THE ROLE OF LOW DENSITY LIPOPROTEINS AS BASED EXTENDER IN BOVINE SEMEN CRYOPRESERVATION AND ITS EFFECT ON FERTILITY

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SUMMARY

The aim of the current study was to evaluate the cryopreservation effect of different concentrations of low density lipoproteins (LDL) (4, 6, 8 and 10%), as a substitute for whole egg yolk (20%) in Holstein bull semen extender and its effect on bull semen freezing and subsequent fertility. Semen was collected from four bulls twice weekly for 12 weeks and the ejaculates with more than 75% progressive motility and 85% normal morphology were pooled prior to cryopreservation. Semen was diluted at 37ºC with Tris-citric acid extender to 8×10^7 motile spermatozoa/ml. Diluted semen was equilibrated at 4ºC for 4h., filled in 0.25 ml French straws and kept on liquid nitrogen vapors for 10 min. Straws were plunged and stored in liquid nitrogen (-196ºC). Sperm motility, plasma membrane integrity and acrosome integrity were assessed at post-dilution, pre-freezing and post freezing-thawing. Results revealed that the 6% LDL concentration in semen extender was more effective ($P<0.05$) in preservation of progressive motility, plasma membrane integrity and acrosome integrity of spermatozoa than control and the other LDL concentrations (8 and 10%) at different cryopreservation processes. Fertility rates were higher ($P<0.05$) in cows artificially inseminated with the semen cryopreserved in 4 and 6% LDL extenders (72.7 and 76.7%) compared with that of control, 8 and 10% LDL extenders (60 , 61.5 and 58.3%, respectively). It could be concluded that the 6% LDL concentration in the extender was better able to protect the bovine sperm cell from cryodamage and improved cryopreserved semen quality and fertility.

Keywords: bovine, semen, LDL, freezing , fertility

INTRODUCTION

Freezing process exposes the spermatozoa to thermal shock, which results in damage to the plasma membrane and acrosome integrity (Celeghini *et al*., 2007). Various extenders have been tested in an attempt to limit cellular injury. Egg yolk is the most widely used in these extenders by A.I centers. Recently, centrifugation techniques have enabled the isolation of the LDL that is responsible for the cryopreservative effect of egg yolk (Moussa *et al*., 2002; Bergeron and Manjunath, 2006). The incorporation of LDL in bovine semen extenders has given an improvement in semen parameters compared with extenders containing whole egg yolk (20%) (Moussa *et al*., 2002; Amirat-Briand *et al*., 2004 and 2010).

In canine semen, Bencharif *et al*. (2008) found that the 6% LDL extenders gave better motility results and did not cause any further damage to the flagella plasma membrane in comparison with the standard extenders containing whole egg yolk. In another study, Bencharif *et al*.(2010) observed that the 6% LDL extender provided the best protection of acrosome integrity in canine spermatozoa due to a direct action through the exchange or repair of acrosomal membrane phospholipids or, simply because the LDL medium is less rich in progesterone than the whole egg yolk because of dialysis. In addition, Hu *et al*. (2010) found that the 8 or 9 % LDL extenders offered the highest intact acrosome and plasma membrane integrity than whole egg yolk (20%) or 7 or 10% LDL extenders. Additionally, in buffalo spermatozoa, Akhter *et al*. (2011); El-Sharawy *et al*. (2012a and 2012b) and El-Shamaa *et al*. (2013) found that LDL extenders have improved motility, plasma membrane integrity and acrosome integrity. The amount of LDL used as a component of semen freezing extender may differently influence the structural and functional parameters of the spermatozoa (Bergeron and Manjunath, 2006). Therefore, the aim of the present study was to demonstrate the cryopretective role of different LDL concentrations in semen extender to formulate an optimum concentration of LDL for better cryopreserved semen quality of Holstein bulls.

MATERIALS AND METHODS

Semen collection:

Four Holstein bulls belonging to the International Livestock Management training center (ILMTC), Sakha station, Animal Research Institute, Ministry of Agriculture, Egypt were used. These bulls were housed in individual boxes and fed on the recommended ration according to Animal production Research Institute (1997). All chemicals used in this study were purchased from Sigma (Sant Luis, MO, USA), unless otherwise indicated. Semen was collected twice weekly using an artificial vagina for a

period of 12 weeks. After semen collection, the ejaculates that had more than 75% visual motility and 85% normal sperm morphology were pooled to have sufficient semen to make a replicates and to eliminate the bull effect (individual differences in bulls). The pooled ejaculates were immediately diluted to 8 x 107 spermatozoa/ml using the five extenders that had been previously prepared and warmed to 37ºC and then subjected to a progressive cooling from 37ºC to 4ºC for 4 h (equilibration period) in a refrigerator unit before being loaded into 0.25 ml straws (IMV, L'Aigle, France). The straws were held for 10 min at 4 cm from the surface of the liquid nitrogen (-120°C) before being immersed and then stored in liquid nitrogen (-196 °C) for 24h of storage.

