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Effect of conjugated linoleic acid isomer -cis-9, trans-11- on the cytological parameters of post-thaw ram spermatozoa

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Abstract

The present study aimed to evaluate the effect of addition of conjugated linoleic acid isomer-cis-9,trans-11- (CLA) to egg yolk-Tris glycerol diluent on the cytological parameters of post-thaw ram spermatozoa. Different concentrations of CLA (50, 100 and 150 µM) were added to the cryopreservation medium of ram semen. Five fat-tailed rams were used and their ejaculates were pooled and processed. Conjugated linoleic acid, which had oily presentation, were prepared by dissolving it in ethanol. Treated diluted semen was cooled, equilibrated and subjected to cryopreservation. The motility characteristics of the post-thaw spermatozoa were analyzed objectively; plasma membrane integrity by hypo osmotic swelling test (HOST), alive and abnormal sperm percentage by eosin negrosin stain and hanocock's mthod and acrosomal integrity by spermac stain. Results showed that CLA (100 and 150 µM) increased significantly ($P< 0.05$) the live sperm percentage, HOST and intact acrosome percentage of frozen thawed ram semen compared to control. Regarding the effect of CLA on the total sperm abnormalities, the 150 µM CLA was the only concentration that decreased significantly ($P <0.05$) the percentage of abnormal sperm cell compared to control (15.0 vs 19.4%). However, concentrations (50, 100 and 150 µM) tested decreased significantly ($P <0.05$) the percentage of abnormal sperm (21.6%, 17% and 15%, respectively compared to control with ethanol 29.2%). The effect of different concentrations (50, 100 and 150 µM) of CLA on the progressive motility percentage was non significant. It could be concluded that, CLA improved the freezability of ram spermatozoa in terms of membrane and acrosome integrity and decreased sperm cell abnormalities.

(Key words: semen cryopreservation, conjugated linoleic acid, ram and fatty acids)

Introduction

Conjugated linoleic acid (CLA) is the nomenclature used to define a group of isomers of octadecadienoic acid with double conjugated bonds, that are most abundant in positions 9, 10, 11 and 12 and can be naturally found in dairy products and ruminant's meat in both cis and trans configurations **(Pariza, 2004; Wahle, Heys and Rotondo, 2004)**. Conjugated linoleic acid, just as essential fatty acids (linoleic and linolenic acids), and other polyunsaturated fatty acids, are known for changing the lipid membrane composition in many cells **(Sampath and Ntambi, 2005)**. These fatty acids can be incorporated by the plasma membrane of cells **(Ringseis, Wen, Saal and Eder, 2008; Amarù and Field, 2009)** provoking modification in its structure and function **(Subbaiah, Gould, Lal and Aizezi, 2011; Zhao, Subbaiah, Chiu, Jakobsson and Scott, 2011)**.

Effects of fatty acids incorporated in maturation and embryonic cultivation media over membrane fluidity of bovine embryos were reported **(Hochi, Kimura and Hanada, 1999)**. An increase of unsaturated fatty acids in the embryonic membrane was observed before freezing, resulting in the modification of membrane fluidity, which may improve the embryo ability to freezing **(Tominaga, Hamada, Yabuue and Ariyoshi, 2000)**.

In ovine semen, the addition of oleic-linoleic acids to the cryopreservation medium resulted a beneficial effect in the preservation of spermatozoa viability **(Pérez-Pé, Cebrian-Pérez and Muio-Blanco, 2001)**. Swine spermatozoa incubated for 4 h at 37 °C in a dilution media containing oleic and linoleic acids demonstrated a significant improvement in motility and viability **(Hossain, Tareq, Hammano and Tsujii, 2007)**. Also, use of linoleic acid in the bovine semen cryopreservation medium caused an improvement in sperm motility after thawing **(Takahashi, Itoh, Nishinomiya, Katoh and Manabe, 2012)**. The composition of the extender as well as the type and the amount of cryo-protectants may have differential effects on spermatozoa depending on species or breed of the animal **(Tasdemir, Büyükleblebici, Tuncer, Coskun, Zgürtas, Aydin, Büyükleblebici and Gürcan, 2013)**. Thus, it is important to investigate

