LOW DENSITY LIPOPROTEINS AS CRYOPROTECTANTS OF RABBIT SEMEN CRYOPRESERVATION

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ABSTRACT

The present study was carried out at the International livestock management training center, Sakha, Kafrelsheikh, belonging to Animal Production Research Institute, Agricultural Research Center, Ministry of Agriculture, Egypt; during the period from March to May 2013. Aim of this study was to evaluate sperm characteristics and fertility of rabbit buck semen cryopreserved in tris-extender containing different levels of low density lipoproteins (LDL) as replacement of egg yolk. Semen was collected twice weekly form twenty APRI rabbit bucks using artificial vagina. Only ejaculates with ≥ 70% mass motility were used for dilution and processing. Four extenders were used: Tris 20% egg yolk extender as a control, and substitution of whole egg yolk with 8, 10 and 12% LDL. Semen was diluted and packaged into 0.25 ml straws, cooled, held at 5°C for 4 h , and then frozen in liquid nitrogen and stored at -196◦C. Percentages of progressive motility, livability, abnormality and intact acrosome were determined at post-dilution, -equilibration, -thawing. Results indicated that LDL extenders were more effective in preservation of rabbit sperm characteristics than whole egg yolk extender. Percentages of progressive motility, livability, abnormality and intact acrosome were much higher (P<0.05) in the 10% LDL extender, being 49.5, 61.6, 10.4 and 71.3%versus 30.5, 38.2, 13.3 and 58.6% in the control extender in post-thawed semen, respectively. Fertility rates were higher in extender containing 10% LDL compared with the control (75 vs. 60%). In conclusion, an extender containing 10% LDL extracted from hen egg yolk could be used as a cryoprotective media because it improved the freezability and fertility of rabbit spermatozoa.

Keywords: Rabbit, semen LDL, sperm motility, freezability, fertility.

INTRODUCTION

Post-thawing the frozen semen, it is important to preserve a large number of spermatozoa with appropriate characteristics in order to obtain good fertility rate. Egg yolk is considered to be an excellent cryoprotectant, and it has long been used for the cryopreservation of spermatozoa at a concentration of 20% ***(Silva et al., 2002)***. This concentration makes results difficult to standardize and may interfere with biochemical assays and metabolic investigations, the problems or negative effects of which could be overcome by removing some of the components of the egg yolk by centrifugation ***(Wall and Foote 1999)***. Numerous authors have proposed that the low density fraction of yolk, which is mainly composed of low density lipoproteins (LDL), could be largely responsible for the resistance against cold shock and the improved motility after storage ***(Demianowicz and Strezek, 1996; Moussa et al., 2002)***. In this respect, LDL could adhere to cell membranes during the freeze–thaw process, thus preserving the membranes of the spermatozoa. However, the respective roles of the protein and lipid components of LDL during interactions with the spermatozoa membrane have yet to be clearly established ***(Polge, 1980; Graham and Foot, 1987)***. Furthermore, the presence of substances in yolk that inhibits the gaseous exchange of spermatozoa or reduces their motility has accelerated the demand to replace whole egg yolk with the cryoprotective fraction alone ***(Watson and Martin, 1975; Haidl and Schilling, 1994)*.**

There have been many attempts to determine which component of egg yolk provides cellular protection in order to prepare a chemically defined extender. Low-density fraction from purified egg yolk using ultracentrifugation had a cryoprotective action ***(Pace and Graham, 1974)***. Therefore, several studies have reported successful results with

the addition and replacement of egg yolk by LDL in the semen freezing process of different species, like bull ***(Moussa et al. 2002; Amirat et al. 2004; Hu et al., 2010)***, buffalo ***(El-Sharawy et al., 2012), ram (Ali Al Ahmad et al., 2008)*** and dogs ***(Varela, 2005)***.