Extenders preparation:

Five extenders were prepared and stored at 4 ºC until use. Tri-egg yolk extender (20% EY, control) containing 20 ml of hen's egg yolk, 3.025 g tris (hydroxylmethyl amino methane), 1.675 g citric acid, 0.75 g glucose, 7 ml glycerol, 0.25mg lincomycin, 0.005 mg streptomycin and 100 ml bi-distilled water. However, LDL extenders containing 4, 6, 8 and 10% LDL were prepared from hen's egg yolk according to the method described by Moussa *et al*. (2002) and diluted in Tris extender control.

Semen quality assays:

All evaluations regarding the sperm parameters such as sperm motility, plasma membrane and acrosome integrity were performed for each extender at post dilution, pre-freezing (post equilibration) and post frozen-thawed spermatozoa. All recorded values representing the average of three evaluations per sample were conducted.

Assessment of sperm motility:

At post-dilution, pre-freezing and post-thawing, four straws from each extender were immersed in water bath at 37°C for 10s and then, sperm motility was assessed using 5 µl semen placed on pre-warmed glass slide and covered by a warmed cover slip. For each sample, at least five microscopic fields were examined for 10µL semen sample at a magnification of 400x using a phase-contrast inverted light microscope (CKX41; Olympus, Tokyo, Japan) with a warm stage maintained at 37°C. The mean of the three successive evaluations at every stage of cryopreservation previously mentioned was recorded as the final motility score. At least 200 spermatozoa were counted per slide.

Assessment of membrane and acrosome integrity:

Membrane integrity was assessed using Hypo-Osmotic Swelling test (HOS) based on curled and swollen tail. The hypo-osmotic solution (150 osm/L) was prepared by dissolving 0.735 g Sodium citrate dihydrate and 1.35 g fructose in 100 ml distilled water. A one ml of hypo-osmotic solution was mixed with 0.1ml of semen and incubated at 37°C for one hour. A drop of diluted semen was placed on a clean dry glass slide and covered by cover slip. A total of 100 spermatozoa were counted under a phase contrast microscope (400x magnification). The percentage of spermatozoa positive to HOS test (having curled and swollen tail) was determined (Jeyendran *et al*., 1984 and Lodhi *et al*., 2008). Whereas, sperm acrosomal integrity was estimated using Giemsa stain (Watson, 1975) and the microscopic examination was carried out using oil immersion lens (x1000).

Fertility trial:

A total of 295 Friesian cows owned by small and medium scale breeding holder in different villages at Kafr-elsheikh and Gharbya Governorates in Egypt were artificially inseminated with random frozen semen doses from the various extenders. Each female was inseminated with a single straw 10h after starting estrous behavior. Using recto-vaginal technique and the universal insemination gun, the thawed semen was deposited in the uterine body just next to the anterior end of the cervix. Conception rate was confirmed by rectal palpation 60 days after insemination.

Statistical analysis:

The experimental data were statistically analyzed using the general model program (SAS, 1999). Data of semen parameters was subjected to repeated measurement according to the following model:

$Y_{ijk} = \mu + T_i + P_j + S(P)_{ijk} + (T*P)_{ij} + e_{ijk}$

Where:

Y_{ijk}= any observation.

μ = overall mean.

T_i = the fixed effect of the estimate.

P_j= the fixed effect of sampling period (1, 2, 3,...).

$S(P)_{ijk}$ = sample within period.

$(T*P)_{ij}$ = the fixed effect of the interaction between Period of sampling and treatment.

e = error.

The least square mean was calculated by modulating the data higher than 70% and less than 30 %.

Differences with $P < 0.05$ were considered significant.