the effect of specific composition of diluents on specific semen samples. Scrutinizing the available literature on the effects of CLA addition to dilution and freezing media used for ovine semen and its interaction with sperm cells have not been reported previously. Therefore, the objective of the present study was to evaluate the cytological parameters of post-thaw ram spermatozoa frozen in media containing different concentrations of cis-9,trans-11 isomer of conjugated linoleic acid.

Materials and Methods

Experimental animals

Five mature (12-16 months) fat-tailed rams were used in the present study. Their body weight ranged from 50 to 60 kg at the commencement of the experiment which lasted for one year (from August, 2015 to August, 2016). Rams were housed in a clean wide box belonging to the Department of Theriogenology, Faculty of Veterinary Medicine, Cairo University. All rams were clinically sound, free from diseases as well as internal and external parasitic infestation. Clinical examination of their genitalia proved to be normal with absence of any palpable abnormalities. Derees, tiben, green forage and water were available during the whole experimental period and were offered adlibitum. In addition, each ram was daily offered 0.5 kg of pelleted concentrates in the morning. A general management schedule for disease prevention and claw trimming was followed.

B-Tested fatty acid

Conjugated linoleic acid isomer (cis-9, trans-11) was added to the freezing media at concentrations of 50, 100 and 150 µM according to **Soares, Brandelli, Celeghini, Arruda and Rodriguez (2013).** As CLA was fat soluble fatty acid, ethanol was used as a solvent.

Experimental procedures

Semen collection regimen

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

Semen processing

The collected ejaculates, with at least 85% initial motility and 3×10^9 sperm cells/ml, were pooled to obtain the needed suitable volume used for dilution and processing.

A- Semen dilution

The egg yolk-Tris diluent was used, in the present study, for cryopreservation 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 (50.000 IU), streptomycin sulphate (50 mg) and glass distilled water to 100 ml. The dilution rate was calculated on the basis that each insemination dose (0.5 ml straw) contained before freezing about 200×10^6 to 300×10^6 alive motile sperm **(Fukui, Hirai, Honda and Havashi, 1993).**

C- Semen cooling

Diluted semen was cooled to +5 °C over a period of two hours by ice cubes in a refrigerator and kept at +5°C for another two hours for equilibration **(Awad and Graham, 2004).**

D- Semen freezing in 0.5 ml straws

The cooled semen was loaded into 0.5 ml straws, sealed by polyvinyl powder and arranged horizontally on cold racks. Racks were then subjected to liquid nitrogen vapor inside a foam **(Darwish, Ziada, Shaker and Mohammed, 2003)** box (54×35×18 cm), containing liquid nitrogen at a height of 6.50 cm for 10 minutes to reach -120 °C. Straws were then immersed in liquid nitrogen and transferred into the liquid nitrogen storage container (-196 °C) until examination.

E- Thawing

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)**. Straws were wiped dry after thawing and de-plugged by cutting off with a scissor at the sealed end side. The thawed semen was then incubated for examination of post-thawing sperm cytological parameters.

Examination of frozen semen

1- Post-thaw progressive motility

Progressive motility was assessed according to **Hafez and Hafez (2000)** using a bright field microscope (× 200 magnification), with a warm stage maintained at 37 °C.

2- Structural membrane integrity

Structural membrane integrity or live sperm percentage was evaluated using eosin-nigrosin stain **(Campbell, Dott and Glover, 1956).**

3- Functional membrane integrity

The hypo-osmotic swelling test (HOST) was used as a complementary test to the viability assessment protocol to evaluate the functional integrity of the sperm plasma membrane. The assay was performed by mixing 30 µl of semen with a 300 µl of 100 mOsm/kg hypo-osmotic solution (9 g fructose plus 4.9 g sodium citrate per liter of distilled water) **(Revell and Mrode, 1994)** and was incubated at 37 °C for one hour. The mixture (20 µl) was placed on a microscope slide, mounted with a cover slip and immediately examined under the bright field microscope (400×). A total of 200 spermatozoa were counted in at least five different microscopic fields. Percentages of spermatozoa with swollen and curled tails were recorded.

