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Exogenous cholesterol modulates oxidative stress and freezability of mithun spermatozoa

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ABSTRACT

Effect of cholesterol loaded cyclodextrin (CLC) on improvement of semen quality parameters (SQPs) and deduction of oxidative stresses following cryopreservation in mithun was explored. Total 50 ejaculates were selected out of 105 collected based on preliminary SQPs. Sperm was treated with 1 mg (Gr II) and 2 mg (Gr III) of CLC/ 120×10^6 spermatozoa and without CLC served as control (Gr I). Diluted semen samples were cryopreserved at ultralow temperature. Frozen thawed samples were evaluated for motility (progressive forward [FPM]; in bovine cervical mucus penetration test [BCMPT] and in computer assisted sperm analyzer [CASA]), viability, total sperm and nuclear abnormality, intactness of plasma membrane and acrosome, intracellular enzymatic leakage and oxidative profile (Malondialdehyde; MDA). Result revealed a significant improvement in motility (FPM, BCMPT and CASA), viability, acrosomal integrity, cholesterol content and reduction of sperm and nuclear abnormalities, leakage of intracellular enzymes and oxidative stressors in 1 mg CLC treated group as compared to control. Moreover, intactness of acrosome and biochemical membrane was protected significantly in extender containing 1 mg CLC. Hence inclusion of mithun spermatozoa with CLC (1 mg/120×10^6) prior to freezing improved the survivability in cryopreservation. The results clearly indicated the beneficial effects of CLC supplementation on freezability by reducing cryodamage and protecting the spermatozoa integrity.

Key words: Cholesterol, Free radicals, Freezability, Mithun, Semen

Mithun semen can be preserved in liquid (Karunakaran *et al*. 2007) or liquid nitrogen (Mondal *et al*. 2010). Cryopreservation exerts cryostresses which cause derangement of membrane fluidity and structure resulting in poor post-thaw sperm survivability and fertilization (Ozkavukcu *et al*. 2008). Different methods or techniques have been developed to minimize the cryocapacitation/ cryostress, oxidative stress and improve post thaw sperm viability. In mithun, 36% good quality ejaculates were non-freezable after freezing thawing (Perumal 2014a). However, a method was standardized to reduce cryoinjury which involved exposure of exogenous cholesterol prior to processing. Inclusion of cholesterol with cyclodextrin (called as CLC), has increased SQPs (Purdy and Graham 2004). Perusal of literature on CLC level on cryo-protection revealed that, 3 mg CLC for buffalo (Rajoriya *et al*. 2016), 0.50 mg for Hariana cattle (Yadav *et al*. 2017), 1.5 mg for equine (Moore *et al*. 2005), 2 mg for donkey jacks (Alvarez *et al*. 2006), 1 mM for boar (Galantino-Homer *et al*. 2006), 1.5 mg for boar (Lee *et al*. 2015) and 2 mg/120×10^6 sperm for ram (Moce *et al*. 2010) were optimum doses for improvement of the SQPs. However, no information is available in mithun. Therefore, present study was conducted to evaluate the effects of CLC on the oxidative stress, SQPs, and biochemical and antioxidant profiles following semen cryopreservation in mithun.

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MATERIALS AND METHODS

Experimental design: Adult healthy mithun bulls (10) of 4–6 yrs, weighing 495–520 kg with good body condition score (5–6 of 10) were selected from the institute. Experimental bulls were reared under homogeneous management as per the farm schedule. Semen ejaculates were collected only twice per week from any mithun bulls by trans-rectal massage method during morning between 08:00 and 10:00 h. Initial transparent secretions were discarded and good quality neat semen portion was collected. Ejaculates with mass activity of 3+ and above were selected for the study whereas ejaculates with wide variation in pH and less volume (0.5 ml and less) were discarded.