RESULTS

Progressive sperm motility:

At post-diluted, post-equilibrated and post-thawed semen, the sperm progressive motility was superior ($P<0.05$) for the semen diluted in the extender containing 6% LDL compared with that of the control (68.64 % *vs.* 62.73 %, Table 1). Similarly, these parameters were found higher in 4% LDL extender than that of the control but the differences were not significant at post-equilibrated and post-thawed semen. On the other hand, sperm progressive motility was slightly lower ($P > 0.05$) in 8 and 10% LDL extenders than that of the control extender (20% EY).

Plasma membrane integrity:

The mean percentages of swollen bull spermatozoa (intact plasma membrane) obtained for each extender during the different stages of cryopreservation are presented in Table (2). The 6% LDL extender gave a significantly ($P < 0.05$) superior percentage of intact plasma membrane than control, 8

and 10% LDL extenders at all of the three cryopreservation processes. However, the percentage of plasma membrane integrity showed slightly higher for spermatozoa cryopreserved in 6% LDL extender than that in 4% LDL extender (P > 0.05) except at the post dilution (P < 0.05) process.

Acrosome integrity:

The mean percentages of acrosome integrity recorded for each extender after the different stages of cryopreservation are presented in Table (3).

The 4 and 6% LDL extenders had the highest percentages of acrosome integrity (P<0.05) when they were compared with that of the control extender at the all stages of cryopreservation. Therefore, the best percentage of acrosome integrity for the spermatozoa at all stages of cryopreservation process was found with 6% LDL extender.

Fertility trial:

Conception rates (%) in Friesian cows inseminated with semen diluted and cryopreserved in 4 and 6 % LDL extenders (72.7 and 76.7%) were significantly higher (P < 0.05) than that of control, 8 and 10% LDL extenders (60, 61.5 and 58.3%, respectively). Furthermore, the best fertility rate was found using the semen diluted and cryopreserved in 6% LDL extender in compared with the other extenders (Table 4). While, the fertility rate in 10% LDL extender was slightly lower than that of control one (58.3.1% *vs*. 60%) but the differences were not significant (P > 0.05). In addition, it was observed that the overall conception rate obtained from the all four LDL extenders were slightly higher than that of the control one (67.1 *vs*. 60%, P > 0.05).

Table 1. Effect of LDL on progressive motility of bull spermatozoa (%) at different stages of cryopreservation (Mean ± S.E)

Item	Treatments					S.E.
	Control EY 20%	LDL				
		4%	6%	8%	10 %	
Post dilution	62.73^{Ba}	66.82^{Aa}	68.64^{Aa}	60.91^{Ba}	60.45^{Ba}	±1.00
Equilibration at 5 ºC	53.18^{BCb}	56.36^{ABb}	59.50^{Ab}	52.27^{BCb}	51.36^{Cb}	±1.40
Post –thaw	43.64^{BCc}	46.36^{ABc}	49.09^{Ac}	43.64^{BCc}	41.82^{Cc}	±0.97

A,B,C,… the different superscripts within the same row and a,b,c,…the different superscripts within the column are significant (P < 0.05). EY= Egg Yolk LDL= Low Density Lipoprotein.
** The number of replicates for the semen evaluation at every stage of cryopreservation in this study was 96 (N=96).

Table 2. Effect of LDL on plasma membrane integrity of bull spermatozoa (%) at different stages of cryopreservation (Mean ± S.E)

Item	Treatments					S.E.
	Control EY 20%	LDL				
		4%	6%	8%	10 %	
Post dilution	72.73^{Ca}	75.9^{Ba}	79.73^{Aa}	2.18^{Ca}	69.55^{Ca}	±1.10
Equilibration at 5 ºC	65.64^{BCb}	69.45^{ABa}	73.91^{Aa}	65.09^{BCb}	62.00^{Cb}	±2.04
Post –thaw	51.36^{Bc}	55.45^{ABb}	60.45^{Ab}	50.91^{Bc}	49.09^{Bc}	±2.71

A,B,C,… the different superscripts within the same row and a,b,c,…the different superscripts within the same column are significant (P<0.05).). EY= Egg Yolk LDL= Low Density Lipoprotein.
** The number of replicates for the semen evaluation at every stage of cryopreservation in this study was 96 (N=96).

