Veterinary World, EISSN: 2231-0916
Available at www.veterinaryworld.org/Vol.7/September-2014/15.pdf
RESEARCH ARTICLE
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Cholesterol loaded cyclodextrin increases freezability of buffalo bull (*Bubalus bubalis*) spermatozoa by increasing cholesterol to phospholipid ratio

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Received: 24-05-2014, **Revised:** 02-08-2014, **Accepted:** 06-08-2014, **Published online:** 20-09-2014

doi: 10.14202/vetworld.2014.702-706. **How to cite this article:** Rajoriya JS, Prasad JK, Ghosh SK, Ramteke SS, Barik NC, Das GK, Pande M (2014) Cholesterol loaded cyclodextrin increases freezability of Buffalo bull (*Bubalus bubalis*) spermatozoa by increasing cholesterol to phospholipid ratio, *Veterinary World* 7(9): 702-706.

Abstract

Aim: The study was conducted to investigate the effect of cholesterol loaded cyclodextrin (CLC) on freezability of buffalo spermatozoa.

Materials and Methods: Murrah buffalo bull semen samples with progressive motility of 70% and greater were used. After the evaluation of motility and livability, four equal fractions of semen samples were made. Group I was kept as control and diluted with Tris, whereas Group II, III and IV were treated with CLC solution at the rate of 2.0, 3.0 and 4.0 mg/ml respectively to obtain 120 × 10^6 sperm/ml as final spermatozoa concentration. The aliquots of all the groups were incubated for action of CLC, followed by dilution and freezing. Evaluation at pre-freeze and post-thaw stage of progressive motility, viability and level of cholesterol and phospholipid was done.

Results: The mean cholesterol content (µg/100 × 10^6 spermatozoa) of Group I, II, III and IV at pre-freeze stage was 21.55±0.63, 49.56±1.38, 55.67±0.45 and 47.79±1.01 and at post-thaw stage were 13.18±0.45, 34.27±0.71, 36.21±0.48 and 33.68±0.56, respectively. At pre-freeze stage, cholesterol content was significantly ($p<0.01$) higher in Group III in comparison to other groups. The mean cholesterol and phospholipids content of fresh sperm was 24.14±0.58 and 51.13±0.66 µg/100 × 10^6 sperm cells, respectively, and C/P ratio of spermatozoa at fresh stage was 0.47±0.067.

Conclusion: CLC treatment maintains the C/P ratio and plays an important role in maintaining membrane architecture of spermatozoa. Hence, addition of CLC may be helpful in increasing freezability of buffalo spermatozoa by increasing the C/P ratio of spermatozoa.

Keywords: buffalo, cholesterol loaded cyclodextrin, cholesterol:phospholipid (C/P) ratio.

Introduction

Buffalo spermatozoa are more susceptible to damages during freezing and average post-thaw motility has been reported to be lower than cattle spermatozoa [1]. There are some specific biochemical factors that affect the ability of spermatozoa to prevent damages caused by the cryogenic procedures. One of the many possible causes of lower freezability of buffalo bull semen compared with cattle bull can be due to the differences in the lipid ratio of the spermatozoa [2]. Sperm sensitivity to cold shock damage is determined by membrane phospholipid composition as well as the membrane C/P ratio [3]. Sperm possessing high C/P ratio (rabbit and human sperm) are more resistant to the cold shock damage than sperm which have low cholesterol: phospholipid ratios (boars, stallions, rams, and bulls) [4]. The integrity of the plasma membrane is important for the spermatozoa to withstand harmful effects of the cryopreservation process. Capacitation can be reduced by adding cholesterol or cholesterol analogs to the medium [5], and can be stimulated by cholesterol acceptors such as β-cyclodextrins [6].

Cholesterol is a hydrophobic molecule and is not soluble in aqueous semen diluents. Cyclodextrin have been used to insert or remove cholesterol from synthetic and cell membranes. Cyclodextrins are cyclic oligosaccharides obtained by the enzymatic degradation of starch, and they possess an external hydrophilic face and an internal hydrophobic core [7] that can encapsulate hydrophobic compounds such as cholesterol.

