EFFECT OF CHOLESTEROL LOADED CYCLODEXTRIN ON SPERM TRAITS, CHOLESTEROL CONTENT AND PROTEIN TYROSINE PHOSPHORYLATION IN CRYOPRESERVED BUFFALO BULL SEMEN

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ABSTRACT

The present study evaluated the impact of cholesterol loaded cyclodextrin (CLC) supplementation (4 mg/ml of extender) in Tris-citric acid egg yolk extender on sperm motility, viability, membrane integrity, cholesterol content and tyrosine phosphorylation of buffalo bull spermatozoa. Thirty ejaculates from 6 buffalo bulls were included in this study. In treatment group, In CLC supplemented extended semen, percent motility, viability and hypo-osmotic swelling increased ($p<0.05$) over the control at pre-freeze and post-thaw stage. Post-thaw cholesterol content was high ($p<0.05$) in CLC supplemented group. The SDS gel electrophoresis and western blotting of buffalo sperm extracts with anti-phosphotyrosine antibody indicated phosphorylation of 75, 65, 60, 55, 50, 45, 40, 35, 30, 25, 20 and 17 kDa proteins at tyrosine residue during cryopreservation of buffalo semen. However, supplementation of CLC decreased tyrosine phosphorylation of 75, 65, 55, 50, 35, 25, 20 and 17 kDa proteins with considerable variation in bulls. In brief, supplementation of CLC to extender increases motility, viability, membrane integrity, cholesterol content and decreases protein tyrosine phosphorylation of buffalo spermatozoa.

Keywords: Buffalo bull, Cholesterol loaded cyclodextrin, Cryocapacitation, Sperm traits, Tyrosine phosphorylation

INTRODUCTION

Cryopreservation is a stressful process, which causes partially irreversible damage to sperm membrane, thereby affecting sperm quality (Moore *et al.,* 2005). Cryopreservation induces cryocapacitation or capacitation like changes in sperm by phosphorylation of protein tyrosine residues (Visconti *et al.,* 1995 and Bailey *et al.,* 2000). A major event in capacitation like changes includes cholesterol efflux that stimulates enzymatic cascade event leading to decrease in fertile life of sperm (Bailey *et al.,* 2008). Further, buffalo bull sperm are more prone to cryopreservation induced membrane damage leading to poor fertility (Dhami *et al.,* 1994). Hence, there is need to minimize the development of capacitation like changes in sperm. The supplementation of extender with cholesterol loaded cyclodextrin (CLC) increased sperm cryosurvival rates in cattle bull (Purdy and Graham, 2004) and Nili Ravi buffalo bull (Qamar *et al.,* 2013). The present study evaluated the impact of CLC on motility, viability, membrane integrity, cholesterol loss and protein tyrosine phosphorylation in buffalo spermatozoa

MATERIALS AND METHODS

Six breeding Murrah buffalo bulls (age 4 yr) of progeny testing program used in present study were maintained at university bull farm under loose housing system (covered area 12x10 ft and uncovered area 25x10 ft) and standard feeding schedule along with *ad lib* green fodder. Semen collection was by artificial vagina method and 5 ejaculates from each bull were collected, evaluated and frozen using standard procedure.

Cholesterol loaded Cyclodextrin (CLC) was prepared as per the standard method (Purdy and Graham 2004). Semen was diluted with Tris egg yolk extender (TYC, control) or same extender supplemented with CLC (4 mg/

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ml) by maintaining final sperm concentration to 120 million sperm/ml. Extended semen was divided into 2 aliquots for evaluation at pre freeze stage and post thaw stage. The aliquot for pre freeze evaluation was cooled at 4^0C for 4 h in cold handling cabinet for equilibration. The aliquot for post thaw stage was filled and sealed by manual filling and sealing method. The filled and sealed straws were also cooled at 4^0C for 4 h and cryopreserved in liquid nitrogen by following standard procedure. The pre freeze and post thaw semen samples were evaluated for motility, viability and HOST (Kumar *et al.,* 2004).

The pre freeze and post thaw spermatozoa were washed thrice with PBS, pH 7.4 at 3000 rpm for 10 min. Sperm proteins were extracted by suspending $250x10^6$ spermatozoa in 0.5 ml of 62.5 mM Tris-HCl buffer, pH 6.8 containing 2% SDS, 10 µl of cock tail protease inhibitors (Serva), sonicated (3 bursts of 20 sec each) and centrifuged at 15000 rpm for 30 minutes (SDS-SE). Cholesterol was estimated in pre frozen SDS-SE and post thaw SDS-SE (Zlatkis *et al.,*1953).

