Acta Scientiae Veterinariae, 2018. 46: 1530.

RESEARCH ARTICLE
Pub. 1530

ISSN 1679-9216

Low Density Lipoproteins at 2% Concentration Can Replace Whole Egg Yolk in TES-Tris-Milk Extender for Freezing Buffalo Sperm Cells

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ABSTRACT

***Background*:** Over the years, the most commonly used extenders for semen cryopreservation contain egg yolk as cryoprotectant. However, more recent studies have used the low density lipoproteins, extract of hen egg yolk which is responsible for the cryoprotective effect. Nevertheless, little was known about its required minimum concentration as well as its interaction with other extra cellular cryoprotectants, like skimmed milk. The present study aimed at investigating the effect of replacing whole egg yolk by adding low density lipoproteins at low concentrations, in TES-Tris-skim milk based extender, on the post-thaw quality of buffalo bull sperm.

***Materials, Methods & Results*:** Eighteen ejaculates were collected from six buffalo bulls and diluted with TES-Tris-skim milk based extender containing LDL, extracted from hen egg yolks, at the concentrations of 2%, 4%, 8% and 14%, against a control extender containing 20% fresh egg yolk. After semen collection, analyses of subjective motility, vigor, force tourbillon, sperm concentration (Neubauer chamber) and sperm morphology (phase contrast microscopy) were performed. The diluted semen was packaged in 0.25 mL straws, and cooling was performed on computerized machine (TK 4000®), using a cooling rate of -0.25°C/min to 5°C. Semen was kept in balance at 5°C for 4 h. The straws were frozen in an ice chest, kept at 5 cm from the surface of liquid nitrogen for 20 min and then immersed in liquid nitrogen. The samples were kept in cryogenic container until thawing. Post-thaw kinetic parameters during incubation at 37°C (CASA), sperm membrane integrity (SYBR-14/PI), membrane functionality (hypo-osmotic swelling test) and DNA fragmentation (%DFI - SCSA) were evaluated after thawing. Immediately post-thaw, total motility was higher in the control (56.53 ± 9.73) than in the tested extenders; however, after 30 min the difference was no longer detected. All other kinetic parameters presented significantly better results in extenders containing 2%, 4% and 8% LDL, compared with the control. There was no difference between treatments regarding the integrity of membranes or fragmentation of the DNA after freezing/thawing procedures.

***Discussion*:** The molecules of low density lipoproteins of egg yolk have the known action of sequestering BSP (binder of sperm proteins) protein, due to the chemical affinity of their main components, phosphatidylcholine and phosphatidylethanolamine. The BSP are responsible for removing cholesterol from the plasma membrane of the sperm, preparing it for the sperm capacitation phase. Low density lipoproteins by inhibiting the action of the BSP increase the stability of the plasma membrane during the freeze-thaw process. The milk caseins micelles have a similar ability to bind the BSP, but with lower affinity. The present study has shown that the use of purified low density lipoproteins has an advantage over the use of whole egg yolk, especially with regard to the kinetic parameters of the spermatozoa after thawing. Furthermore, it was observed that low concentrations of low density lipoproteins, such as 2%, in extenders containing skim milk, could preserve the sperm cell during the freeze-thawing process like that higher LDL concentrations and whole egg yolk. Further studies are needed to determine if the cryoprotectant action found in this study was a result of the synergistic action of skim milk with lipoproteins or even at low concentrations like 2% the lipoproteins can provide protection to the buffalo spermatozoa during freezing, as it has been reproduced in other domestic species. In conclusion, our results indicated that the extender TES-Tris-skim milk containing 2% LDL extracted from egg yolk could be used successfully in the cryopreservation of buffalo sperm cells.

Keywords: LDL, cryopreservation, *Bubalus bubalis*, flow cytometry, sperm kinetic parameters.