Semen collection and processing: Every day, two good ejaculates were obtained per bull as a minimum quantity and collected ejaculates were preserved in a water bath at 37°C immediately after collection and evaluated for preliminary SQPs. Ejaculates meeting the following selection criteria were selected for further processing:

concentration >500 million/ml, mass activity >3+ or above and total abnormality <10%. Based on the preliminary screening, 50 of 105 ejaculates were selected and utilized for the experiment. Two consecutive ejaculates of same bull were pooled together (termed "sample" hereafter, n=25) and subjected to the two fold initial dilution with pre-warmed (37°C) Tris diluent without egg yolk and glycerol. Thus, from 105 initial collections, 50 selected ejaculates were pooled to obtain 25 samples for the experiment. Partially diluted samples were brought to the laboratory in an insulated flask containing warm water for further processing.

Cholesterol loaded methyl-β-cyclodextrin (CLC) was prepared as per the method described by Purdy and Graham (2004). Semen samples were diluted in the EYTG extender in such a way to yield approximately 60×10^6 motile sperm cells/ml. The pH and osmotic pressure (osmolality) of 7.0 and 320 mOsm/kg, respectively was maintained for the extender finally used. The extender (Egg yolk-Tris-Glycerol, EYTG extender) used in this study contained Tris [Tris (hydroxyl methyl) amino methane], 3.028 g; fructose, 1.250 g; citric acid monohydrate, 1.675 g; penicillin G sodium (IU/ml), 1000; streptomycin sulphate (µg/ml), 1000; double distilled water, up to 100 ml; with 20% egg yolk and 7% glycerol added finally and different concentrations of CLC (1 (Gr II) and 2 mg/120×10^6 spermatozoa (Gr III)) whereas the extender without CLC served as control (Gr I).

Following incubation with CLC, each semen sample was extended with EYTG to maintain the concentration of sperm 120×10^6 spermatozoa/ml in each sample. Diluted semen samples of each group were processed and preserved in ultralow temperature as per the standard procedure. SQPs such as sperm motility (Nikon, Eclipse 80i, 400×; Salisbury *et al*. 1985), mobility and velocity by CASA (Perumal 2014b), viability and total sperm abnormality by eosin–nigrosin staining (Tomar 1997), intactness of acrosome by Giemsa staining (Watson 1975), plasma membrane integrity by hypo-osmotic swelling test (HOST; Jeyendran *et al*. 1984), nuclear integrity by Feulgen staining technique (Barth and Oko 1989) and vanguard distance travelled by BCMPT (Matouseket *et al*. 1989) were determined. The biochemical assays such as total antioxidant capacity (TAC), oxidative stress (MDA), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and total cholesterol content were estimated with commercially available kits.

Statistical analysis: Statistical analysis of the data was done as per standard methods. Analysis of variance (ANOVA) was performed using a generalized linear model (SPSS, version 15.0; Chicago, IL), and treatment means were separated using Student–Newman-Keuls multiple range test. The data used in the study were tested for normality before analysis using Shapiro-Wilk statistics. The per cent data were subjected to arcsine (angular) transformation before proceeding to general linear model and differences with values of P<0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

Semen ejaculates of mithun bulls were almost creamy white in colour with 2.38±0.32 ml as the average volume and concentration was $864.12\pm5.32\times10^6$ sperm/ml. Results revealed a significant (P<0.05) improvement in SQPs of ejaculates extended with 1 mg of CLC. Post thaw motility (9%), viability (7%), intact acrosome (5%), plasma membrane integrity (6%), sperm nuclear integrity (10%) and vanguard distance travelled by the sperm (13%) were significantly (P<0.05) higher in Gr II than control whereas total abnormal sperm was 14% lesser in Gr II than Gr I (control) (Fig. 1). There was linear increase of SQPs from control to Gr II and then decrease to Gr III in post thaw whereas total sperm abnormalities decreased linearly from control to Gr II and then increased to Gr III (Fig. 1). Activity of intra-cellular enzymes, viz. AST (5%) and ALT (7.3%) was significantly (P<0.05) decreased in Gr II as compared to Gr I and Gr III (Fig. 2). Total cholesterol of spermatozoa and TAC of seminal plasma were significantly (P<0.05) improved with simultaneous decrease of MDA (Fig. 3). Similar to SQPs, TAC and total cholesterol were 12% and 43% (respectively) higher in Gr II as compared to control whereas LPO (MDA) was 18% lower in Gr II than Gr I in frozen thawed semen. TAC and total cholesterol increased linearly, and AST, ALT and LPO decreased linearly from control to Gr II and then decreased to Gr III similar to SQPs in the present study. Motility and velocity profiles measured by CASA revealed significantly (P<0.05) higher FPM (11%), total motility (6%), straightness (5%), linearity (6%), amplitude of lateral head displacement (6%) and other velocity profiles. Significantly (P<0.05) lower percentage of static motility (8%) was observed in Gr II as compared