Cholesterol content of sperm membranes can be modified using cholesterol loaded cyclodextrin (CLC) [8]. Since cholesterol efflux from the sperm membranes plays an important role in sperm capacitation, it is possible that increasing sperm cholesterol content, using CLC technology, may reduce premature sperm capacitation thereby increasing the lifespan of a cryopreserved sperm cell, in addition to increasing the number of sperm that survive cryopreservation. Cholesterol also decreases the capacitation like changes (cryocapacitation) that occurs when sperms are frozen. CLC have been used in several species like

bull, ram, stallion, boar and donkey's semen cryopreservation [9].

The objective of this study was aimed to assess the effect of CLC treatment on C/P ratio and freezability of buffalo spermatozoa.

Materials and Methods

Climate and experimental animals

Geographically Bareilly is located at 28°10' North latitude and 78°23' East latitude at an altitude of 172 m above the mean sea level. Bareilly is known to have a moderate climate. Summer season goes up to 40°C, whereas winter goes down up to 8°C. The rainy season starts in June and extends up to September with humid and warm conditions. Four healthy breeding buffalo bulls maintained at Germ-Plasm Center of Animal Reproduction Division, IVRI, Izatnagar, Bareilly were utilized for the study.

Chemicals

All chemicals were reagent grade and purchased from Sigma-Aldrich (St. Louis, MO, USA).

CLC preparation

Methyl-β-cyclodextrin was loaded with cholesterol as described [8]. Briefly, 200 mg of cholesterol was dissolved in 1 ml of chloroform in a glass tube. In second glass tube, 1 g of methyl-β-cyclodextrin was dissolved in 2 ml of methanol. A 0.45 ml aliquot of the cholesterol solution was added to the cyclodextrin solution, and the mixture was stirred until the combined solution appeared clear. This was followed by pouring of mixture into a glass petri dish and removing of solvents using a stream of nitrogen gas. The resulting crystals were allowed to dry for an additional 24 h and then were removed from the dish and stored in a glass container at 22°C. A working solution of CLC was prepared by adding 50 mg of CLC to 1 ml of Tris diluent at 37°C and mixing the solution briefly using a vortex mixer.

Collection of semen and its processing

Semen was collected by using an artificial vagina as per the standard method. A total of 40 ejaculates, ten from each bull (10 × 4 = 40) were collected. The percentage of progressively motile sperm in each sample was determined by semen quality analyzer (SQA-Vb; MES, Israel). Only ejaculates containing >70% motile spermatozoa were used in the study. At fresh stage evaluation for progressive motility, viability and level of cholesterol and phospholipid were done.

After the initial semen evaluation, four equal fractions of the samples were made. The Group I (control) was diluted with Tris, while Group II, III and IV were treated with CLC solution [10] at the rate of 2.0, 3.0 and 4.0 mg/ml respectively to obtain 120 × 10^6 sperm/ml as final spermatozoa concentration. The aliquots of all the groups were incubated at 37°C for 15 min for action of CLC. After incubation, each sample was diluted to 60 × 10^6 sperm/ml in Tris-egg yolk-glycerol dilutor.

Semen freezing and its evaluation

The extended semen was packaged in 0.5 ml French straws and subjected to a combined cooling with equilibration period of 3 h at 5°C. The straws were kept in automatic programmable biological cell freezer (IMV technology, France) until temperature of straws reached −145°C. Then straws were plunged into liquid nitrogen (−196°C) for storage. Semen samples were evaluated at pre-freeze and post-thaw stage for progressive motility, viability and level of cholesterol and phospholipid. Viability of spermatozoa was assessed using fluorescent dye Hoechst (H33258). Pre-freeze evaluation was done immediately after the equilibration period. Frozen straws were thawed at 37°C for 30 s after 24 h of freezing for post-thaw evaluation.

Cholesterol and phospholipid estimation in spermatozoa

Washing of spermatozoa

Washing of spermatozoa was necessitated for estimation of cholesterol and phospholipid content of spermatozoa. Immediately after evaluation fresh, pre-freeze and frozen-thawed spermatozoa were washed using Percoll density gradient [11] to remove egg yolk particles, dead cells, and debris as described below:

- 1 ml layer of 40% Percoll (v/v, Sigma Aldrich, USA) in a non-capacitating medium (NCM) was pipetted carefully over 1 ml layer of 80% Percoll (v/v in NCM) in a disposable 15 ml centrifuge tube.
- 1 ml fresh or thawed semen was gently layered on top of the two steps Percoll column.
- This test tube was centrifuged at 400 g for 30 min.
- After centrifugation, the pellets were washed once again with NCM and resuspended in NCM to make desired concentration of sperm depending upon experiments.