DOI: 10.22456/1679-9216.81815

Received: 25 November 2017 Accepted: 3 February 2018 Published: 17 February 2018

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INTRODUCTION

Artificial insemination, with freezing semen, has been widely used for genetic improvement in buffalo production systems. The most commonly used extender to preserve buffalo sperm contains 20% egg yolk or skimmed milk. However, due to the large variation in its constitution and presence of certain components which have noxious effect on spermatozoa [17], other substitutes have been proposed, such as low density lipoprotein (LDL), extracted from hen egg yolk.

The concentration of lipoproteins added to the extender, and its interaction with other medium components, may cause different effects on the structural and functional parameters of the sperm [14]. Furthermore, the chemical composition of the seminal plasma and sperm of the studied species influences both freeze resistance and the interaction with medium compounds, and consequently the choice of the extender to be used [2]. Therefore, the selection of species specific-extender must be carefully investigated.

For buffalo sperm, it has been suggested that concentrations between 10-12% of LDL, using Tris-Citric acid-based medium [1,8]. However, superior results for buffalo sperm quality after thawing were found when extenders containing skim milk were used [7]. The combination of milk and LDL could allow reduction of the concentration of one or both components, reducing the participation of the animal origin compounds in the diluents. Experiments of this approach have not been reported for buffalo sperm.

Therefore, the objective of this experiment was to evaluate the suitable LDL concentration to replace whole egg yolk in a TES-TRIS-milk extender for the cryopreservation of buffalo sperm cells.

MATERIALS AND METHODS

Preparation of extenders

Low-density lipoproteins were extracted from hen egg yolk according to the method described by [14] with some modifications proposed by [16]. The extracted LDL presented qualitative characteristics as reported by [14], when submitted to electrophoretic run 1D SDS PAGE.

The TES-Tris-milk extender was prepared as follows: 4.9 g N-[tris(hydroxymethyl) methyl]-2-aminoethanesulfonic acid[1], 1 g Tris-(hidroxymethyl)-aminomethane[1], 0.216 g Fructose[1], 0.14 mg/mL Penicillin[1] and 1.40 mg/mL Streptomycin[1] in 100 mL distilled water (Solution A). For preparation of the final solutions, the same proportion of Solution A and ultra-heat-treated skim milk (v/v), and 7% glycerol were added. LDL were added to the extenders at concentrations of 2% (LDL2), 4% (LDL4), 8% (LDL8) and 14% (LDL14) (v/v), and the extender with 20% fresh egg yolk was used as control (EY). All extenders were corrected for pH 7.

Semen collection and processing

Semen was collected from six healthy Murrah buffalo bulls (*Bubalus bubalis*), aged 30 to 36 months, maintained under similar food and health conditions at the Center of Biotechnology in Buffaloes - EV-UFMG, in Pedro Leopoldo, State of Minas Gerais, Brazil.

Only ejaculates presenting ≥ 70% individual sperm motility, ≥ 3 mass motility and ≤ 30% morphologically abnormal spermatozoa were used. A total of 18 ejaculates (three ejaculates from each bull) collected using an artificial vagina (at 42-45°C) were used. Each ejaculate was split into five aliquots. Aliquots were diluted to obtain 50 x 10^6 spermatozoa/mL using extenders in test. Sperm concentration was determined using a Neubauer haemocytometer chamber.

Diluted semen samples were filled into 0.25 mL French straws and cooled to 5°C in a programmable cell freezer (TK 4000®)[2], at a cooling rate of -0.25°C/min. The semen was maintained in equilibrium for 4 h at 5°C. The straws were then held 5 cm above the surface of the liquid nitrogen for 20 min, before being plunged into the liquid nitrogen for storage (-196°C).

Quality assays of post-thawed sperm cells - Longevity of sperm kinetic characteristics

Evaluations of post-thawed sperm kinetics parameters (CASA; SCA® v. 4.0, Microptic, Spain) were carried out immediately post thaw (37°C for 30 s) and every 30 min on samples incubated in water bath at 37°C, for a maximum of 2 h. Five fields were chosen at random over the slide/coverslip and analyzed, an average was then taken. Twenty images per second were taken. The following parameters were assessed: overall motility (Mot, %), curvilinear velocity (VCL, μm/sec), straight-line velocity (VSL, μm/sec), average path velocity (VAP, μm/sec), linearity (LIN, %) and beat cross frequency (BCF, Hz).