Generally, good results for fertility rate of frozen rabbit semen (70-80%) were reported by ***O'Shea and Wales (1969); Weitze et al. (1976)*** and ***Viudes-de-Castro and Vicente (1996)***, being lower than that of fresh semen. However, only ***Viudes-de-Castro and Vicente (1996)*** found similar results for fresh and frozen semen in prolificacy (8.1 vs. 8.0 total born/rabbit does, respectively). However, when frozen rabbit spermatozoa were used to perform inseminations on commercial farms, results in both fertility and prolificacy were lower than those obtained with fresh semen***(Theau-Clement and Roustan 1982;Moce et al.2003)***.

The aim of this study is to assess the effect of diluent composed of LDL extracted from chicken egg yolk on sperm characteristics including percentages of motility, livability, abnormality and acrosome integrity during different processing stages of rabbit semen.

MATERIALS AND METHODS

The present study was carried out at the International livestock management training center, Sakha, Kfrelsheikh belonging to Animal Production Research Institute, Agricultural Research Center, Ministry of Agriculture, Egypt, during the period from March to May 2013.

Animals:

Total of 20 APRI buck rabbits (12 months old and 3.5 kg live body weight) were used in this study for semen collection. Bucks were individually housed in cages under and fed *ad libitum* on a commercial diet (17.5% crude protein, 3.5% ether extract, 16.7% crude fiber on DM basis). Also, total of 80 APRI rabbit does (multiparous non-lactating).

All animals were kept under the same managerial and hygienic conditions. Fresh tap water was freely available at all times from stainless steel nipples in each cage.

Semen collection and evaluation:

Semen was collected twice weekly using artificial vagina for rabbits. Gel plug was removed immediately after semen collection and the collected semen was kept at 35-37ºC in water bath until evaluation. Only ejaculates with mass motility ≥70% were used for dilution and processing. On day of semen collection, semen was pooled and dived into four portions; the 1st portion was extended with traditional tris-egg yolk (20%) extender (control). Meanwhile in the other three portions, egg yolk in tris-extender was replaced by 8, 10 and 12% of LDL.

The LDL was extracted from egg yolk of hens according to the method described by ***Moussa et al. (2002)***.

Freezing protocol:

After dilution, semen was packaged in 0.25 ml straws and then left at 5°C for four hours as an equilibration period. Straws were then exposed to liquid nitrogen vapor at 4 cm distance above liquid nitrogen level for ten minutes at about -90 to -100°C. Straws were then dipped in liquid nitrogen (-196°C). After 24 hours of cryopreservation, straws were removed from liquid nitrogen container and thawed by dipping them in water bath at 37°C for 30 seconds.

Semen evaluation:

Progressive motility, livability, abnormality and acrosome integrity of spermatozoa were estimated post-dilution, post-equilibration and post-thawing. The percentage of sperm livability was determined according to ***Hancock (1951)***; however assessment of the percentage of intact acrosome was conducted according to ***Watson (1975).***

Artificial insemination:

Total of 80 does were subcutaneously injected with 75 IU of PMSG: (Folligon, Intervet, Holland) and ovulation was induced with 100 IU of a HCG analogue (Superefact, Hoechst Roussel, Madrid, Spain) given at the insemination time. Total of 20 does were artificially inseminated by semen extended with each level of LDL and control semen immediately post-thawing at 37ºC for 30 sec) using filled plastic AI gun close to the cervical canal. Pregnancy diagnosis was performed by abdominal palpation on day 12 after AI and parturition was subsequently recorded.

Statistical analysis:

Data were statistically analyzed using general linear models procedure adapted by ***SPSS (2008)*** for user's guide with one-way ANOVA. Duncan test within program SPSS was done to determine the significant differences among means ***(Duncan, 1955)***.