This procedure was followed to make an aliquot of 1 ml (in duplicate) of all the four groups in cryovials and stored at −20°C till used for cholesterol and phospholipid estimation.

Cholesterol assay

The cholesterol content in spermatozoa was estimated as per the method of [12].

Phospholipid assay

Phospholipid in the lipid extract of spermatozoa was estimated by the method of [13].

Statistical analysis

All the data were statistically analyzed using SPSS 16.0 (SPSS Inc., Chicago) using one-way analysis of variance and significant means were compared using Duncan's multiple range test.

Results and Discussion

CLC has been used for semen cryopreservation of animal species such as bull, ram, stallion, boar, donkey with varying degree of its beneficial effect on

semen cryopreservation. It was further postulated that CLC can be useful to certain extent for cryopreservation of buffalo bull spermatozoa.

Seminal attributes of buffalo spermatozoa at fresh stage

Sperm progressive motility and viability

The initial progressive motility of a semen sample gives a good indication of the fertility of the bull and ability of spermatozoa to withstand the stress of the cryopreservation process. Furthermore, the viability of spermatozoa of a semen sample has been significantly correlated with motility and fertility of spermatozoa. In the present study, initial progressive motility and viability were 83.28±2.71 and 84.43±2.45%, which were comparable to the values previously reported by various researchers [10,14], but higher than the values reported by [15]. The difference may be attributed to the various factors that affect motility and viability like bull's age [10], season, frequency of collection and sexual excitement before semen collection.

Cholesterol-phospholipids content and C/P ratio of spermatozoa

The mean cholesterol and phospholipid content of fresh sperm were 24.14±0.58 and 51.13±0.66 μg/100 × 10^6 sperm cells, respectively and C/P ratio of spermatozoa at fresh stage was 0.47±0.067. The result was in agreement with some previous report [16,10]. The cholesterol content of spermatozoa varies widely between bulls as well as different ejaculates of the same bull. Many studies have shown that spermatozoa capacitation is accompanied by a change in the lipid composition of the plasma membrane involving a decrease in the membrane C/P ratio [17]. These changes appear to be a reversible phenomenon that influences the fluidity and ionic permeability of the plasma membrane [18]. The lipid composition of the sperm plasma membrane plays an important role in determining the membrane fluidity, sperm motility and viability [19]. Lipids in the sperm play important role in sperm physiology and metabolism. Phospholipids are important structural component of membranes. The intrinsic importance of phospholipids in membrane structure and the expression of enzyme activity indicate their significant role in cellular reactions to environmental change. Sperm sensitivity to cold shock damage is determined by the membrane C/P ratio and phospholipid composition [3]. Species that possess sperms with high C/P ratio (human and rabbit) are more resistant to cold shock than species presenting membrane low C/P ratio such as stallion, ram and bull [4]. C/P ratio in buffalo spermatozoa at fresh and post-thaw stage was 0.44±0.05 and 0.16±0.063, respectively [10]. Hence, loss in C/P ratio was about 63.63%. However, the C/P ratio in cattle bull spermatozoa varied from 0.42 to 0.45 [20]. There is an active participation of sperm plasma membrane in the process of capacitation, mainly through loss of cholesterol [5]. Cholesterol efflux leads to changes in membrane architecture and fluidity that gives rise to the capacitation of the frozen sperm cells.