Quality assays of post-thawed semen - Hypoosmotic swelling test

Functional integrity of the plasmalemma was evaluated by the hypoosmotic swelling test using sodium citrate and a fructose solution, with 30 min of incubation at 37°C [18]. Two hundred spermatozoa per slide were counted at 1000x magnification, through a phase contrast microscope. The percentage of typical tail coiling/swelling was determined.

Quality assays of post-thawed semen - Flow Cytometric assay

Flow cytometer analyses were carried out by using FACScan[3] equipped with 15 mW air-cooled Argon laser. After acquisition, data were evaluated with FlowJo software version 7.6.14. Sperm viability was analyzed by two DNA-binding fluorescent stains: Sybr green[1], a membrane-permeant stain, and Propidium iodide - PI[1], a conventional dead-cell stain. The Sybr green was diluted 1:10 with phosphate buffered saline (PBS). Five microliters of this work solution were added to 1 mL of diluted semen to a final concentration of 4 x 10^6 spermatozoa/mL. Next, 15 µL of PI solution (0.01 mg/mL) were added and incubated for 10 min before analysis. Each sample was analyzed after acquisition of 30,000 total events, and results were expressed in percentage of viable sperm (Sybr green$^+$/PI$^-$).

Sperm nuclear integrity was assessed using the sperm chromatin structure assay (SCSA) [9]. A sample of 400,000 sperm was diluted into 100 µL of TNE buffer (0.01 M Tris/HCl, 0.15 M NaCl, 1 mM EDTA, pH 7.4) and incubated with 200 µL of detergent acid solution (0.17% Triton 100X, 0.15 M NaCl, 0.08 N HCl, pH 1.4) for 30 s at room temperature. Then, 1.2 mL of final solution (0.15 M NaCl, 1 mM EDTA, 0.1 M citric acid, 0.2 M Na_2HPO_4, pH 6.0) were added, followed immediately by adding 10 µL of a solution of acridine orange (0.5mg/mL), and signals from 30,000 events were read after 5 min of incubation at room temperature. The DNA fragmentation index (DFI) was obtained by the ration of red to total fluorescence intensity.

Statistical analysis

The experimental design used completely randomized blocks with split plot, where each animal was considered a block and the reading times as subplots. Variables presented normal distribution, according to Fisher's test. The data on semen quality parameters were analyzed using ANOVA and were presented as mean (± SD). Tukey's test was used to compare the means. Differences with values of $P < 0.05$ were considered as statistically significant.

RESULTS

After the freezing-thawing processes of the sperm cells, an average drop of 43.4% for total motility was observed. The longevity of sperm kinetics parameters post-thaw is displayed in Figure 1. Higher total sperm motility ($P < 0.05$) was observed immediately post-thawing in cells frozen in the EY (56.53 ± 9.73%), compared to others with LDL (LDL2 47.16 ± 11.06%; LDL4 45.64 ± 7.46%; LDL8 45.71 ± 6.56; LDL14 43.33 ± 5.97%). However, this difference disappeared after 30 min of incubation at 37°C.

Moreover, the EY led to a significant decrease in all the other kinetic parameters ($P < 0.05$), regardless of the evaluation time, when compared to LDL2, LDL4, LDL8 and LDL14 (except for VCL at 0 and 30 min, and VAP at 0 min for LDL14) [Figure 1].

The functionality and structural integrity of plasma membranes and the DNA fragmentation of post-thawing buffalo sperm were evenly preserved ($P > 0.05$) in the extender containing whole egg yolk as they were in the extenders containing 2 to 14% LDL (Table 1).

Table 1. Incidence of functional plasma membrane by the hipoosmotic swelling test, and of intact plasma membrane and integrity of DNA by flow cytometric evaluation, of post-thawed buffalo sperm diluted in TES-Tris-skim milk extender containing different LDL concentrations (LDL2, LDL4, LDL8, LDL14) and 20% whole egg yolk (EY).

