



**Influence of linoleic acid supplementation on the quality of frozen-thawed ram semen**

Elsherbiny, H.<sup>\*</sup>; Ghallab, A. M.<sup>\*</sup> and Abou-Ahmed, M. M.<sup>\*</sup>

<sup>\*</sup>Theriogenology Department,  
Faculty of Vet. Medicine, Cairo University; Giza, Egypt

**Abstract**

The present study was conducted to evaluate the effect of linoleic acid (LA) supplementation in the freezing diluent on the quality of frozen-thawed ram semen. Semen was collected from five mature fat tailed rams once weekly using the conventional A.V. of rams. Semen samples were processed as a pool, divided into 5 aliquots, each aliquot was diluted with egg yolk-Tris-glycerol diluent and three concentrations (50, 100 and 150  $\mu$ M) of LA were used. Diluted semen was then cooled, equilibrated and subjected to cryopreservation. Evaluation of post-thaw semen quality included progressive sperm motility, alive and abnormal sperm percentages, functional membrane integrity (HOST) and acrosome integrity. Results showed that the percentages of alive sperm (51.4 vs 40.2%;  $P < 0.05$ ) and HOST (53.2 vs 39.6%;  $P < 0.05$ ) were significantly increased only at concentration of 50  $\mu$ M of LA compared to control, respectively. Also, LA (50  $\mu$ M) decreased significantly ( $P < 0.05$ ) the percentage of abnormal sperm compared to control with ethanol (20.6 vs 29%). Regarding the intact acrosome percentage, only the 150  $\mu$ M LA decreased significantly ( $P < 0.05$ ) the intact acrosome (75 vs 80.4%) compared to control. The effects of the other concentrations on the other post-thaw semen quality parameters were non significant. It could be concluded that, addition of LA (50  $\mu$ M) to Tris based diluent improved the post-thaw quality of ram semen in terms of sperm membrane integrity and sperm morphology.

**Key words;** semen cryopreservation, n-6 fatty acids, linoleic acid and ram)

**Introduction**

Semen cryopreservation is essential for its efficient use in artificial insemination program; however, freeze-thaw damages are inevitable that induce detrimental effects on sperm functional parameters (Yoshida, 2000). The plasma membrane, a major determinant of motility and overall viability (Hammerstedt, Graham and Nolan, 1990), is greatly affected by alteration in lipid composition during cryopreservation. Loss of total phospholipids possibly from plasma membrane during cooling, freezing and thawing has been reported in bull, ram and boar spermatozoa (Darin-Bennett, Poulos and White, 1973).

The plasma membrane is a highly dynamic structure that regulates not only extracellular exchanges, but the process of fertilization as well (Flesch, Gadella, 2000). Differences in lipid composition of the spermatozoon plasma membrane have been suggested as a key factor in the freezability of spermatozoa (Parks and Lynch, 1992). In many mammalian species, up to 60% of the total fatty acids are long-chain polyunsaturated fatty acids (LCPUFA) of the n-

3 series (Poulos, Darin-Bennett and White, 1973; Nissen and Kreysel, 1983). This specific lipid composition confers a greater fluidity on the plasma membrane due to the presence of the many double bonds (Erickson, 1998).

Poly unsaturated fatty acids are extremely vulnerable to oxidative damage generated by reactive oxygen species (ROS) (Aitken and Baker, 2006; Koppers, Gargb and Aitken, 2010). Although ROS are involved in cell signaling at low levels, excessive production of ROS can lead to oxidative stress, lipid peroxidation and shortage of these fatty acids in the spermatozoal plasma membrane (De Iuliis, Thomson, Mitchell, Finnie, Koppers and Hedges, 2009). Moreover, fatty acids are responsible for changing the lipid membrane composition in many cells (Sampath and Ntambi, 2005). These fatty acids can be incorporated by the plasma membrane of the cells causing modifications in its structure and function (Zhao, Subbaiah, Mintzer, Chiu, Jakobsson and Scott, 2011).