Proteins (pre frozen and post thawed SDS-SE) separated by SDS-PAGE under reducing conditions were transferred to nitrocellulose membrane using wet electrophoresis transfer apparatus at 100V for 2.30 h. Transfer quality was checked by 0.2 % ponceau dye and proteins were blocked in 3% BSA as blocking solution for overnight at 4^0C. After washing the membrane with PBS+0.05% Tween-20, it was incubated in 1:1000 diluted anti-phosphortyrosine (Bioscience Inc) antibody for 2.5 h. Again washed thrice with PBS+0.05% Tween-20 and incubated with 1:10000 anti-rabbit IgG as secondary antibody for 45 min. Washed thrice with PBS+Tween-20 and incubated with substrate (0.05% Diaminobenzidine+0.015% 4- ChloroNapthol+0.06% Hydrogen Peroxide) for 10 min. Gel images were captured on Syngene gel doc using Gene Snap image acquisition software and analyzed for molecular weight and concentration of proteins by using GeneTools gel analysis software (Syngene). The Arc sine transformation of percent data was carried out. The data were analysed using students 't' test.

RESULTS AND DISCUSSION

In CLC supplemented group at pre freeze and post thaw stage, the percent motility, viability and membrane integrity (HOST) was higher in comparison to control ($p<0.05$, Table). Similar observations were reported in Nili Ravi buffalo bulls (Qamar *et al.,* 2013) and cattle bulls (Moraesa *et al.,* 2010). Usually, the sperms exposed to osmotic stress or repeated changes in osmolality during cryopreservation results in a significant loss of functional integrity (Guthrie *et al.,* 2002). The supplementation of sperms with CLC alters membrane permeability, thereby extending the osmotic tolerance limits of sperm (Walters *et al.,* 2008). Thus, the observed increase in percent motility, viability, membrane integrity following the supplementation of CLC might be due to increased osmotic tolerance of sperm.

In the present study, sperm cholesterol content in CLC supplemented and control group was similar at pre freeze stage, however, at post thaw stage, the cholesterol content was higher in CLC group ($p<0.05$, Table). Cholesterol efflux activates capacitation like changes in

Table: Impact of cholesterol loaded cyclodextrin (CLC) on motility, viability, membrane integrity and cholesterol content at pre freeze and post thaw stage in buffalo bull spermatozoa

Parameters	Pre freeze		Post thaw	
	Control	CLC	Control	CLC
Motility, %	74.6 ± 1.5^a	79.9 ± 1.3^b	48.8 ± 1.8^c	56.9 ± 1.9^d
Viability, %	85.3 ± 1.03^a	91 ± 0.90^b	65.8 ± 1.2^c	72.6 ± 1.5^d
HOST, %	74.4 ± 1.8^a	81.4 ± 1.7^b	55.9 ± 1.82^c	64.8 ± 1.6^d
Cholesterol content (µg/billion sperm)	503±31	532±38	424 ± 26^c	480 ± 34^d

$^{a\ vs\ b,\ c\ vs\ d}p<0.05$, within a row

the sperm membrane due to the activation of several enzymatic cascades (Visconti *et al.,* 1999). The addition of CLC before freezing may have given structural stability to buffalo sperm and prevented the cholesterol efflux during freezing process which might have reduced the capacitation like changes in sperm membrane during the freezing-thawing process.

The SDS-PAGE of sperm extracts revealed polymorphism in the freshly ejaculated, pre freeze and post thaw spermatozoa. The cross reactivity of anti-phosphotyrosine antibody indicated phosphorylation of 75, 65, 60, 55, 50, 45, 40, 35, 30, 25, 20 and 17 kDa proteins at tyrosine residue. Tyrosine phosphorylation of more number of post thaw sperm proteins as compared to pre freeze indicated cryocapacitation of buffalo bull semen during freeze thaw process. However, a considerable variation was observed in the phosphorylation of proteins during cryopreservation of semen of different bulls. About 28 major polypeptides ranging from 15-24 bands in each individual Brangus and Brown Swiss bull were reported (Chacur *et al.,* 2010). Contrary to our results, the addition of CLC did not alter the protein profile of buffalo spermatozoa during the process of cryopreservation in a previous study (Marques *et al*., 2000).

The supplementation of CLC decreased tyrosine phosphorylation of 75, 65, 55, 50, 35, 25, 20 and 17 kDa proteins in post thaw spermatozoa of 5 buffalo bulls in the range of 3.2-46.2, 67.3-75.2, 9.5-66.7, 67.7-90 and 8.3-40.7%. Literature is not available on effect of CLC on tyrosine phosphorylation of proteins during the cryopreservation of semen. However, i*n vitro* induction of cholesterol efflux from sperm plasma membrane results in an increase in capacitation and protein tyrosine phosphorylation through the cAMP / PKA pathway (Visconti *et al.,*1999).

In brief, the supplementation of cholesterol-loaded cyclodextrin to semen extender increases the motility, viability, membrane integrity, cholesterol content and minimizes protein tyrosine phosphorylation of cryopreserved buffalo bull spermatozoa.

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