	Functional plasma membrane (%)	Intact plasma membrane (%)	Unfragmented DNA (%)
LDL2	55.39 ± 3.66	61.88 ± 2.99	99.20 ± 0.54
LDL4	51.22 ± 7.82	39.73 ± 9.72	98.74 ± 0.96
LDL8	52.39 ± 4.24	40.33 ± 7.38	98.44 ± 1.04
LDL14	49.39 ± 5.21	49.33 ± 28.10	98.01 ± 2.05
EY	53.44 ± 3.83	49.15 ± 12.02	98.69 ± 0.62

Values are expressed as a mean ± SD of 18 ejaculates from six buffaloes (3 per bull). [$P > 0.05$].

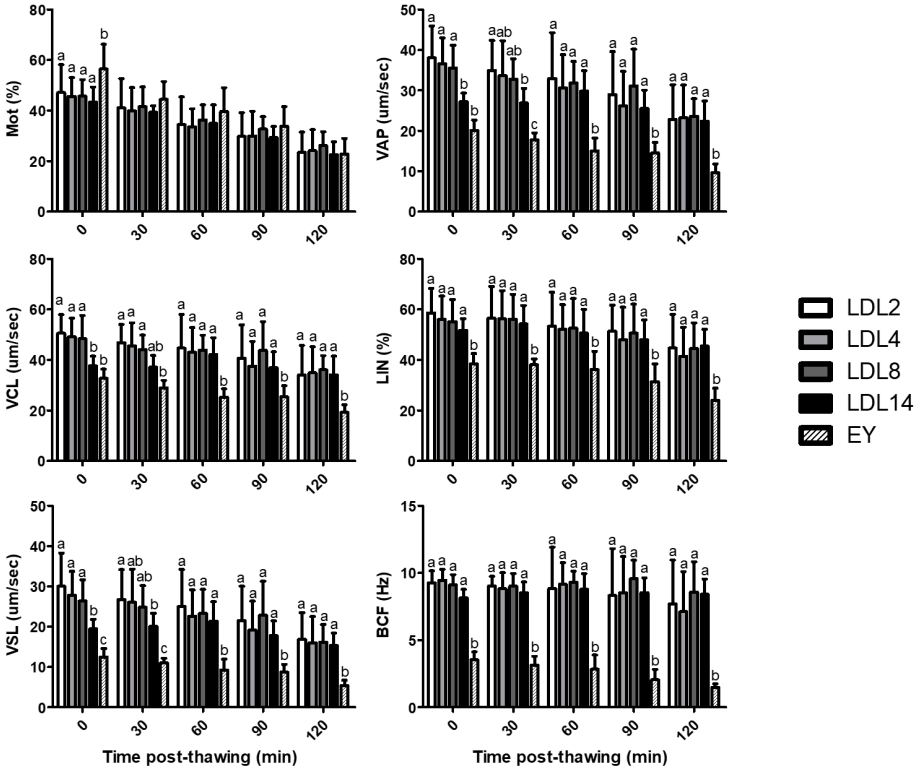

Figure 1. Effect of different concentrations of low density lipoprotein (LDL2, LDL4, LDL8 and LDL14) and 20% egg yolk (EY) in TES-Tris-skim milk extenders on the post-thawing kinetic parameters of buffalo spermatozoa. Mot= overall motility; VCL= curvilinear velocity; VSL= straight-line velocity; VAP= average path velocity; LIN= linear motility; BCF= beat cross frequency. Values are expressed as a mean ± SD of 18 ejaculates from six buffaloes (3 per bull). Different letters in the same evaluation time are significantly different ($P < 0.05$).

DISCUSSION

Numerous researches [1,8,13,14,20] have demonstrated that LDL possessed protective action for freezing-thawing mammalian spermatozoa. Nevertheless, little was known about its required minimum concentration or its interaction with other extra cellular cryoprotectants, like skimmed milk. The results of the present study show that LDL at concentrations of 2%, 4% or 8%, in extender with skimmed milk, improve buffalo spermatozoa kinetic parameters following cryopreservation in comparison to the same extender containing 20% egg yolk with skimmed milk ($P < 0.05$).

