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The Effect of LDL from Different Bird's Eggs on the Freezability of Buffalo Spermatozoa

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Bstract: The present study aimed to investigate the effect of adding LDLs, from different avian species, at different concentrations in Tris-based extender, on the post-thaw quality of buffalo bull sperm. Semen samples were collected from 4 buffalo bulls and diluted with tris-based extender containing LDLs extracted from turkey, hen and quail yolks at the rate of 5%, 7.5%, 10% or 15%, against a control extender containing 20% fresh egg yolk. Semen was processed for cryopreservation. Post-thaw motility, membrane and acrosome integrities were recorded. The results showed that, the extraction rates of turkey, hen and quail LDLs were 71.17, 67.30 and 56.25%, respectively. Post-thaw motility, viability indices as well as membrane and acrosome integrities of buffalo spermatozoa were highest (P = 0.05) in diluents containing 10% turkey and hen LDLs as compared to other LDL concentrations and 20% egg yolk. On the other hand, adding quail LDL resulted in the significantly lowest post-thaw results. In conclusion, the addition of turkey and hen LDLs, at concentration of 10%, to buffalo bull semen diluents improves the frozen-thawed semen quality in terms of motility, viability, sperm membrane and acrosomal integrities.

Key words: Buffalo · Semen · Cryopreservation · LDL · Hen · Quail · Turkey · Egg yolk

INTRODUCTION

Since the late 1930's [1], egg yolk was considered an essential additive to maintain viability of cooled frozen sperm. Among 9000 species of birds throughout the world, domestic chickens (*Gallus gallus domesticus*) egg yolk is a common component as a cryoprotectant agent for sperm storage in different animals [2]. There have also been numerous reports that egg yolk from avian species such as the Turkey, quail, duck or chicken has different combinations of fatty acids, phospholipids and cholesterol, which could result in different cryopreservation effects on the sperm [3-5]. In recent years, there has been frequent opinion against the use of egg yolk due to the increased risk of microbial contamination and the subsequent production of endotoxin, which may reduce the potential fertilizing capacity of spermatozoa [6, 7]. Moreover, the protective action of yolk was largely attributed to the LDL [8, 9]. In recent years, centrifugation techniques have enabled the isolation of the LDL that is responsible for the cryopreservative effect of egg yolk [8, 10]. The protective mechanism of LDLs is either through the stabilization of sperm membranes, or by the replacement of membranes phospholipids that are lost during the cryopreservation process or via seizure of deleterious proteins present in bovine seminal plasma [11].

Better post-thaw sperm motility in extender containing LDLs than egg yolk has been reported in bull [12-14], buffalo bull [15], equine [16], caprine [17], boar [18] and dog [19, 20]. The amount of LDLs used as a component of semen freezing extender may differently influence the structural and functional parameters of the spermatozoa [8]. Therefore there is need to study and formulate optimum concentration of LDL for better cryopreserved semen quality.

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The aim of the present study was to investigate the effect of adding LDLs, from different avian species, at different concentrations in Tris-based extender on the freezability and post-thaw sperm quality of buffalo bull semen.

MATERIALS AND METHODS

Extraction of LDL from Egg Yolk: Fresh turkey, hen and quail eggs were thoroughly cleaned and rinsed with distilled water. The egg shells were cracked and the yolk and albumen were separated. The vitellin membrane was perforated, the egg yolk was aspirated, diluted 1:2 (v:v) in 0.17 M sodium chloride solution, homogenized with a magnetic stirrer for 1 h at 4°C and pH was measured. The homogenate was then centrifuged (10,000g for 45min at 4°C; all centrifugations subsequently described used these conditions). The pellet (granular portion of the egg yolk) was discarded and the supernatant was again centrifuged. Ammonium sulphate solution (40%) was added dropwise (20 to 30 min) to the cooled plasma [4°C; 21]. The mixture was maintained at 4°C, with continuous stirring, for another hour. The mixture was then centrifuged and the supernatant dialyzed in cellophane membrane against MilliQ water over night, followed by centrifugation of the dialysis membrane content. The upper floating part containing the LDL was carefully withdrawn from the centrifuge tube, avoiding contamination by the fluid portion located at the bottom of the tube. The extracted LDL was preserved at -20°C until analysis [8].