RESULTS

Effect of LDL concentrations on sperm characteristics during freezing process:

Data in Table (1) indicated that percentage of progressive sperm motility in post-diluted semen was significantly ($P<0.05$) improved only with tris extender containing 10% LDL as compared to 8 and 12% LDL and control extenders, while percentage of sperm livability was significantly ($P>0.05$) improved with tris-extender containing 8 or 10% LDL as compared to 12% LDL and the control extenders. However, percentages of abnormality and intact acrosome did not differ significantly with LDL at levels of 8, 10 and 12% as compared to the control extender, but the significant ($P<0.05$) differences in percentage of spermatozoa with intact acrosome were found between 10 and 12% LDL.

Table (1): Effect of LDL on sperm characteristics in post-diluted rabbit semen.

Sperm characteristics (%)	Control	LDL concentration		
		8%	10%	12%
Progressive motility	71.0 ± 1.78^{b}	73.5 ± 1.07^{ab}	77.0 ± 1.33^{a}	70.5 ± 0.90^{b}
Livability	75.0 ± 2.04^{c}	80.2 ± 1.02^{b}	86.3 ± 1.51^{a}	74.8 ± 2.20^{c}
Abnormality	10.0 ± 0.88	10.4 ± 0.67	9.0 ± 0.54	10.9 ± 0.53
Intact acrosome	78.8 ± 1.43^{ab}	81.6 ± 1.64^{ab}	83.1 ± 1.35^{a}	78.0 ± 1.84^{b}

The subscripts a, b and c in the same row are significant at P<0.05.

In post-equilibrated semen (Table 2), percentages of progressive motility, livability and intact acrosome of spermatozoa significantly (P>0.05) increased in semen diluted with tris-extender containing 10% LDL, while only motility and livability percentages significantly (P<0.05) increased by 8% LDL. However, percentage of sperm abnormality did not differ significantly by all LDL levels as compared to control. It is of interest to note that increasing LDL level from 10 to 12%, significantly (P<0.05) increased percentage of sperm abnormality.

Table (2): Effect of LDL on sperm characteristics in post-equilibrated rabbit semen.

Sperm characteristics (%)	Control	LDL concentration		
		8%	10%	12%
Progressive motility	58.5 ± 1.83^{b}	64.5 ± 2.03^{a}	68.5 ± 1.30^{a}	59.5 ± 1.67^{b}
Livability	65.0 ± 1.84^{c}	71.5 ± 1.01^{b}	76.8 ± 1.90^{a}	66.2 ± 1.53^{c}
Abnormality	11.5 ± 0.89^{ab}	10.4 ± 0.58^{ab}	9.9 ± 0.55^{b}	12.1 ± 0.59^{a}
Intact acrosome	71.5 ± 1.93^{b}	76.0 ± 2.24^{b}	82.3 ± 1.79^{a}	77.2 ± 1.37^{ab}

The subscripts a, b and c in the same row are significant at P<0.05.

In post-thawed semen (Table 3), percentages of progressive motility, livability and intact acrosome of spermatozoa significantly (P<0.05) increased in post-thawed semen diluted by all LDL levels. While, only sperm abnormality significantly (P<0.05) improved only by 10% LDL. These results mean that all sperm characteristics studied showed significantly (P<0.05) the best results in post-thawed semen diluted with 10% LDL.

Table (3): Effect of LDL on sperm characteristics in post-thawed rabbit semen.

Sperm characteristics (%)	Control	LDL concentration		
		8%	10%	12%
Progressive motility	30.5 ± 1.74^{c}	35.5 ± 1.57^{b}	49.5 ± 1.89^{a}	36.0 ± 1.45^{b}
Livability	38.2 ± 1.78^{c}	45.5 ± 1.72^{b}	61.6 ± 1.61^{a}	46.5 ± 2.38^{b}
Abnormality	13.3 ± 0.93^{a}	12.5 ± 0.72^{ab}	10.4 ± 0.75^{b}	12.6 ± 0.98^{ab}
Intact acrosome	58.6 ± 2.18^{c}	63.5 ± 1.26^{ab}	71.3 ± 2.16^{a}	65.3 ± 1.71^{b}

The subscripts a, b and c in the same row are significant at P<0.05.