4- Sperm cell abnormalities

For the assessment of sperm abnormalities **(Schafer and Holzmann, 2000),** three drops of each sample were added to Eppendorf tubes containing 1 ml of Hancock solution [(62.5 ml formalin (37%), 150 ml physiological saline solution (0.9% Na Cl), 150 ml buffer solution (sodium citrate dehydrate 2.9%) and 500 ml distilled water)]. One drop of this mixture was put on a slide and covered with a cover slip. The percentage of total sperm abnormalities was determined by counting a total of 200 spermatozoa under phase-contrast microscope (magnification 1000x, oil immersion).

5- Acrosome integrity

Acrosome integrity was estimated using a specific stain (Spermac stain, FertiPro N.V., Beernem, Belgium) according to **Chan, Corselli, Jacobson, Patton and King (1999).** A total of 200 sperm were counted in several microscopic fields using a bright field microscope (1000×) and the percentage of the intact acrosome (dark green acrosome with faint green head and tail) was recorded.

Statistical analyses

The obtained data were expressed as mean± SEM. The effect of different concentrations of conjugated linoleic acid on the studied semen parameters were tested by one-way analysis of variance (ANOVA). If the F-value was significant, differences in means amongst the studied parameters were evaluated by the least significant difference (LSD) using SPSS/PC version 20 software (SPSS, Chicago). Differences with values at least at $P < 0.05$ were considered to be statistically significant **(Daniel, 1991).**

Results

The obtained results are presented in Table 1. Inclusion of egg yolk-Tris diluent with conjugated linoleic acid (100 and 150 µM) increased significantly ($P< 0.05$) the live sperm percentage, functional membrane integrity (HOST) and intact acrosome percentage of post-thaw ram spermatozoa compared to control. Regarding the effect of CLA on the total sperm abnormalities, the 150 µM CLA was the only concentration that decreased significantly ($P <0.05$) the percentage of sperm defects compared to control (15.0% vs 19.4%). However, all concentrations (50, 100 and 150 µM) tested decreased significantly ($P <0.05$) the percentage of sperm abnormalities (21.6%, 17.0% and 15.0%, respectively compared to control with ethanol 29.2%). The effect of different concentrations (50, 100 and 150 µM) of CLA on the progressive motility percentage was non significant.

Table 1: Effect of different concentrations of conjugated linoleic acid isomer (cis-9 trans-11) on the studied semen parameters of post-thaw ram spermatozoa (Mean±SEM).

Conjugated linoleic acid isomer (cis-9 trans-11)	Progressive motility (%)	Alive percentage (%)	Functional membrane integrity (%)	Abnormal sperm (%)	Acrosome integrity (%)
Control (n=7)	36±2.91^a	40.2±3.33^b	39.6±3.17^b	19.4±1.32^b	80.4±1.20^b
Control with ethanol (n=7)	37±1.22^a	38.2±1.35^b	37.8±1.71^b	29.2±1.01^a	79.4±1.16^b
50 µM (n=7)	37±2.00^a	38.6±2.37^b	38.6±2.15^b	21.6±1.96^b	77.6±1.50^b
100 µM (n=7)	36±1.87^a	48.0±2.98^a	49.0±2.42^a	17.0±1.37^c	86.0±1.04^a
150 µM (n=7)	37±1.22^a	50.2±2.85^a	52.4±2.20^a	15.0±1.14^c	87.2±0.86^a

Means with different alphabetical superscripts (a, b, c, ...) within columns are significantly different at least at $P< 0.05$.
HOST= Hypo-osmotic swelling test. n= number of replicates

