EFFECT OF CHOLESTEROL LOADED CYCLODEXTRIN ON CRYOSURVIVABILITY OF BUFFALO SPERMATOZOA

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ABSTRACT

The study was carried out to understand the effect of various concentrations of cholesterol loaded cyclodextrin (CLC) on cryosurvivability of buffalo spermatozoa. Twenty four ejaculates with mass motility ≥ 3+ from 4 bulls (6 from each bull) were collected as per standard procedure. Each ejaculate was split into four groups *viz*., Group I (control: extended with Tris-egg yolk-glycerol extender upto 80×10^6 sperm/ml), Group II (treated with CLC 1 mg/ml), Group III (treated with CLC 2 mg/ml) and Group IV (treated with CLC 3 mg/ml). Semen samples of Group II, III or IV were incubated with CLC at 37°C for 15 minutes for the entry of cholesterol into sperms and finally diluted upto 80×10^6 sperm/ml. The results revealed that post-dilution and after freezing at 0.7 and 30 days of cryopreservation motility, live and dead, acrosomal integrity and hypo-osmotic swelling test were significantly ($P<0.05$) higher in Group III as compared to all other groups. However, significantly ($P<0.05$) higher percentage of abnormal sperm was observed in control group as compared to other groups. Therefore, addition of CLC 2 mg/ml in extender during buffalo semen cryopreservation has beneficial role in improvement of semen quality.

Keywords: *Bubalus bubalis*, buffaloes, cholesterol, cyclodextrin, concentration, cryosurvival, spermatozoa

INTRODUCTION

In tropics, despite providing milk, meat and draft power for human consumption, artificial insemination using frozen semen for breeding of buffalos is limited. The major reasons are poor freezable quality semen and sperm fertility of buffalo in comparison to cattle due to higher susceptibility of buffalo spermatozoa to steps involved in cryopreservation and ultra-low temperatures (Andrabi, 2009; Rajoriya *et al*., 2016). One of the major factors of sperm motility and fertility reduction during freezing and thawing process is damage of plasma membrane (Chakrabarty *et al*., 2007), which occurs in during transition of plasma membrane from liquid crystalline to a gel phase (Darin-Bennett and White, 1977). During cryopreservation destabilization of plasma membrane also occurs due to cholesterol efflux from the membrane (Bailey *et al*., 2008). Therefore, maintenance of plasma membrane integrity helps spermatozoa

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to withstand adverse effects of cryopreservation (Lone *et al*., 2016a). Addition of cholesterol or its analogues in the extender during cryopreservation process decreases capacitation like changes (Serin *et al*., 2011; Rajoriya *et al*., 2016). Hydrophobic nature of cholesterol is responsible for its poor solubility in semen diluents (Klein *et al*., 1995). Enzymatically degraded products of starch called cyclodextrins have been used for incorporation of cholesterol in the cell membrane as a hydrophobic core along with a hydrophilic face is present. In buffalo semen cholesterol loaded cyclodextrin (CLC) increases semen freezability (Lone *et al*., 2016a), reduces capacitation like changes (Rajoriya *et al*., 2016), enhances seminal antioxidant activity (Lone *et al*., 2016b), reduces oxidative stress (Lone *et al.,* 2016c). However, scanty information is available regarding the cryosurvival of buffalo spermatozoa treated with CLC after various days of cryopreservation. Therefore, the objective of the present study was designed to investigate the effect of various levels of CLC on cryosurvivability of buffalo spermatozoa after various days of cryopreservation.

MATERIALS AND METHODS

Experimental design

The study was carried out at Artificial breeding research centre (ABRC), ICAR-National Dairy Research Institute. Semen of four Murrah buffalo bulls, 4 to 6 year old was used for the experiment. The bulls were maintained under standard feeding and management conditions.

Preparation of cholesterol loaded cyclodextrin (CLC)

As per Purdy and Graham (2004) Methyl-β-cyclodextrin was loaded with cholesterol. In Brief, in one glass tube 200 mg cholesterol was added in 1 ml chloroform and dissolved. In another glass tube 1 g methyl-β-cyclodextrin was added in 2 ml methanol and dissolved. Then a mixture was made by adding 0.45 ml cholesterol solution with to the cyclodextrin solution and stirred till the mixture became clear. The mixture was poured into a glass petri dish and using a stream of nitrogen gas solvents was removed to form crystals, which was dried for an additional 24 h in the glass petri dish. Then the cholesterol loaded cyclodextrin (CLC) was stored in a glass container at 22^{o}C after removal from the dish. At 37^{o}C, 50 mg of CLC was added to 1 ml of Tris extender to form working solution after proper mixing using a vortex mixer.

Semen collection

Semen collection was carried out in the morning as per standard practice followed in the semen station using an artificial vagina. 24 Ejaculates from 4 bulls, 6 ejaculate from each bull were selected based on semen samples having +3 and above mass activity and 70% and above individual motility. The semen samples were evaluated immediately after semen collection.