Results of recovery rate of progressive motility, livability and intact acrosome presented in Table (4) revealed that semen extended with LDL at levels of 10 and 12% significantly (P<0.05) improved sperm freezability in rabbit semen, being the better with 10 than 12% LDL.

Table (4): Effect of LDL on recovery rate of sperm characteristics in post-thawed rabbit semen.

Sperm characteristics (%)	Control	LDL concentration		
		8%	10%	12%
Progressive motility	43.2 ± 2.62^{c}	48.4 ± 214^{ab}	64.3 ± 2.35^{a}	51.1 ± 2.15^{b}
Livability	51.4 ± 2.80^{c}	56.7 ± 1.85^{ab}	71.4 ± 1.70^{a}	62.6 ± 3.54^{b}
Intact acrosome	74.5 ± 2.88^{b}	78.1 ± 2.43^{ab}	86.1 ± 3.16^{a}	84.3 ± 3.49^{a}

The subscripts a, b and c in the same row are significant at P<0.05.

Effect of LDL concentrations on fertility:

Insemination of synchronized does with frozen/thawed semen diluted with tris-extender containing 10% LDL significantly increased conception rate to 75% as compared to 60% in the control semen. However, conception rate was 70% and 65% for semen extended with tris-extenders containing 8 and 12% LDL, respectively.

DISCUSSION

The present results regarding the effect of tris-extender containing different levels of LDL (8, 10 and 12%) on different sperm characteristics including progressive motility, livability, abnormality and intact acrosome in post-dilution, -equilibrated and –thawed semen, show clearly that LDL can replace egg yolk in tris-extender with better results in terms of sperm motility, live sperm, abnormal sperm and intact acrosome of rabbit spermatozoa. In this respect, the optimum concentration of LDL has been determined to be 10%. This level was higher than 8% LDL gives the best results in bull semen ***(Moussa et al., 2002; Amirat et al., 2004)***. However, the obtained results on rabbit semen are in agreement with those reported on buffaloes by ***Akhter et al. (2011)***, who demonstrated that the percentage of motile spermatozoa following different stages of cryopreservation in 10% LDL give the best results in Nili-Ravi buffalo bulls. On the other hand, ***El-Sharawy et al. (2012)*** indicated that the extender containing 12% LDL showed the best results as compared to extenders containing other LDL level or 20% egg yolk in Egyptian buffalo bull semen.

The results presented in this study suggested that the use of LDL instead of egg yolk in tris extender could enhance the ability of rabbit spermatozoa against cold shock and improve sperm quality during the freeze-thaw process. Egg yolk is reported to contain some deleterious components which are potent to reduce sperm motility in bull semen ***(Pace and Graham, 1974)*** and porcine semen ***(Demianowicz and Strezek, 1996)***. The control medium is composed of 20% egg yolk, which itself contains 50% dry matter, 6.6% of which is LDL ***(Moussa et al., 2002; Bencharif et al., 2008)***. It is known that some components in egg yolk play an antagonistic role to the cryoprotective effect of LDL. This postulate may explain the higher post-thawed motility in extender containing 10% LDL compared with egg yolk containing extender.

Amirat et al. (2004) also reported in bulls that LDL did not induce more plasma membrane damage during the cryopreservation procedure than egg yolk. Moreover, ***Bencharif et al. (2008)*** reported better preservation of flagellar plasma membrane integrity for dog spermatozoa cryopreserved in LDL compared to egg yolk. Furthermore in earlier reports, ***Courtens et al. (1989)*** reported that LDL showed less aggressive effect on cell than egg yolk; there was alteration of the plasma membranes and very little acrosome disruption. They emphasized the possible adverse effect of calcium, present in high concentration in egg yolk. According to the later authors, the acrosomes were modified or damaged, which could result from a rapid calcium influx into spermatozoa when the temperature is below 30ºC, another interesting aspect that could be evaluated in impact of oxidation on membrane stability ***(Pillet et al., 2011)***.