Linoleic acid (LA) is an essential, short chain fatty acid and a precursor to long chain fatty

acids such as, ecosapentanoic acid, docosahexanoic acid and arachidonic acid (Lands, 1992). Linoleic acid improved the motility of frozen thawed bovine spermatozoa with poor freezability (Takahashi, Itoh, Nishinomiya, Katoh and Manabe, 2012).

The present study was, therefore, aimed to evaluate the effect of LA (50, 100 and 150  $\mu$ M) supplementation in the dilution medium (Tris based) on the post thaw progressive sperm motility, alive and abnormal sperm percentages, functional membrane integrity (HOST) and acrosome integrity of frozen-thawed ram semen.

### Materials and Methods

#### Animals and semen collection

Several weeks before the commencement of the experiment, five mature fat tailed rams were trained to mount an anestrous ewe as a teaser. Throughout the study, once a week ejaculate was collected from each ram early in the morning using the conventional artificial vagina of rams. This regime began three weeks prior to initiation of the experiment in order to stabilize epididymal sperm reserves and semen characteristics of rams.

#### Diluent composition

The egg yolk-Tris diluent was used in the present study for long term (-196 °C) preservation of ram semen (Evans and Maxwell, 1987). It is composed of Tris (hydroxyl methyl amino methane; 3.634 g), glucose (0.5 g), citric acid monohydrate (1.99 g), fresh chicken egg yolk (15 ml), glycerol (7 ml), penicillin G sodium (100.000 IU), streptomycin sulphate (100 mg) and glass distilled water to 100 ml. three concentrations (50, 100 and 150  $\mu$ M) of LA were supplemented to the freezing semen diluent (Sampaio, Bender, Silva and Zuccari, 2015) and ethanol was used as a solvent. The dilution rate was calculated on the basis that each insemination dose (0.5 ml straw) contains about 200 to 300 $\times$ 10<sup>6</sup> alive motile sperm pre-freezing (Fukui, Hirai, Honda and Havashi, 1993).

#### Processing of semen

Diluted semen was cooled to 5 °C over a period of 1 to 2 hours in a refrigerator and kept at 5°C for another 1 to 2 hours for equilibration (Awad and Graham, 2004). The cooled semen was loaded into 0.5 ml straws, sealed by polyvinyl

powder and arranged horizontally on cold racks. The cold racks were then lowered into liquid nitrogen vapor inside a foam (Darwish, Ziada, Shaker and Mohammed, 2003) box (54 $\times$ 35 $\times$ 18 cm), containing 10 liters of liquid nitrogen at a height of 6.50 cm above the level of liquid nitrogen for 10 minutes. The straws were then immersed in liquid nitrogen and transferred into the liquid nitrogen storage container (-196 °C). Frozen ram semen was thawed by removing two straws from liquid nitrogen container and dropping them in a water bath at 40 °C for 30 seconds (Kumar, Millar and Watson, 2003).

#### Evaluation of frozen thawed semen

Progressive motility percentage was assessed subjectively using a bright field microscope ( $\times$  200 magnification), with a warm stage maintained at 37 °C. Structural membrane integrity or alive sperm percentage and total sperm abnormalities were evaluated using eosin-nigrosin stain (Campbell, Dott and Glover, 1956). The hypo-osmotic swelling test (HOST) was used to evaluate the functional integrity of the sperm plasma membrane (Revell and Mrode, 1994). Acrosome integrity percentage was estimated using spermac stain as described by Chan, Corselli, Jacobson, Patton and King (1999).

#### Statistical analyses

The obtained data were expressed as mean $\pm$  SEM. The effect of different concentrations of LA on the studied semen parameters were tested by one-way analysis of variance after angular transformation of percentages to their corresponding arc-sin values (Snedecor and Cochran, 1989). If the F-value was significant, differences in means amongst the studied parameters were estimated by the least significant difference (LSD) using SPSS/PC version 20 software (SPSS, Chicago). Differences with values at least at  $P \leq 0.05$  were considered to be statistically significant (Daniel, 1991).