Table 3. Effect of LDL on acrosome integrity of bull spermatozoa (%) at different stages of cryopreservation (Mean ± S.E)

Item	Treatments					S.E.
	Control EY 20%	LDL				
		4%	6%	8%	10 %	
Post dilution	74.18^{Ca}	79.82^{Ba}	83.55^{Aa}	76.18^{BCa}	75.09^{Ca}	±1.01
Equilibration at 5 ºC	70.16^{Bb}	76.55^{Aa}	77.18^{Ab}	71.09^{Bb}	70.36^{Bab}	±1.53
Post –thaw	65.00^{Cb}	72.00^{ABb}	73.91^{Ab}	67.27^{Bb}	63.90^{Cb}	±1.85

A,B,C,… the different superscripts within the same row and a,b,c,…the different superscripts within the same column are significant (P < 0.05). EY= Egg Yolk LDL= Low Density Lipoprotein.
** The number of replicates for the semen evaluation at every stage of cryopreservation in this study was 96 (N=96).

Table 4. Conception rate of Friesian- cows inseminated with frozen semen cryopreserved in different concentrations of LDL.

Item	No. of inseminated females	No. of conceived females	Conception rate (%)
Control	55	33	60.0^{B}
4% LDL	55	40	72.7^{A}
6% LDL	60	46	76.7^{A}
8% LDL	65	40	61.5^{B}
10% LDL	60	35	58.3^{B}
Overall of LDL treatment	240	161	67.1^{B}

A, B,… the different superscripts within the same column are significant (P<0.05) among treatments**.**

DISCUSSION

The results of the present study clearly reveal a significant effect of LDL concentrations derived from hen′s egg yolk on the cryopreserved semen quality and the 6% LDL concentration was better able to protect the sperm cell from cryodamage than the other LDL concentrations tested or with the whole egg yolk. In fact, the whole egg yolk has been widely used in semen freezing extenders and proven to offer resistance against cold shock during the process of sperm freezing and thawing in several animal species (Holt, 2000; Moussa *et al*., 2002; and Mocé and Viecente 2009). However, the use of egg yolk has several drawbacks besides being a source of bacterial contamination (Moreno *et al*., 2013) and some of its constituents could have detrimental effects on spermatozoa. Accordingly, the granules found in egg yolk were observed to reduce respiration and motility of bull spermatozoa (Amiart-Briand *et al*., 2004). In addition, progesterone in egg yolk is responsible for the capacitation of spermatozoa and thus harmful for the preservation of spermatozoa during freezing (Bowden *et al*., 2001).

The presence of these detrimental substances in yolk could explain why in vitro preserved sperm quality was not as good as that recorded for the LDL extenders. These postulates may explain the higher cryopreserved sperm motility diluted and cryopreserved in 6% LDL extender compared with the egg yolk extender. The present results are consistent with that of Moussa *et al*. (2002) and Hu *et al*., (2010 and 2011) in bull; Akhter *et al*. (2011) in buffalo; Moustacas *et al*. (2011) in ram; Moreno *et al*. (2013) in horse, and Bencharif *et al*. (2010) in canine semen.

The results also showed that after semen freezing-thawing, the percentages of spermatozoa with acrosome integrity and plasma membrane integrity in bull semen were higher using the 6% LDL extender than that for the control and the other LDL extenders. These results agree with the findings of Hu *et al*. (2010) who reported a higher proportion of bovine spermatozoa with plasma membrane integrity in the extender containing LDL than egg yolk. Cryopreservation is known to affect the lipid organization and chemical composition of the spermatozoa plasma membrane (Amann and Pickett, 1987). It has been assumed that LDL directly or indirectly reduces these sperm membrane modifications (Pace and Graham, 1974; Manjunath *et al*., 2002; Moussa, *et al*., 2002; Bergeron *et al*., 2004 and Vera-Munoz *et al*., 2009). It is possible that, LDL interacts specifically with bovine seminal plasma proteins (Bergeron *et al*., 2004). This would decrease the binding of the major proteins of bovine seminal plasma to the sperm and would prevent lipid efflux from the sperm membrane, which could explain its beneficial effects.

In the present study, when the LDL concentration was increased from 4 to 6% LDL, an improvement was noted in bull sperm cryopreservation. In contrast, the use of 10% LDL led to reduce post thaw sperm quality. We attribute this finding to a drop in the osmotic pressure of the extender when the LDL concentration is increased (Laffaldano *et al*., 2014). Moussa *et al*. (2002) attributed the reduction in bull sperm quality after freezing- thawing in the presence of LDL above 10% to the precipitation of fructose and salts in the extender or to LDL aggregation with consequent neutralization of its cryoprotective effect.