Seminal attributes of buffalo spermatozoa at pre-freeze and post-thaw stage

Sperm progressive motility and viability

The mean value of motility at pre-freeze (81.25±2.39%) and post-thaw (69.50±0.29%) spermatozoa was significantly ($p<0.01$) higher in Group III when compared with other three groups. (Tables 1 and 2). In the present study, an approximate of 17.57% improvement in the progressive motility was observed after treatment with 3 mg CLC, which was higher than the values (12.5%) after treatment with 2 mg CLC as reported [10]. The exact mechanism by which cholesterol improves sperm cryosurvival is still not known. The possible mechanism behind increased progressive motility of spermatozoa following cryopreservation after incorporation of cholesterol in sperm membrane is due to protection of mitochondria [21]. The per cent livability among control and treatment groups in pre-freeze stage (Table-1) did not show any significant difference ($p<0.01$) however, at post-thaw stage it showed significant difference ($p<0.01$) amongst all the groups with highest in Group III followed by II, IV and I (Table-2). Higher viability at post-thaw stage after CLC treatment may be due to stabilizing effect of sperm plasma membrane [22] and hence reduced loss of sperm during cryopreservation was observed.

Cholesterol-phospholipids content and C/P ratio of spermatozoa

The mean cholesterol content (μg/100 × 10^6 spermatozoa) of Group I, II, III and IV at pre-freeze stage was 21.55±0.63, 49.56±1.38, 55.67±0.45 and 47.79±1.01 and at post-thaw stage were 13.18±0.45, 34.27±0.71, 36.21±0.48 and 33.68±0.56, respectively. At pre-freeze stage, cholesterol content was significantly ($p<0.01$) higher in group III in comparison to other groups. At post-thaw stage, it was also significantly ($p<0.01$) higher than Group I and IV and non-significantly higher than Group II (Table-2).

The mean values of phospholipids content of spermatozoa (μg/100 × 10^6 spermatozoa) in Group I, II, III and IV at pre-freeze stage were 90.48±0.54, 90.33±0.53, 90.23±0.59 and 92.15±0.52 and at post-thaw stage were 79.45±0.38, 79.09±0.34, 82.35±0.51 and 85.17±0.45, respectively. There was no significant difference observed in the phospholipids content among different groups in the pre-freeze stage, however, in the post-thaw stage, Group IV (treated with 4.0 mg dose of CLC) showed significantly ($p<0.01$) higher values followed by Group III.

An appreciable reduction in phospholipid content of spermatozoa from pre-freeze to post-thaw stage was observed in the study that might be due to cold shock and freeze-thaw lead to phospholipid efflux in buffalo spermatozoa [23]. The present study indicated that pre-freeze and post-thaw values

Table-1: Seminal attributes in buffalo bulls spermatozoa at pre-freeze stage (mean±SE).

Group	Initial progressive motility (%)	Livability (%)	Cholesterol (µg/100×10⁶ spermatozoa)	Phospholipid (µg/100×10⁶ spermatozoa)	C/P
Control	76.13^{b}±1.81	80.65^{a}±2.49	21.55^{c}±0.63	90.48^{a}±0.54	0.24^{c}±0.044
CLC 2.0 mg/120×10⁶ spermtaozoa	77.63^{b}±1.47	81.95^{a}±1.36	49.56^{b}±1.38	90.33^{a}±0.53	0.54^{b}±0.098
CLC 3.0 mg/120×10⁶ spermtaozoa	81.25^{a}±2.39	81.78^{a}±1.61	55.67^{a}±0.45	90.23^{a}±0.59	0.62^{a}±0.039
CLC 4.0 mg/120×10⁶ spermtaozoa	76.75^{b}±1.55	80.98^{a}±1.33	47.79^{b}±1.01	92.15^{a}±0.52	0.52^{b}±0.074

$^{a, b, c}$Means with dissimilar superscript in a column differ significantly ($p<0.01$). SE=Standard error

Table-2: Seminal attributes in Buffalo bulls spermatozoa at post-thaw stage (mean±SE).

Group	Initial progressive motility (%)	Livability (%)	Cholesterol (µg/100×10⁶ spermatozoa)	Phospholipid (µg/100×10⁶ spermatozoa)	C/P
Control	51.93^{d}±0.51	58.25^{d}±0.86	13.18^{c}±0.45	79.45^{c}±0.38	0.17^{c}±0.036
CLC 2.0 mg/120×10⁶ spermtaozoa	66.38^{b}±0.53	72.10^{b}±0.53	34.27^{ab}±0.71	79.09^{c}±0.34	0.43^{a}±0.057
CLC 3.0 mg/120×10⁶ spermtaozoa	69.50^{a}±0.29	76.85^{a}±0.51	36.21^{a}±0.48	82.35^{b}±0.51	0.44^{a}±0.037
CLC 4.0 mg/120×10⁶ spermtaozoa	63.88^{c}±0.42	69.28^{c}±0.31	33.68^{b}±0.56	85.17^{a}±0.45	0.39^{b}±0.047