### Results

The effect of different concentrations of LA on the studied semen parameters of frozen-thawed ram semen is presented in table (1). Results showed that the percentages of alive sperm (51.4 vs 40.2%;  $P < 0.05$ ) and HOST (53.2 vs 39.6%;  $P < 0.05$ ) were significantly increased only at

concentration of 50  $\mu\text{M}$  of LA compared to control, respectively. Also, LA (50  $\mu\text{M}$ ) decreased significantly ( $P<0.05$ ) the percentage of abnormal sperm compared to control with ethanol (20.6 vs 29%). Regarding the intact acrosome percentage, only the 150  $\mu\text{M}$  LA

decreased significantly ( $P<0.05$ ) the intact acrosome (75 vs 80.4%) compared to control. The effects of the other concentrations on the other post-thaw semen quality parameters were non significant.

**Table (1):** Effect of different concentrations of linoleic acid on the studied semen parameters of frozen-thawed ram semen (Mean $\pm$ SEM).

Linoleic Acid	Progressive motility (%)	Alive percentage (%)	HOST (%)	Abnormal sperm (%)	Acrosome integrity (%)
Control (n=7)	36 $\pm$ 2.91 <sup>a</sup>	40.2 $\pm$ 3.33 <sup>b</sup>	39.6 $\pm$ 3.17 <sup>b</sup>	19.4 $\pm$ 1.32 <sup>b</sup>	80.4 $\pm$ 1.20 <sup>a</sup>
Control with ethanol (n=7)	37 $\pm$ 1.22 <sup>a</sup>	38.2 $\pm$ 1.35 <sup>b</sup>	37.8 $\pm$ 1.71 <sup>b</sup>	29 $\pm$ 0.44 <sup>a</sup>	79.4 $\pm$ 1.16 <sup>a</sup>
50 $\mu\text{M}$ (n=7)	40 $\pm$ 1.58 <sup>a</sup>	51.4 $\pm$ 2.87 <sup>a</sup>	53.2 $\pm$ 2.67 <sup>a</sup>	20.6 $\pm$ 1.96 <sup>b</sup>	79 $\pm$ 1.84 <sup>ab</sup>
100 $\mu\text{M}$ (n=7)	36 $\pm$ 1.58 <sup>a</sup>	40.6 $\pm$ 2.87 <sup>b</sup>	41.6 $\pm$ 2.67 <sup>b</sup>	26.8 $\pm$ 1.96 <sup>a</sup>	77.6 $\pm$ 1.84 <sup>ab</sup>
150 $\mu\text{M}$ (n=7)	35 $\pm$ 1.58 <sup>a</sup>	38.8 $\pm$ 1.88 <sup>b</sup>	40.2 $\pm$ 1.28 <sup>b</sup>	27.8 $\pm$ 2.57 <sup>a</sup>	75 $\pm$ 2.00 <sup>b</sup>

Means with different alphabetical superscripts within columns are significantly different at least at  $P<0.05$ .

HOST= Hypo-osmotic swelling test

n = number of replicates

### Discussion

In the present study, addition of LA (50  $\mu\text{M}$ ) significantly ( $P<0.05$ ) increased the quality of frozen thawed ram semen in terms of membrane integrity and sperm cell morphology. Cryopreservation imposes irreversible damage to sperm, including swelling and disruption of sperm plasma membrane (Graham and Foote, 1987). Phospholipids, which are cell membrane components, have ensured membrane fluidity, in the event they have protected ram spermatozoa from cold shock (Kasamo, Kagita, Yamanishi and Sakaki, 1992). Unsaturated fatty acid such as linoleic acid is high level participation in phospholipid of cell membrane and the supplementation of linoleic acid to the extender for sperm freezing is enhanced the sperm cryopreservation (Zhao and Buhr, 1995).