The medium containing 10% LDL provides the best protection of acrosome integrity, possibly via a direct action through the exchange or repair of acrosomal membrane phospholipids or possibly simply because the medium is less rich in progesterone than egg yolk due to the filtering effect of the dialysis membrane ***(Benchsrif et al., 2008)***. The progesterone found in egg yolk plays a role in the capacitation of spermatozoa in cattle ***(Witte and Schafer- Somi., 2007)***, horses ***(Aitken and McLaughlin, 2007)*** and man ***(Meyers et al., 1995)***, it appears to act via an extra-genomic action on human spermatozoa, via the secondary activation of calcium channels leading to an increase in intracellular Ca^{++}, which may be responsible for the capacitation of spermatozoa.

CONCLUSION

The current study recommended that LDL extracted from hen egg yolk may be use as a cryoprotective media in tris-extender of frozen rabbit semen because it improved the freezability and fertility of rabbit spermatozoa.

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الليبوبروتينات منخفضة الكثافة كواقيات للتجميد لحفظ السائل المنوي للأرانب

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تم إجراء هذه الدراسة بالمركز الدولي للتدريب على رعاية الحيوان بسخا التابع لمعهد بحوث الإنتاج الحيواني –وزارة الزراعة و تهدف هذه الدراسة الى تحسين جودة السائل المنوى والخصوبة فى الأرانب باستخدام الليبوبروتينات منخفضة الكثافة (LDL) بديلا عن صفار البيض الكامل. .. تم جمع السائل المنوي باستخدام المهبل الصناعي من عشرون ذكر أرنب مرتين أسبوعيا ثم توضع العينات في حمام مائي على درجة حرارة 35– 37 مْ. تم استخدام العينات التي تزيد حيويتها عن 70% ثم يتم تقسيم العينات إلى 4 أجزاء، الأولى يتم تخفيفها باستخدام مخفف الترس مع 20% صفار البيض الكامل (مقارنة) بينما الأجزاء الأخرى تم استبدال صفار البيض بمستويات 8، 12،10% من LDL. بعد تخفيف السائل المنوي توضع العينات فى الثلاجة لمدة 4 ساعات على درجة 5مْ (فترة الموازنة) ثم تعبئتها في قصيبات سعتها 0.25 مل وتجميدها وتخزينها في النتروجين السائل على درجة حرارة –196 مْ.

بعد ذلك تم إسالة العينات المجمدة بوضعها في حمام مائي لمدة 30 ثانية على درجة حرارة 38م . تم تقدير النسبة المئوية للحيوية، النسبة المئوية للحيوانات المنوية الحية و النسبة المئوية للحيوانات المنوية الشاذة وسلامة الأكروسوم وذلك بعد التخفيف، فترة الموازنة والتجميد والإسالة, وتم اختيار عينات عشوائية من االقصيبات المجمدة الممثلة لكل المخففات وتلقيح عدد من الإناث صناعيا.

أشارت النتائج إلى أن المخفف المحتوي على 10% LDL حسن معنويا (عند مستوى 5%) النسبة المئوية للحيوية، النسبة المئوية للحيوانات المنوية الحية و النسبة المئوية للحيوانات المنوية الشاذة وسلامة الأكروسوم (49.5، 61.6 ، 10.4، 71.3 % ،على التوالي) مقارنة بمجموعة المقارنة (30.5، 38.2 ، 13.3، 58.6 % ،على التوالي)، أيضا أعطى المخفف المحتوى على 10% LDL أعلى معدل خصوبة مقارنة بمجموعة المقارنة (75% مقابل 60% ،على التوالي).

الخلاصة:

يمكن استخدام مخفف LDL بديلا عن صفار البيض الكامل لأنه يؤدى إلي تحسين خصائص السائل المنوى المجمد والخصوبة فى الأرانب.