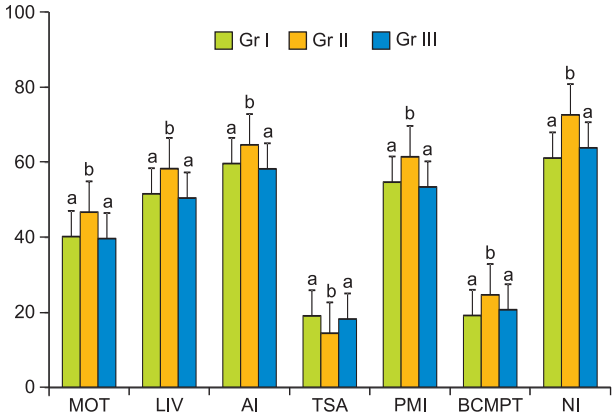

Fig. 1. Semen quality parameters following treatment with cholesterol loaded cyclodextrin in mithun (mean±SEM). Vertical bar on each point represents standard error. Gr I, control; Gr II, 1 mg/120×10^6 sperm; Gr III, 2 mg/120×10^6 sperm; MOT, forward progressive motility (%); LIV, livability (%); AI, acrosomal integrity (%); TSA, total sperm abnormality (%); PMI, plasma membrane integrity (%); BCMPT, bovine cervical mucus penetration test (vanguard distance travelled by sperm in estrous bovine cervical mucus; mm/h) and NI, nuclear integrity (%) (*indicates P<0.05 between treatment and control groups) (n=25).

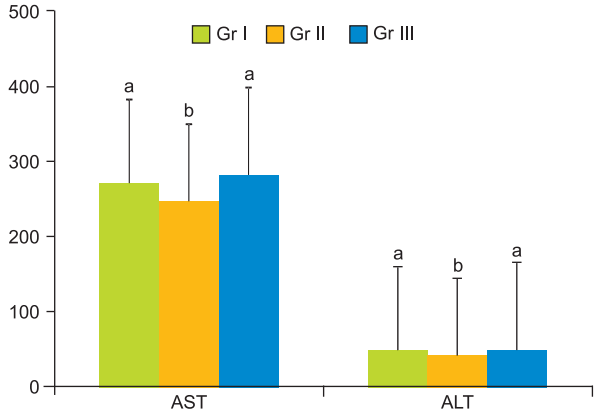

Fig. 2. Leakage of intracellular enzymes of sperm following treatment with cholesterol loaded cyclodextrin in mithun (mean±SEM). Vertical bar on each point represents standard error. Gr I, control; Gr II, 1 mg/120×10^6 sperm; Gr III, 2 mg/120×10^6 sperm; AST, aspartate aminotransferase (µM/l); ALT, alanine aminotransferase (µM/l) (*indicates P<0.05 between treatment and control groups) (n=25).