Moreover, the present results support the findings of Badr, Abdel-Malak and Shaker (2004) that linoleic acid supplementation in citrate extender increased viability of frozen-thawed ram spermatozoa. In addition, Takahashi et al. (2012) mentioned that addition of the linoleic acid albumin to the dilution medium used to cryopreserve bull spermatozoa of low freezability enhanced the membrane integrity parameters after long term equilibration that may last for 30 h. in bulls, Buyukleblebici, Tasdemir, Tuncer, Durmaz, Ozgurtas, Buyukleblebici, Coskun, and Gurcan (2014), reported improvement in the viability and

membrane integrity by addition of LA to the freezing extender of frozen-thawed bull semen and a decrease in the percentage of total abnormal sperm. The improvement in the sperm membrane integrity and sperm cell morphology reported herein could be attributed to the increment of LA into the membrane lipid bilayer which affect the function and increase the membrane fluidity which in turn increase sperm cryoresistance (Takahashi et al., 2012). Moreover, this improvement may be due to increase the total antioxidant activity of the sperm cells by addition of LA to the dilution medium that decrease the lipid peroxidation cascade which in turn protect the plasma membrane integrity and morphology Buyukleblebici et al. (2014).

Regarding the effect of LA fortification on the post-thaw progressive sperm motility of frozen-thawed ram semen, the current results revealed a non significant effect. The present results are in agreement with the findings of Buyukleblebici et al. (2014) and Sampaio et al. (2015) who did not find any significant improvement in the quality of frozen-thawed bull sperm by addition of LA to the dilution medium. Moreover, Takahashi et al. (2012) reported an improvement in the post-thaw motility of the sperm of low freezability after long term equilibration at 4 °C but didn't find any significant effect after short term equilibration for 4 h. the later authors suggested that the

increase in the sperm motility after long term equilibration may be due to the effect of time that gave the chance to the fatty acid to be incorporated into the lipid bilayer and exert its effect.

In agreement with **Buyukleblebici et al. (2014)**, inclusion of LA (150  $\mu$ M) in the freezing diluent exerted a significant decrease in the percentage of acrosome integrity which may be attributed to

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- the higher amount of ethanol solvent used in this concentration which in turn affect negatively the post-thaw acrosome integrity percent.
- Conclusion**
- In conclusion, addition of LA (50  $\mu$ M) to egg yolk-Tris diluent used for cryopreservation of ram semen could improve the post-thaw semen quality in terms of sperm membrane integrity and sperm morphology.
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### الملخص العربي

تهدف هذه الدراسة الى تقييم تأثير اضافة حمض اللينوليك الي مخفف الترس علي جوده السائل المنوي المجمد للكباش. استخدمت 3 تركيزات في هذه الدراسة (50 و 100 و 150 ميكرومول). 5 كباش تم استخدامهم. يتم خلط القنفاة لاستبعاد الاختلافات الفرديه. يتم اذابه الحمض الدهني باستخدام الايثانول. السائل المنوي المخفف المعالج يتم تبريده وتجميده. بعد الاساله يتم فحص حركه الحيامن، قوه الجدار الخلوي، نسبة التشوهات وكفائه القلنسوه. ادي استخدام حمض اللينوليك (50 ميكرومول) الي تحسن ملحوظ في قوه الجدار الخلوي للحيامن مقارنة بالكنترول. ايضا نفس التركيز ادي الي خفض ملحوظ في نسبة التشوهات مقارنة بالكنترول مضافا اليه ايثانول. بالنسبه لكفائه القلنسوه، تركيز 150 ميكرومول هو الوحيد الذي ادي الي خفض ملحوظ في نسب كفائه القلنسوه مقارنة بالكنترول. تأثير التركيزات الثلاثه علي حركه الحيامن كان غير ملحوظ. في النهايه اضافة حمض اللينوليك (50 ميكرومول) ادي الي تحسن ملحوظ في جوده السائل المنوي المجمد للكباش في جوده الجدار الخلوي وخفض نسبة التشوهات .

الكلمات الداله: السائل المنوي المجمد، حمض اللينولك، الكباش.