the same column are significantly different at least ($P<0.05$).

The current results demonstrated that, supplementation of Tris – based extender with low concentration of fatty acids existed a significantly high correlation between the measured semen parameters (post – thawing motility, viability index and acrosomal defect) and in vitro fertilization rate and embryo development to the morula and blastocyst stages. However, this correlation was not recorded when high concentrations of fatty acids were added to the Tris – based extender.

DISCUSSION

The main characteristic feature of the plasma membrane of mammalian spermatozoa is the asymmetric transbilayer distribution of lipid (Nolan *et al.* 1995), which has been suggested to play an important role in the fertilization process of spermatozoa (Nolan and Hammerstedt, 1997). The results in the current study emphasize that, addition of appropriate concentrations of fatty acids to diluted ram semen can play an important role in improving freezability and vitro fertilizing potential of ram spermatozoa. These results are entirely consistent with Quinn *et*

al. (1980); Watson and Anderson (1983); Hinkovska – Galcheva *et al.* (1988) and Perez-Pe *et al.* (2001). They found that, the addition of phospholipids to suspension of ram semen prior to freezing, decreased membrane damage, increased the percentage of motile spermatozoa and those with intact acrosome post- thawing. There is a growing evidence that fatty acid composition of sperm membranes especially their unsaturated components determine their biophysical characteristics such as fluidity and specific sperm function, including sperm motility and fertilizing capacity (Ladha, 1998).

Cryopreservation of ram spermatozoa altered severely the fatty acids composition of sperm membranes and these variation were mainly due to a decrease of polyunsaturated fatty acids as well as an increase of the diacyle derivatives of phosphatidyl choline and phosphatidyl ethanolamine (Hinkovska- Galcheva *et al.*, 1988; Drokin *et al.*, 1999 and Cerolini *et al.*, 2001) .The susceptibility of spermatozoa to damage during freezing is linked with a high ratio of polyunsaturated: saturated fatty acids in the phospholipids of plasma membranes of ram spermatozoa (White, 1993). Additionally, the high unsaturated fatty acid content of sperm also make them susceptible to damage from lipid peroxidation which adversely affects sperm motility, metabolism, ultra structure and fertility (Maxwell and Watson, 1996; Iwata *et al.*, 1998 and Badr *et al.*, 2003). In agreement with previous results, the current results reveal a highly significant reduction in the motility, viability, acrosomal integrity and in vitro fertilizing potential of untreated frozen semen. Recently, there is a strong evidence that, the production of total polyunsaturated fatty acids decreased over the period of storage and is balanaced by a complementary increase in the proportion of total saturated fatty acids (Cerolini *et al.*, 2000). Moreover, the components and surfactants in the freezing diluents have also been shown to affect membrane fluidity and the viability of spermatozoa after cryopreservation (Pettitt and Buhr, 1998). Therefore, it is reasonable to assume that modification of membrane fluidity before freezing might be linked to the damage caused during freezing and thawing. Moreover, it has been demonstrated that when the yeast cell membrane was more fluid, the cells survived frozen – thawed damage significantly better (Kruuv *et al.* 1978). Additionally, Watson (1979) suggested that, if the cell is sufficiently permeable to water, the differential vapor pressure for water across the plasma membrane remains small during freezing and dehydration results as water moves out of the cell to freeze extra cellulary. By increasing membrane fluidity and permeability, the amount

of intracellular ice formation, which is lethal to cell in cryopreservation, will be decreased (Mazur, 1985). Therefore, the improvement of ram semen freezability and fertilizing capacity with palmitic acid and linoleic acid observed in the current study may be attributed to its ability to modify plasma membrane fluidity and permeability in such a way as to increase membrane stability during cooling.

Moreover, the current results underlines that, the protective effects of fatty acids on sperm functions during freezing are dose-dependent. These results are in agreement with Pellicer- Rubio and Combarnous (1998) who found that, the major unsaturated fatty acid (Oleic acid) but not the major saturated fatty acid (palmitic acid) exhibited dose- dependebt determintal effects on goat spermatozoa.

The present results strongly suggest that cholesterol addition to semen extender drastically inhibit sperm motility, viability, acrosomal integrity and sperm fertilizing potential. These results are parallel to previous studies on cholesterol: phospholipid ratio (Grippo *et al.* 1994 and Zeng and Terada, 2000). Cholesterol, a major structural: constituent of plasma membranes, is one of the important regulators of membrane fluidity (Hartel *et al.*, 1998) and permeability (McGrath, 1988). Membrane cholesterol efflux induces an enhanced membrane fluidity and permeability (Cooper *et al.* 1978). Conversely, elevation of membrane cholesterol is associated with decreased membrane fluidity (Hartel *et al.*, 1998) and therefore, increased susceptability of sperm to cryoinjury.

The relationship between sperm motility, viability, acrosomal integrity and in vitro fertilization rates and embryo production was demonstrated in the present study, as the percentage of post- thawing sperm motility increased, so did the chances of morula and blastocyst production in vitro. These results are in agreement with the findings of Aziz *et al.* (2000) and Lee *et al.* (2001). They recorded high relationship between sperm motility and IVF rates.

In conclusion, the current study provides novel evidence that, the addition of

1mg/ ml palmitic acid or 5mg / ml linoleic acid to the dilutedram semen enhance ram semen freezability, in vitro fertilizing potential, presumably by modifying the plasma membrane fluidity. Whereas, the addition of cholesterol to ram semen diluent result in a drastic decrease in the percentage of ram sperm freezability and their fertilizing ability. The current results demonstrate that, supplementation of Tris – based extender with low concentration of fatty acids exists a significantly high

correlation between the measured semen parameters (post – thawing motility, viability index and acrosomal defect) and in vitro fertilization rate and embryo development to the morula and blastocyst stages.

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