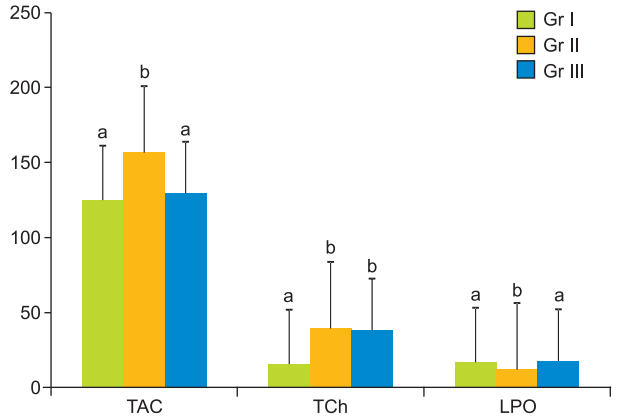

Fig. 3. Total antioxidant capacity (TAC; µM/L), total cholesterol (TCh; µg/120×10^6sperm) and lipid peroxide (LPO or MDA, Malondialdehyde; µM/ml) following treatment with cholesterol loaded cyclodextrin in mithun (mean±SEM). Vertical bar on each point represents standard error. Gr I, control; Gr II, 1 mg/120×10^6 sperm; Gr III, 2 mg/120×10^6 sperm (*indicates P<0.05 between treatment and control groups) (n=25).

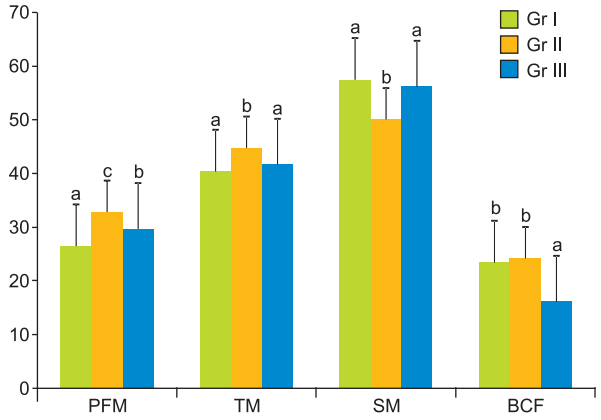

Fig. 4. Progressive forward motility (PFM; %), total motility (TM; %), static motility (SM; %) and beat cross frequency (BCF; Hz) estimated by computer assisted sperm analyser (CASA) following treatment with cholesterol loaded cyclodextrin in mithun (mean± SEM). Vertical bar on each point represents standard error. Gr I, control; Gr II, 1 mg/120×10^6 sperm; Gr III, 2 mg/120×10^6 sperm (*indicates P<0.05 between treatment and control groups) (n=25).

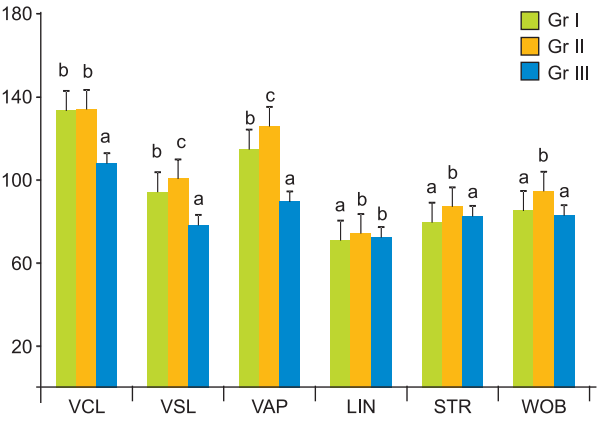

Fig. 5. Comparison of curvilinear velocity (VCL; µm/sec), straight-line velocity (VSL; µm/sec), average path velocity (VAP; µm/sec), linearity (LIN; %), straightness (STR; %) and wobble (WOB; %) estimated by computer assisted sperm analyser (CASA) following treatment with cholesterol loaded cyclodextrin in mithun (mean±SEM). Vertical bar on each point represents standard error. Gr I, control; Gr II, 1 mg/120×10^6 sperm; Gr III, 2 mg/120×10^6 sperm (*indicates P<0.05 between treatment and control groups) (n=25).

to control. CASA parameters increased linearly from control to Gr II and then decreased to Gr III (Figs 4, 5). Results of the present study clearly indicated that CLC at 1 mg/ 120×10^6 sperm is optimum or threshold concentration for semen cryopreservation in mithun species.