Low density lipoproteins (LDL) can serve as a source for the replacement of phospholipids in the sperm membrane, which are lost during the cooling/freezing process [3]. Another described protection mechanism refers to the known action of sequestering BSP (binder of sperm proteins). Hereby, LDL prevents the insertion of BSP proteins into the phospholipid layer of spermatozoa [11], reducing the efflux of cholesterol and thus increasing the resistance of the cells to freezing-thawing processes [12]. The casein micelles, present in skimmed milk, produce an effect like LDL. They are also able to bind to BSP of the seminal plasma, reducing the efflux of cholesterol and phospholipids in the spermatozoa plasma membrane [6]. Although, this protein-lipid interaction suffering by the skimmed milk, presenting relatively less binding affinity. Moreover, it has been shown that the presence of lactose in freezing media acts beneficially in post-thaw parameters, though the mechanism of action is unknown [4].

As concentration as low as 2% were effective in cryoprotection, we suggest that LDL and skimmed milk act in synergism to protect sperm cells during criopresevation with a clear advantage over the use of whole egg yolk. Further research is needed to analyze if the results obtained with 2% of LDL would be maintained in extender without skimmed milk. In studies with other species using media composed by LDL in Tris-citric acid media, without skimmed milk, low concentrations such as 2% were as be able to protect sperm cells during the freezing process than higher concentrations or whole egg yolk [5,15] (boar and ram, respectively).

During the LDL purification of hen egg yolk, some deleterious substances present in whole egg yolk, which serves as substrate for forming a potent inhibitor of sperm respiration and motility, are removed [19]. Therefore, the elimination of those substances using purified LDL may have contributed to the improvement of VCL, VSL, VAP, LIN and BCF and longer longevity of these parameters found in sperm cells cryopreserved with LDL in replacement of whole egg yolk. Considering that VCL, VSL, VAP, LIN, and BCF are kinetics parameters positively correlated with fertility *in vivo* index [10] and oocyte penetration *in vitro* fertilization, it can be foreseen that buffalo frozen sperm in extenders containing LDL could present satisfactory fertility results. However, more specific tests are required to confirm this hypothesis.

Spermatozoa with a swollen tail (flagellum) have a functional plasma membrane. Similarly, spermatozoa that do not present red fluorescence when in presence of propidium iode have a structurally sound plasma membrane. Present results show that all the extenders containing LDL provided similar protection of the membrane than when whole egg yolk was used. Considering that the function of providing greater stability to the membranes is assigned to LDL, it seems that none of the LDL concentrations accounted for any improvement of membrane integrity [3].

The integrity of the DNA or chromatin is an important factor when assessing spermatozoa fertility. The results obtained herein are encouraging as more than 98% of DNA was unfragmented, independent of the LDL concentration used. This suggests that LDL also provide protection for cellular DNA during the freeze-thaw process of buffalo semen.

CONCLUSIONS

Based on the results of this study, it was shown that LDL possess remarkable cryoprotective properties for freezing-thawing buffalo spermatozoa. Higher kinetics parameters were achieved with the use of 2%, 4% and 8% LDL as compared to 20% whole egg yolk, in a TES-Tris-milk extender. Thus, it can be concluded that low concentration of LDL such as 2% associated with skimmed milk can be used for buffalo semen freezing. However, further studies are needed to verify whether such a low concentration of LDL without skimmed-milk is sufficient for promoting acceptable protection during the freezing - thawing processes of buffalo sperm.

MANUFACTURERS

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Ethical approval. All procedures involving animals were performed in accordance with the Ethical Committee of Animal Use of the Federal University of Minas Gerais (CEUA-UFMG – 357/2015).

Acknowledgements. The authors sincerely thank INCT Pecuária/CNPq/FAPEMIG for their research grant and financial support, and PRPq of the UFMG for their financial support for language services.

Declaration of interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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http://dx.doi.org/10.22456/1679-9216.81815