Protein composition of LDL extracted was analyzed by SDS-PAGE. The stacking and running gels held 4 and 12.5% polyacrylamide respectively. Approximately 25µg of proteins were set on the gel. Electrophoretic migration was performed at 200 watts, 48 mA for 50 min at 21-23°C. Molecular weights of proteins and apoproteins were determined using reference standard proteins: 8, 15, 24, 31, 57 and 72 KDa. Polyacrylamide gel was stained with Comassie Blue.

Dry matter contents of the whole egg yolk (control) and of the LDL extracted from different egg sources were obtained after 24 h incubation in a muffle furnace at 104°C [8]. The lipid content was determined by the Soxhlet method [22] and the protein content was determined as described by Lowry *et al*. [23].

Preparation of Extenders: Tris-based egg yolk extender was prepared according to Reddy *et al.* [24], which comprised Tris 33.2 g/l, citric acid 18.3 g/l, dextrose 7.8 g/l, egg yolk 20% (v/v), glycerol 6.4% and supplemented with 100 mMol of trehalose [24] and antibiotics (gentamycin sulphate 500µg/mL, tylosin tartrate 100µg/mL, lincomycin HCl 300µg/mL and spectinomycin HCl 600µg/mL, [15]). The LDLs extracted from the turkey, hen and quail egg yolks were added to the experimental extenders at the rate of 5%, 7.5%, 10%, or 15% and the extender with 20% fresh egg yolk was used as control [8].

Animals and Semen Collection: Four healthy buffalo bulls of known fertility, maintained on the experimental farm of Animal Reproduction Research Institute, were used in the present study. Their age and body weight ranged between 3-4 years and 400-550 Kg, respectively. Early in the morning, using a pre-warmed artificial vagina (40-42 °C), twice a day ejaculates were collected from each bull once per week for 5 consecutive weeks (a total of 40 ejaculates).

Semen Processing: Semen samples were diluted with tris-based extender (containing LDLs added at the rate of 5%, 7.5%, 10% or 15% and a control extender containing 20% fresh egg yolk as control) at 37°C in incubator in an appropriate dilution rate to obtain a final concentration of 50 x 10^6 sperm cell/ml. Diluted semen was then cooled slowly to 5°C in a cold cabinet for a period of 1.5 h. Semen was loaded in 0.25 ml straws (IMV, France) and placed 4 cm above liquid nitrogen in the vapor phase in a foam box for 15 minutes before being plunged into the liquid phase [25]. Straws were stored in liquid nitrogen until thawing (one week after freezing) at 37°C in a water bath for 30 sec.

Two straws of each treatment (N =13) and replicate (N= 5) were thawed at 37°C in a water bath for 30 sec and pooled (for every treatment alone) for evaluation (motility, plasma membrane and acrosome integrities).

Evaluation of Frozen-thawed Semen: Motility estimations were done at hourly intervals for a period of 3 h. The viability index was calculated according to Milovanov [26] to be equal to half of the post-thaw motility in addition to the summation of recorded motility at 1st, 2nd and 3rd h post-thawing. The procedure described by Jeyendran *et al*. [27] was used to determine the percentage of hypo-osmotic swelling test (HOST) positive sperm cells in each semen sample. A 100 ìl aliquot of each semen sample was mixed in 1.0 ml of a pre-warmed hypo-osmotic solution (0.735 g of sodium citrate dihydrate and 1.351 g of fructose in 100 ml of sterile, de-ionized water). The mixture was incubated at 37°C for

30 min in a 1.5 ml micro-centrifuge tube. Following incubation, a small drop of sample was placed on a clean microscope slide and cover-slipped for examination using phase contrast microscopy (400X) to evaluate 100 spermatozoa for evidence of swelling and curling changes of the sperm tail. Acrosome integrity was estimated using fast green stain [28].

Statistical Analysis: Two way analysis of variance and Duncan's multiple range tests were done for the obtained data after angular transformation of percentages to their corresponding arc-sin values [29]. Data were analyzed using the 1984-version of Costat [30] and the level of statistical significance was set at $P \leq 0.05$.