Various reports mentioned that CLC has protected the sperm at different concentrations, viz. 3 mg for buffalo (Rajoriya *et al*. 2016), 0.50 mg for Hariana cattle (Yadav *et al*. 2017), 1.5 mg for equine (Moore *et al*. 2005), 2 mg for donkey jacks (Alvarez *et al*. 2006), 1 mM for boar (Galantino-Homer *et al*. 2006), 1.5 mg for boar (Lee *et al*. 2015) and 2 mg for ram (Moce *et al*. 2010) improved the SQPs after freeze-thawing. However, no information is available on use of CLC in ultralow temperature storage of mithun semen. In our earlier study, 36% of good quality semen turned out to be non-freezable after cryopreservation in mithun. It is possible that modification or manipulation of the spermatozoal lipid content of mithun sperm could improve their structural and functional competence following freezing-thawing. Therefore, this study was planned in mithun. Results revealed that CLC at 1 mg/ 120×10^6 sperm is optimum for sperm cryopreservation in mithun. It demonstrated that addition of CLC to freezing media has improved the SQPs of frozen-thawed spermatozoa by maintaining the stability of spermatozoal plasma and mitochondrial membranes while also decreasing the nuclear damage and minimized oxidative stress and leakage of intracellular enzymes in CLC treated groups than control. Thus, CLC methodology could be very useful to

minimize the cryocapacitation damages particularly for high genetic merit bulls with poor freezability of sperm that are not able to maintain the optimum level of SQPs after freezing thawing (Moce *et al*. 2010) and this technology may enhance the number of bulls as semen donors.

Cholesterol of spermatozoa plays an important role in determination of cryoresistance of sperm in cryo-preservation. Exposure of spermatozoa with seminal plasma protein (BSP) on ejaculation leads to modification of sperm membrane structure which inturn influence efflux of cholesterol from sperm membrane (Srivastava *et al*. 2012). Cholesterol efflux is an important step in sperm capacitation, acrosomal reaction and finally fertilization (Desnoyers and Manjunath 1992). Whereas higher quantity than threshold level of BSP leads to higher efflux of cholesterol which result in disintegrity of membrane structure and membrane fluidity alteration and sperm death (Srivastava *et al*. 2012). There were reports that stated that higher the membrane cholesterol content; proportionally the sperms have higher membrane stability and fertility (Amann and Graham 1992). Cholesterol: phospholipid ratio and cholesterol content are playing key roles in resistance to cold shock damage (Watson 1981). It had been reported that CLC-treated spermatozoa has higher cholesterol content in their plasma membrane (between 1.93 and 2.7 folds) (Moce *et al*. 2010). In present study, cholesterol content of sperm was 43% higher (2.5 folds) in CLC treated sperm than control. Loss of cholesterol content in mammalian spermatozoa is associated with higher percentage of sperm with plasma membrane degeneration (Leahy and Gadella 2011) which is an important and critical stage for induction of apoptosis and sperm death (Paasch *et al*. 2003). Several reports outlined that sperm cholesterol increased two to three times in cattle, buffalo, trout, sheep after incorporation with CLC and this additional cholesterol improves the cholesterol content as well as the C:P ratio (>0.8) which inturn enhances the cryoresistance to cold shock during cryopreservation (Rajoriya *et al*. 2016). Therefore, supplementation of CLC in extender medium is an effective and suitable method to manipulate and modify the cholesterol and lipid content of sperm plasma membrane. The optimum concentration of cholesterol in CLC differs with species because of varied biochemical structure and composition of sperm membrane which inturn influences the sensitivity to cold shock.

During the cryopreservation process, there is alteration of osmotic pressure across the sperm plasma membranes which inturn creates osmotic stress/mechanical stress to sperm. Concentration of solute in unfrozen portion of extracellular medium increases and marked adjustments occur in the cell volume (Hammerstedt *et al*. 1990). Water molecules pass from the sperm cells to maintain the osmotic equilibrium between intra and inter cellular concentrations of solute which causes dehydration of sperm. CLC improved integrity of the plasma membrane which enhances the osmotic tolerance of sperm (Salmon *et al*. 2017). Exogenous cholesterol incorporated in the sperm membrane play an important role in maintaining the membrane integrity as well as reducing permeability to water at relatively lesser temperatures, thereby regulating the water transfer across the membranes during freezing-thawing process (Li *et al*. 2006). The cholesterol uptake reinforces the sperm membranes which minimize the water transfer across the sperm membrane during temperature as well as osmotic alterations and therefore, cryosurvival of sperm is enhanced (Salmon *et al*. 2017). Similar results were observed in the present study that CLC incubation improved the integrity of plasma membrane, acrosome and nucleus.