Semen processing and preservation

Immediately after semen collection, each ejaculate was divided into four groups *viz*., Group I (control: diluted with Tris-egg yolk-glycerol extender up to 80×10^6 sperm/ml), and Group II, Group III and Group IV treated with CLC 1 mg/ml, 2 mg/ml and 3 mg/ml, respectively. To allow entry of cholesterol CLC treated groups were incubated at 37^{o}C for 15 minutes and finally diluted up to 80×10^6 sperm/ml. Individual motility, viability, acrosomal integrity and hypo-osmotic swelling test was performed for all the groups.

In all the groups 20 million sperms were filled in French mini (0.25 ml) straw using filling and sealing machine. The filled straws were spread in rack and kept at 5°C for 4 h equilibration. The rack along with the straws was shifted to biological cell freezer and the freezing was carried out 5°C/ minutes for 4 to -10°C; 40°C/minutes for -10 to -100°C and 20°C/minutes for -100 to -140°C. Finally the Straws were kept into liquid nitrogen (-196°C) till further assessment. Post thaw sperm quality in terms of individual motility, live and dead, acrosomal integrity and hypo-osmotic swelling test was carried out at 0.7 and 30 days after freezing.

Semen analysis

The semen samples were evaluated for Individual motility using phase contrast microscope equipped with a warm stage (37°C) at 400× magnification. Semen samples were assessed as per method described by Campbell *et al*., 1953 for live sperm percentage using Eosin-Nigrosin stain; by Watson, 1975 for Acrosomal intactness using Giemsa stain and by Jeyendran *et al*. (1984) for Hypo-osmotic swelling test (HOST).

Statistical analysis

One way ANOVA was used to analyze the data using Statistical Analysis System (SAS 2011; version 9.3), and the results were presented as mean ± SD.

RESULTS AND DISCUSSION

The various post dilution seminal attributes in all the four groups are presented in Table 1. Group III had significantly (P<0.05) higher individual motility (79.06±0.30 vs. 75.47±0.23, 76.84±0.35, and 77.37±0.25), viability (79.81±0.29 vs. 78.09±0.25, 78.44±0.28, and 78.88±0.26), HOST (73.32±0.22 vs. 69.94±0.23, 71.54±0.18, and 72.33±0.21) and acrosomal integrity (87.46±0.27 vs. 83.54±0.25, 85.28±0.32, and 86.55±0.81) in comparison to other groups. The post-thaw motility, sperm viability, hypo-osmotic swelling response (HOS) and acrosomal integrity at 0.7 and 30 days of cryopreservation was significantly (P<0.05) higher in Group III comparison to other groups (Table 2). Group III had around 8 to 9% higher motility on 0.7 and 30 days after freezing as compared to control. The higher motility in Group III may be due to reduced levels of lipid peroxides and reactive oxygen species by cholesterol loaded cyclodextrin as reported by Lone *et al.* (2016b). In Group III, around 10% higher motility was reported as compared to Group I. The percent reduction in viability was 13, 9, 8 and 10, in Group I, Group II, Group III and Group IV, respectively. The reduction in viability from 0 to 30 days was least in Group III and highest in control group. The enhanced motility and viability in CLC treated samples may be due to enhanced seminal antioxidant activity (Lone *et al*., 2016b) or reduced lipid peroxidation and reactive oxygen species levels in the spermatozoa (Lone *et al*., 2016c). Around 10% higher HOS responsive spermatozoa were reported in Group III as compared to control. The improvement in percentage of HOS responsive spermatozoa in CLC treated group was in agreement with the findings of Lone *et al.* (2016b), who reported significantly higher percentage of HOS responsive spermatozoa in CLC treated spermatozoa. The increased HOS response of CLC treated sperm may be due to enhanced membrane integrity and reduced oxidative stress level (Lone *et al*., 2016c) or increased activity of seminal antioxidants (Lone *et al*., 2016b).