RESULTS

A purer fraction of LDL was obtained when extraction was done according to the above mentioned. Four stained bands with approximate molecular weights of 42, 57, 125 and 165 KDa for turkey LDL, three stained bands of 57,72 and 125 KDa for hen LDL and three stained bands of 42, 57 and 125 KDa for quail egg yolk were obtained (Fig. 1; SDS-PAGE).

Data regarding the effects of adding different concentrations of turkey, hen and quail LDLs to semen extender on post-thaw characteristics of buffalo bull frozen-thawed semen were represented in tables 2, 3 and 4, respectively. The spermatozoa motility at 0, 1, 2 and 3 h after thawing as well as viability indices were highest ($P \leq 0.05$) for 10%, intermediate ($P \leq 0.05$) for 7.5% and lowest ($P \leq 0.05$) for 5 and 15% concentrations of turkey and hen LDLs. Concerning the quail LDL, the post-thaw motility of buffalo spermatozoa for the extenders containing 7.5% and 10% LDL was significantly ($P \leq 0.05$) higher than those of 5% and 15% concentrations. The viability index of buffalo spermatozoa for the extenders containing 15% quail LDL was significantly ($P \leq 0.05$) lower than those containing 5%, 7.5 and 10% concentrations. The percentage of spermatozoa with intact membranes (HOS +ve) and intact acrosomes were highest ($P \leq 0.05$) for the 10% concentrations, intermediate ($P \leq 0.05$) for the 7.5% concentrations and lowest ($P \leq 0.05$) for the 5 and 15% concentrations of turkey and hen LDLs.

Data regarding the effects of adding 10% turkey, hen and quail LDLs, as well as 20% hen egg yolk, to semen extender on post-thaw characteristics of frozen buffalo bull semen were summarized in table 5. The post-thaw spermatozoa motility, viability indices as well as the percentage of spermatozoa with intact membranes (HOS +ve) and intact acrosomes were significantly higher ($P \leq 0.05$) in diluents containing 10% turkey LDL and 10% hen LDL as compared to those containing 20% hen egg yolk. Adding 10% quail LDL resulted in the significantly ($P \leq 0.05$) lowest results in terms of post-thaw motility, viability index, the percentages of spermatozoa with intact membranes and acrosomes.

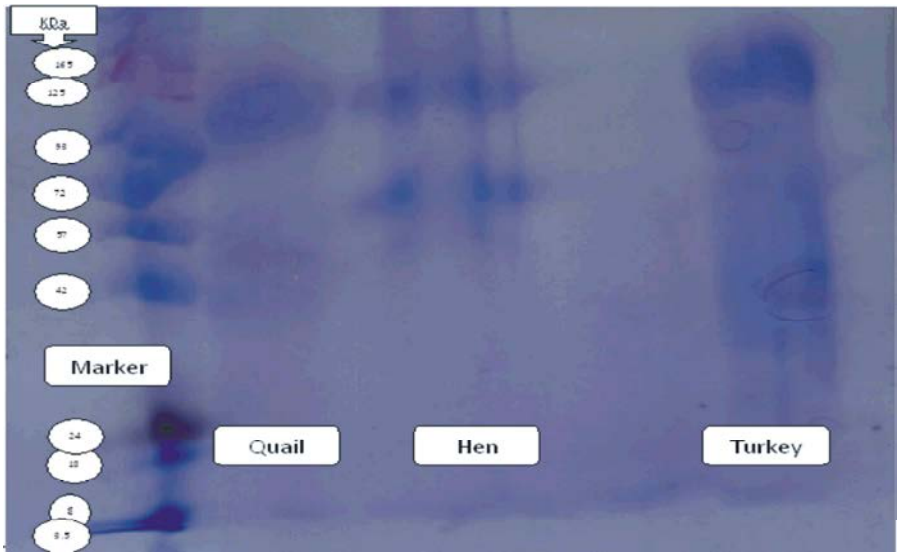

Fig. 1: SDS-PAGE profiles of proteins of quail, turkey and hen LDLs As shown in table 1, the dry matter content was highest ($P \leq 0.05$) in turkey LDL, intermediate ($P \leq 0.05$) in hen LDL and lowest ($P \leq 0.05$) in quail LDL. There were no significant differences in the protein and lipid contents between the LDL of the three avian species. The extraction rate of quail LDL was significantly ($P \leq 0.05$) lower than those of turkey and hen LDLs.