Regarding the results of the fertility trial, the conception rates were significantly higher (P<0.05) in cows artificially inseminated with semen diluted and cryopreserved in 4 and 6% LDL extenders compared with that of the control one. However, the conception rate obtained from the cows that were inseminated with the semen diluted and cryopreserved in 10% LDL extender was slightly lower than that obtained from the cows inseminated with semen cryopreserved in the control extender (58.3% *vs*. 60%), however, the difference was not significant. The success rates with artificial insemination obtained from the semen diluted and cryopreserved in 4 and 6% LDL extender (72.7% and 76.7%) in compared with Tris-egg yolk extender (control, 60%), are satisfactory. In an *in vitro* study with bovine semen, a higher cleavage rates were observed with the semen cryopreserved in extender containing LDL than with egg yolk-based prepared extender Optidyl (Amiart-Briand *et al*., 2004). Whereas, in the Nili-Ravi buffalo bulls, Akhter *et al*. (2011) obtained a higher (P<0.01) fertility rate with semen cryopreserved in 10% LDL extender compared with that cryopreserved in 20% egg yolk extender.

CONCLUSIONS

On the basis of the present results, it could be concluded that the 6% low density lipoproteins in semen extender improves the freezing ability and fertilizing capacity of bovine bull semen. Furthermore, applying this study on a large scale would make it possible to specify the impact of the LDL extender on the success of artificial insemination programs in cows. It can also be concluded that the 6% LDL concentration in extender is better to protect the bovine sperm cell from cryodamage, improves cryopreserved semen quality and fertility.

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دور مخفف الليبوبروتينات منخفضة الكثافة في حفظ السائل المنوي لطلائق الأبقار بالتجميد وتأثيره علي الخصوبة

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الهدف من هذه الدراسة هو تقييم تأثير الحفظ بالتجميد لتركيزات مختلفة من الليبوبروتينات منخفضة الكثافة (٤، ٦، ٨ و ١٠%) كبديل لصفار البيض الكامل (٢٠%) في مخفف السائل المنوي علي تجميد السائل المنوي لطلائق الأبقار وخصوبته. تم تجميع السائل المنوي من أربعة طلائق مرتين أسبوعيا لمدة ١٢ أسبوع وجمعت القذفات ذات الحركة التقدمية أكثر من ٧٥% و ٨٥% طبيعية الشكل مع بعضها قبل الحفظ بالتجميد ثم خففت بمخفف الترس – حمض الستريك علي درجة حرارة ٣٧°م الي تركيز ٨ × ١٠ ^{٧} حيوان منوي / مل. تم تعبئة السائل المنوي في قصيبات ٠.٢٥ مل فرنسية الصنع وحفظت معرضة لبخار النيتروجين السائل لمدة ١٠ دقائق ثم غمرت في النيتروجين السائل (-١٩٦°م). تم تقييم وتقدير الحيوية ، سلامة الغشاء البلازمي وسلامة الأكروسوم للحيوانات المنوية بعد التخفيف ، قبل التجميد (بعد فترة الموازنة) وبعد التجميد والإسالة.

أظهرت النتائج أن مخفف السائل المنوي المحتوي علي ٦% ليبوبوتينات منخفضة الكثافة كان أكثر فعالية (عند مستوي معنوية ٠,٠٥) في الحفاظ علي الحركة التقدمية وسلامة الغشاء البلازمي والأكروسوم للحيوان المنوي مقارنة بالمجموعة الضابطة والتركيزات الأخري المستخدمة من الليبوبروتينات منخفضة الكثافة (٨ و ١٠%) عند مختلف خطوات الحفظ بالتجميد.

كانت معدلات الخصوبة عالية (عند مستوي معنوية ٠,٠٥) في الأبقار الملقحة إصطناعيا بعينات من السائل المنوي المخفف والمجمد في ٤ و ٦% ليبوبروتينات منخفضة الكثافة (٧٢.٧% و ٧٦.٧%) مقارنة بتلك المتحصل عليها من المجموعة الضابطة والتركيزات الأخري (٨ و ١٠%) من الليبوبروتينات منخفضة الكثافة (٦٠ ، ٦١ و ٥٨% علي التوالي). نستنتج أن إستخدام مخفف السائل المنوي المحتوي علي ٦% ليبوبروتينات منخفضة الكثافة كبديل لصفار البيض الكامل أكثرقدرة وفعالية علي حماية خلية الحيوان المنوي من الآثار الضارة للتجميد وحسَّنَ من جودة السائل المنوي المحفوظ بالتجميد وخصوبته.