Acrosomal integrity in Group III was about 10%, 9% and 7% higher as compared to Group I, at 0.7 and 30 days, respectively. Structural and functional intactness of acrosome is essential for fertility (Srivastava *et al*., 2012). In our study, we observed CLC increased the acrosomal integrity, so it is presumed that by increasing the acrosomal integrity, the fertility of CLC treated semen may be enhanced. At low temperatures, cholesterol maintains the membrane fluidity and structure and therefore the sensitivity of membrane to cooling injuries is reduced (White, 1993). At low temperature cholesterol incorporation in sperm membrane enhances sperm membrane regulation and decrease water permeability, thus help in regulating the water transfer across the membranes (Glazar *et al*., 2009). The improvement of sperm quality treated with cholesterol loaded cyclodextrin may be due to decrease in capacitation status (Longobardi *et al*., 2017; Rajoriya *et al*., 2016), reduced oxidative stress (Lone *et al*., 2016c) or enhanced antioxidant levels (Lone *et al*., 2016b). Studies have also revealed that CLC treatment of semen leads to increase in the percentage of noncapacitated sperm (Longobardi *et al*., 2017) and better freezability of sperm may be due to higher cholesterol: phospholipid ratio (Rajoriya *et al*., 2016) helps in membrane stability and decrease in capacitation changes (Longobardi *et al*., 2017). The increase in semen freezability may also be due to increased membrane stability due to cholesterol. It is evident from the reports in buffalo (Rajoriya *et al*., 2014), boar (Cerolini *et al*., 2001) and stallion (Moore *et al*., 2005) sperm that loss of cholesterol during cryopreservation process responsible for capacitation like changes. It is known that cholesterol efflux results in capacitation, leading to increased bicarbonate, calcium and cAMP levels (Therien *et al*., 1995). Higher susceptibility of buffalo spermatozoa to premature capacitation is due to low cholesterol, phospholipids ratio (Rajoriya *et al*., 2014). Providing cholesterol through the agency of cyclodextrins reduces capacitation by increasing the cholesterol: phospholipid ratio. Thus cholesterol depletion which occurs during cryopreservation of buffalo spermatozoa is countered by cholesterol loaded cyclodextrin treatment (Rajoriya *et al*., 2016).

CONCLUSION

It is concluded that adding cholesterol loaded cyclodextrin to buffalo semen 2 mg/ml resulted significantly better enhancement in motility, viability, acrosomal integrity, and HOST response and reduced sperm abnormality. The enhanced semen freezability due to cholesterol loaded cyclodextrin may be due to reduced capacitation like changes, reduced reactive oxygen species levels, increased antioxidant levels and increased membrane cholesterol: phospholipid ratio. It can be used in buffalo semen preservation for better cryosurvivability.

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Table 1. Effect of different levels of CLC on post-dilution seminal attributes (%).

Seminal attributes (%)	Group I (control)	Group II	Group III	Group IV
Individual motility	75.47^{a}±0.23	76.84^{b}±0.35	79.06^{c}±0.30	77.37^{b}±0.25
Viability	78.09^{a}±0.25	78.44^{b}±0.28	79.81^{c}±0.29	78.88^{b}±0.26
HOST	69.94^{a}±0.23	71.54^{b}±0.18	73.32^{c}±0.22	72.33^{d}±0.21
Acrosomal integrity	83.54^{a}±0.25	85.28^{b}±0.32	87.46^{c}±0.27	86.55^{d}±0.81

Means bearing different superscripts within the same row differ significantly (P<0.05).

Table 2. Effect of different levels of CLC on seminal attributes (%) after various days of cryopreservation.

Seminal attributes (%)	Period	Group I	Group II	Group III	Group IV
Individual motility	0 day	37.42^{a}±0.30	41.31^{b}±0.34	48.25^{c}±0.31	41.48^{b}±0.34
	7 day	35.73^{a}±0.31	38.91^{b}±0.34	44.85^{c}±0.29	39.04^{b}±0.32
	30 day	34.85^{a}±0.34	36.98^{b}±0.27	43.42^{c}±1.05	38.06^{d}±0.29
	Overall Post thaw	36.00^{a}±0.20	39.09^{b}±0.22	45.50^{c}±0.40	39.52^{b}±0.21
Viability	0 day	46.09^{a}±0.24	51.45^{b}±0.27	56.12^{c}±0.43	52.50^{d}±0.24
	7 day	44.12^{a}±0.24	49.64^{b}±0.28	54.61^{c}±0.42	50.11^{b}±0.25
	30 day	42.07^{a}±0.25	46.72^{b}±0.27	51.61^{c}±0.42	47.41^{d}±0.25
	Overall Post thaw	44.09^{a}±0.18	49.27^{b}±0.21	54.12^{c}±0.28	50.01^{d}±0.20
HOS response	0 day	37.89^{a}±0.32	42.58^{b}±0.25	47.51^{c}±0.31	43.91^{d}±0.29
	7 day	35.04^{a}±0.33	40.03^{b}±0.26	44.10^{c}±0.32	41.16^{d}±0.30
	30 day	34.06^{a}±0.33	37.73^{b}±0.25	40.96^{c}±0.32	38.64^{d}±0.30
	Overall Post thaw	35.65^{a}±0.22	40.11^{b}±0.20	44.18^{c}±0.26	41.23^{d}±0.23
Acrosomal integrity	0 day	51.57^{a}±0.30	56.84^{b}±0.29	61.55^{c}±0.29	57.49^{d}±0.36
	7 day	48.00^{a}±0.30	52.38^{b}±0.29	57.34^{c}±0.22	53.67^{d}±0.36
	30 day	46.59^{a}±0.30	50.62^{b}±0.28	53.97^{c}±0.21	51.43^{d}±0.37
	Overall Post thaw	48.72^{a}±0.22	53.29^{b}±0.24	57.64^{c}±0.26	54.20^{d}±0.27

Means bearing different superscripts within the same row differ significantly (P<0.05).

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