Table 1: Characterization of LDL fraction extracted from different avian egg yolk

	LDL		
Frozen-thawed semen characters	Turkey	Hen	Quail
Dry matter (DM%)	44.20 ± 0.97 [a]	42.10 ± 1.21 [ab]	37.57 ± 1.43 [b]
Protein (g/100g DM)	12.73 ± 1.19 [a]	10.24 ± 1.08 [a]	8.76 ± 0.53 [a]
Lipids (g/100g DM)	86.33 ± 1.27 [a]	83.53 ± 2.07 [a]	81.60 ± 0.94 [a]
Extraction rate (%)	71.17 ± 1.54 [a]	67.30 ± 0.84 [a]	56.25 ± 1.48 [b]

Means with different alphabetical superscripts within row are significantly different at P ≤ 0.05

Table 2: Effects of adding different concentrations of turkey LDL to semen extender on post-thaw characteristics of frozen buffalo bull semen

		Concentration of turkey egg LDL			
Frozen-thawed semen characters		5%	7.5%	10%	15%
Post-thaw motility (%) at	0 h	29.88 ± 1.63 [d]	47.50 ± 1.62 [b]	55.00 ± 1.58 [a]	34.75 ± 1.60 [c]
	1 h	25.00 ± 1.32 [c]	40.00 ± 1.99 [b]	47.38 ± 1.64 [a]	27.88 ± 1.51 [c]
	2 h	20.00 ± 1.62 [c]	35.00 ± 1.66 [b]	40.00 ± 1.66 [a]	20.00 ± 1.50 [c]
	3 h	10.25 ± 1.34 [d]	27.38 ± 1.17 [b]	32.63 ± 1.28 [a]	14.75 ± 1.64 [c]
Viability index		70.19 ± 3.75 [d]	126.13 ±2.64[b]	147.50 ±1.98[a]	80.00 ± 4.47 [c]
Swollen spermatozoa (HOS +ve %)		27.50 ± 1.16 [c]	43.50 ± 1.03 [b]	50.00 ± 1.41 [a]	30.00 ± 0.91 [c]
Normal acrosome (%)		41.00 ± 0.76 [d]	52.05 ± 1.08 [b]	63.10 ± 0.78 [a]	48.01 ± 0.61 [c]

Means with different alphabetical superscripts within row are significantly different at P ≤ 0.0

Table 3: Effects of adding different concentrations of hen LDL to semen extender on post-thaw characteristics of frozen buffalo bull semen

		Concentration of hen egg LDL			
Frozen-thawed semen characters		5%	7.5%	10%	15%
Post-thaw motility (%) at	0 h	34.75 ± 1.39 [d]	45.00 ± 1.62 [b]	57.63 ± 1.63 [a]	40.00 ± 1.32 [c]
	1 h	27.63 ± 1.64 [c]	37.38 ± 1.64 [b]	50.00 ± 2.19 [a]	30.25 ± 1.97 [c]
	2 h	20.00 ± 1.54 [d]	30.00 ± 1.66 [b]	40.00 ± 1.89 [a]	25.00 ± 1.36 [c]
	3 h	10.25 ± 1.17 [d]	22.88 ± 1.11 [b]	35.00 ± 1.66 [a]	15.00 ± 1.66 [c]
Viability index		75.25 ± 3.38 [d]	112.75 ±4.20[b]	153.81 ±3.99[a]	90.25 ± 4.75 [c]
Swollen spermatozoa (HOS +ve %)		30.15 ± 1.02 [d]	40.25 ± 0.99 [b]	52.00 ± 0.78 [a]	32.95 ± 0.78 [c]
Normal acrosome (%)		50.10 ± 1.52 [bc]	55.45 ± 1.20 [b]	68.00 ± 1.38 [a]	47.00 ± 1.05[c]

Means with different alphabetical superscripts within row are significantly different at P ≤ 0.05