$^{a, b, c, d}$Means with dissimilar superscript in a column differ significantly ($p<0.01$). SE=Standard error

of phospholipid content of spermatozoa were higher than fresh spermatozoa. This happened because of phospholipid present in egg yolk dilutor (contain 80% phospholipid) used in the study, incorporated into the sperm membrane thereby increasing the phospholipid content of spermatozoa. Similar type of finding indicated that phospholipid content of spermatozoa increased after egg yolk based dilutor for cryopreservation of boar semen [24]. The phospholipid content of spermatozoa increased significantly after freezing and thawing in high and average class viability when compared to low-class viability. This result indicated active sperm lipid metabolism might be responsible for the increase in lipid content [24].

Treatment with CLC in Group III increased cholesterol content by 2.3 fold at pre-freeze and 1.5 fold at post-thaw stage. However, phospholipid content was increased by 1.7 fold at pre-freeze and 1.6 fold at post-thaw stage in comparison to a fresh stage. CLC-treated sperm presented high cholesterol content in the membrane (between 1.93 and 2.7 folds) than control sperm in bulls, stallions and rams have been reported [25]. This may be attributed to the reason that with an increase in cholesterol content in the plasma membrane the sperm resistance to cold shock during freeze-thawing increases. High membrane cholesterol levels inhibit crystallization of membrane hydrocarbon chains at low temperature, thereby eliminating the phase transition [1]. On the other hand, CLC-treated sperm exhibited wider osmotic tolerance limits than control sperm in bulls, stallion, rams and boars [1,25-27]. This could provide resistance to increase post-thaw survival of sperm.

Significantly higher ($p<0.01$) C/P ratio at pre-freeze stage in the spermatozoa of Group III (0.62±0.039) was observed in comparison to other groups (Table-1). At post-thaw stage C/P ratio was significantly higher ($p<0.01$) in both Group II and III in comparison to Group I and IV. There was no significant difference in the C/P ratio of Group II and III was observed (0.43±0.057 vs. 0.44±0.037). It is well-documented that sperm with higher C/P ratio is more resistant against cryodamage. In the present study incubation with CLC increase, the cholesterol content at post-thaw stage and also significantly increase in the phospholipid content possibly from the source of egg yolk leads to an increase in C/P ratio at post-thaw stage. As no significant difference in C/P ratio at post-thaw stage between Group II and III was noticed in the present study, however, significantly higher post-thaw motility and viability in the spermatozoa of Group III is an indication of improvement in seminal parameters after treatment with CLC. Comparing the C/P ratio at fresh stage and post-thaw stage between Group I and III; in the control group a reduction in C/P of about 64% was noticed in comparison to about 6% in Group III (treated with 3 mg CLC/120 × 10^6 spermatozoa). This clearly indicates that CLC treatment may maintain the C/P ratio unlike fresh stage and play important role in maintaining membrane architecture of spermatozoa. Hence, addition of CLC may helpful in increasing freezability of buffalo spermatozoa by increasing the C/P ratio of spermatozoa.

Conclusion

Maximum beneficial effect on semen cryopreservation was observed at dose rate of 3.0 mg CLC/120 × 10^6 spermtaozoa. Addition of CLC significantly improved semen freezability as revealed by higher progressive motility, livability, and C/P ratio.

Authors' Contributions

JSR planned and carried out research work for his PhD thesis programme in collaboration with guide (JKP). Author is thankful SKG and GKD for providing lab facility during my research work. SSR and NCB: collection of sample and estimation of parameters during my research work. MP helped

during preparation of manuscript. All authors read and approved the final manuscript.

Acknowledgments

The authors are thankful to Director, Indian Veterinary Research institute, Izatnagar - 243122, Bareilly for providing facilities and fund during thesis research work of first author. Also, we thankfully acknowledge, Dr. Amit Dohare, PhD Scholar, Division of LES, IVRI for his help during statistical analysis.

Competing Interests

The authors declare that they have no competing interests.

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