Discussion

In the current study, parameters of post-thaw ram spermatozoa frozen in the presence of conjugated linoleic acid were examined. Sperm motility showed no differences among treatments after thawing, suggesting that the presence of CLA does not improved the motility of cryopreserved ram spermatozoa. These results were in agreement with **Soares et al. (2013)** who reported that, no significance differences were observed on the post thaw progressive motility of frozen thawed bull semen by addition of CLA (50, 100 and 150 µM) to the cryopreservation medium. Although effects of fatty acids during freezing of ovine spermatozoa have not been described previously, **Hossain et al. (2007)** observed an increase in swine sperm motility after the addition of linoleic acid into the dilution medium under refrigeration. These contradictory results may be due to the different technique of preservation and species difference. moreover, bull semen, with low sperm freezing tolerance treated with 1 mg/ml linoleic acid albumin prolonging the equilibrium time before freezing for 30 h at 4 °C showed high motility. Addition of linoleic acid albumin to the extended media for long-term equilibrium of bull spermatozoa might improve the motility of freeze-thawed sperm with poor freezability **(Takahashi et al., 2012).**

Regarding the effect of CLA on membrane and acrosome integrity, the present results revealed that, CLA (100 and 150 µM) improved significantly ($P< 0.05$) the live sperm percentage, functional membrane integrity and intact acrosome percentage and decreased the abnormal sperm of frozen thawed ram spermatozoa compared to control. These results are matched with those obtained by **Teixeira, Chaveiro and Silva (2015)** who found a significant increase in the membrane and acrosome integrity by addition of CLA (50 and 100 µM) to the medium used for preservation of swine semen after long term refrigeration at 17 °C. This improvement may be due to the antioxidant effect of CLA and decreasing the lipid peroxidation of the membrane lipids that resulted in an increase in the resistance of the sperm membrane against the effect of reactive oxygen species **(Teixeira, 2015).** The results of the current work are in contrast with **Soares et al. (2013),** who did not observe any significance improvement in the membrane and acrosome integrity by fortification of the cryopreservation medium (egg yolk-Tris) with CLA (50 and 100, 150 µM). These contradictory results may be due to the difference in solvent used (DMSO with 1 % Sod lauryl sulphate) which may had some determintal effects on the sperm cell membrane that masked the positive effect of CLA.

Conclusion

It could be concluded that, inclusion of egg yolk Tris diluent with CLA-cis-9, trans- 11- improved the cytological parameters of post-thaw ram spermatozoa except progressive sperm motility. The best results obtained at concentrations of 100 and 150 µM.

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الملخص العربي

تهدف هذه الدراسه الي تقييم تأثير اضافه شبيه حمض اللينوليك (سيس 9-ترانس 11) الي مخفف الترس علي جوده السائل المنوي المجمد للكباش. استخدمت 3 تركيزات في هذه الدراسه (50و 100 و 150 ميكرومول). 5 كباش تم استخدامهم. يتم خلط القذفات 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، نسبه التشوهات وكفائه القلنسوه. ادي استخدام شبيه حمض اللينوليك (50 و 100 ميكرو مول) الي تحسن ملحوظ في قوه الجدار الخلوي للحيامن وكفائه القلنسوه مقارنه بالكنترول. بالنسبه للتشوهات، تركيز 150 ميكرومول هو الوحيد الذي ادي الي خفض ملحوظ في نسب التشوهات مقارنه بالكنترول ولكن كل التركيزات ادت الي خفض ملحوظ في نسبه التشوهات مقارنه بالكنترول مضاف اليه الايثانول. تأثير التركيزات التلاته علي حركه الحيامن كان غير ملحوظ. في النهايه اضافه شبيه حمض اللينوليك (سيس 9-ترانس 11) الي تحسن ملحوظ في جوده السائل المنوي المجمد للكباش في جوده الجدار الخلوي وكفائه القلنسوه.

الكلمات داله: السائل المنوي المجمد، شبيه حمض اللينولك، الكباش.