Table 4: Effects of adding different concentrations of quail LDL to semen extender on post-thaw characteristics of frozen buffalo bull semen

		Concentration of quail egg LDL			
Frozen-thawed semen characters		5%	7.5%	10%	15%
Post-thaw motility (%) at	0 h	20.00 ± 1.46 [b]	25.00 ± 1.71 [a]	26.13 ± 1.65 [a]	15.50 ± 1.91 [b]
	1 h	15.00 ± 1.36 [a]	17.63 ± 1.17 [a]	17.38 ± 0.91 [a]	11.00 ± 1.08 [b]
	2 h	7.88 ± 1.11 [b]	10.75 ± 1.27 [ab]	12.63 ± 1.17 [a]	7.50 ± 1.04 [b]
	3 h	5.50 ± 1.08 [a]	5.25 ± 0.99 [a]	5.00 ± 0.83 [a]	2.50 ± 0.54 [a]
Viability index		38.39 ± 3.46 [a]	46.13 ± 3.39 [a]	48.06 ± 3.19 [a]	28.75 ± 2.90 [b]
Swollen spermatozoa (HOS +ve %)		23.01 ± 1.03 [c]	28.00 ± 0.77 [b]	35.05 ± 0.99 [a]	24.20 ± 0.89 [c]
Normal acrosome (%)		43.80 ± 0.99 [bc]	50.35 ± 1.35 [a]	46.30 ± 0.97 [b]	41.85 ± 0.88 [c]

Means with different alphabetical superscripts within row are significantly different at P ≤ 0.05

Table 5: Effects of adding 10% concentrations of turkey, hen and quail LDLs or 20% hen egg yolk to semen extender on post-thaw characteristics of frozen buffalo bull semen

		10% LDL			
Frozen-thawed semen characters		20% Egg yolk	Turkey	Hen	Quail
Post-thaw motility (%) at	0 h	49.65 ± 1.60 [b]	55.00 ± 1.58 [a]	57.63 ± 1.63 [a]	26.13 ± 1.65 [c]
	1 h	42.88 ± 1.27 [b]	47.38 ± 1.64 [a]	50.00 ± 2.19 [a]	17.38 ± 0.91 [c]
	2 h	37.65 ± 1.47 [a]	40.00 ± 1.66 [a]	40.00 ± 1.89 [a]	12.63 ± 1.17 [b]
	3 h	32.25 ± 1.60 [a]	32.63 ± 1.28 [a]	35.00 ± 1.66 [a]	5.00 ± 0.83 [b]
Viability index		137.58 ±4.07[b]	147.50 ± 1.98[a]	153.81 ±3.99[a]	48.06 ± 3.19 [c]
Swollen spermatozoa (HOS +ve %)		44.35 ± 1.55 [b]	50.00 ± 1.41 [a]	52.00 ± 0.78 [a]	35.05 ± 0.99 [c]
Normal acrosome (%)		57.70 ± 1.29[c]	63.10 ± 0.78 [b]	68.00 ± 1.38 [a]	46.30 ± 0.97 [d]

Means with different alphabetical superscripts within row are significantly different at P ≤ 0.05

DISCUSSION

The purpose of this study was to determine the cryoprotective effect of different concentrations of LDLs extracted from different egg yolk sources (turkey, hen and quail) on buffalo bull sperm. The extraction rates of LDLs recorded herein (ranged from 56.25% to 71.17%) was more or less similar to 67.0% that was recorded by Moussa *et al*. [8]. The later authors also recorded that the purity of the extracted LDL was 97.0%. The protein and lipid content of hen LDL recorded herein (10.24% and 83.53%, respectively) was in accordance with the findings of Demianowicz and Strezek [10] who recorded corresponding values of 10-15% and 85-90%, respectively. Moreover, It has been reported that there are different proportions of LDLs in commercial hen egg yolk depending on the hybrid line selected, management and nutritional practices adopted [31].

In the present study, the post-thaw motility and viability index of spermatozoa were significantly higher in the extender containing 10% turkey and hen LDLs compared with the control. Better post-thaw sperm motility in extender containing LDLs than that containing egg yolk has also been reported in buffalo bull [15, 43], bull [9, 13, 32], boar [18] and dog semen [20]. This suggests that egg yolk could contain some deleterious components which are potent to reduce semen motility [8].

Protection exerted by LDL may be attributed to association of these molecules with the cell membrane, increasing its stability during cryopreservation [33]. Moreover, it has been shown in bovine that LDL protect sperm membranes by associating with seminal plasma proteins [12] preventing them from promoting cholesterol efflux of the membrane and thereby triggering capacitation [13] which is unwanted during cryopreservation. In bull, Kampshmidt *et al.* [34] demonstrated that the granules found in egg yolk can reduce the respiration and motility of spermatozoa. Furthermore, egg yolk can be a source of bacterial contamination [35]. Amirat *et al*. [36] demonstrated that the extraction process used to obtain LDL from egg yolk reduces bacterial contamination by 10^7 colony forming units/ml.

Rauch [37] found that after the freeze-thawing process of bull sperm, quail LDL improved the percentage of motile sperm, compared to chicken yolk. However in the current study, quail LDL gave the worse sperm motility and viability results post thawing. The discrepancies in the present results may also be related to the freezing method used and species-specific factors. For quail egg yolk, Kulaksiz *et al*. [38] found that it resulted in suboptimum post-thaw motility. Also, Moreno *et al*. [4] reported that adding quail egg yolk in the diluents have no advantage over chicken egg yolk in the cryopreservation of Spanish ibex epididiymal sperm. Contrarily, Trimeche *et al.* [39] found that quail egg yolk improved the percentage of motile donkey sperm, compared to chicken yolk.

It is suggested that the reported improvement or decline in post-thaw quality of buffalo spermatozoa with LDL of different avian species in freezing extender is attributed to the differences in biochemical composition of the yolks. However, the analysis of the fat and protein content of the various LDLs used in this experiment (Table 1) did not help in the explanation of observed treatment differences. The high level of cholesterol in the turkey yolk reported by Kulaksiz *et al*. [38] could be beneficial, as Purdy and Graham [40] reported that addition of cholesterol to the bull sperm resulted in better post-thaw sperm quality.

In the present study, the post-thaw functional integrity of plasma membrane (HOS +ve) and acrosome of buffalo bull spermatozoa was higher in extender containing turkey and hen LDLs compared with the control. In a similar study, a higher proportion of buffalo bull sperm with intact sperm membranes was observed in extender containing hen LDL than in the extender containing egg yolk [15]. Moreno *et al.* [16] stated that LDL provided good protection of acrosome integrity, possibly via a direct action through the exchange or repair of acrosomal membrane phospholipids or possibly simply because the extender has lower progesterone content than egg yolk because of the filtering effect of the dialysis membrane. The progesterone found in egg yolk plays a role in the capacitation of spermatozoa in cattle [41] and horses [42].

According to all measured parameters herein, the extender containing 10% turkey and hen LDLs showed beneficial cryoprotective effects on frozen-thawed buffalo bull spermatozoa. In buffaloes, Akhter *et al*. [15] and El-Sharawy *et al.* [43] concluded that 10% and 12% LDL (respectively) in extender improved spermatozoa freezability and fertility. It appeared that 8% LDL was found to be more suitable for cryopreservation of bull [8, 32], ram [44] and caprine [17] sperm. Based on work with canine sperm, Bencharif *et al.* [45] demonstrated that 6% LDL gave the best post-thaw results.

In our study, at 5%, the LDLs, of different egg yolk sources, may not have been quite enough to interact with sperm plasmalemma to provide cryoprotection. Whereas

at 15% the osmotic pressure of the extender may not have been within the range specifically required for buffalo spermatozoa. Similar findings were observed for lower and higher LDLs concentrations by Akhter *et al.* [15]. It is reported by Moussa *et al.* [8] that with the increase in LDLs concentration above 10%, there is a decline in the osmotic pressure of extender, because of salt precipitation or the LDL aggregation effect, with the end result of a decrease in spermatozoa performance after freezing [8, 18].

In conclusion, the inclusion of 10% Turkey and hen LDLs, but not quail LDL, in the semen diluents improves the frozen-thawed semen quality in terms of post-thaw motility, viability, sperm membrane and acrosomal integrity.

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