There are different methodologies that could describe the cryoprotective effect of CLC on cryopreservation induced nuclear or DNA damage in sperm. First, cryopreservation induces the mitochondrial membrane damage (O'Connell *et al*. 2002) which results in liberation of cytochrome C as well as proapoptotic agents into cytosol causing cascade DNA damage and apoptosis, cell death (Martin *et al*. 2004). Therefore, it is believed that supplementation of CLC during cryopreservation protects mitochondrial membrane from damage that, in-turn, prevents DNA damage. This indicated higher motility in general and distance travelled in cervical mucus in particular in the present study. While CLC supplementation decreased significantly the spermatozoal plasma membrane enzymes or intra-cellular enzymes (ALT and AST) in the seminal plasma of cryopreserved semen which indicates that CLC has capability to prevent sperm membrane damages. Similar result was observed in the present study. Further, the mammalian spermatozoa easily suffers from osmotic as well as oxidative stresses during freezing-thawing cycle and that oxidative stress is one of the major causes for damage of DNA and sperm membrane (Walters *et al*. 2005). It is plausible that increasing the TAC and decreasing the MDA in CLC supplemented semen could supply an alternative method to increase the post-thawing quality of semen and fertility of sperm. Similar result was observed in the present study.

Parks and Graham (1992) observed that freezing-thawing cycle induces ultra-structural changes in plasma membrane of sperm as well as membrane reorganization may lead to membrane phospholipid bi-layer faults that could favour or facilitate extra cellular calcium influx into the spermatozoa. Reduction of plasma membrane stability in cryopreserved spermatozoa of control group might be due to the higher efflux of cholesterol from plasma membrane and influx of calcium into the cell in our study. This was supported by significant negative association between cholesterol content and lower plasma membrane integrity and stability in control group which indicates cholesterol content of spermatozoa and its membrane directly and/or indirectly alter the plasma membrane permeability and stability. Thus, lower the cholesterol content, higher the increased intra-cellular Ca^{2+} concentration and subsequently the survivability has reduced after cryopreservation. However when germ cells were incubated with CLC which caused high membrane cholesterol content in Gr II, a linear

increase in plasma membrane stability proportionate to cholesterol content was observed as compared to control spermatozoa in the present study.

Membrane damage in sperm is an unavoidable phenomenon in freezing-thawing cycle of cryopreservation. Different damages occur throughout the sperm membrane including the acrosome region of most of the mammalian spermatozoa (Blottner *et al*. 2001). Supplementation of CLC in semen extender has reduced the acrosomal damage in cattle bull (Purdy and Graham 2004) and in buffalo bull (Kumar 2012) indicating that CLC has protected the acrosomal cavity and its contents. The higher per cent of spermatozoa showing intact acrosome in CLC treated group than control in this study was in agreement with the earlier findings (Purdy and Graham 2004). Increased membrane cholesterol content through inclusion of CLC perhaps maintained a more fluid state to the acrosomal membrane, thus prevented the damage to acrosome which resulted in to more number of spermatozoa with intact acrosome in this study.

Cholesterol loaded cyclodextrin significantly ($P<0.05$) increased the FPM and viability at post-thaw stage in CLC treatment group which might be due to decreased LPO and reactive oxygen species (ROS) levels in spermatozoa by CLC. In our study, CLC increased percentage of sperm with plasma membrane intactness which was similar to reports of Kumar (2012). The increase in integrity of plasma membrane by inclusion of CLC may be due to decreased plasma membrane damage by minimizing the levels of LPO and ROS. Purdy and Graham (Purdy and Graham 2004) reported that inclusion of CLC might enhance the cholesterol content of the mitochondrial membrane; stabilization of mitochondria membrane cholesterols as well as it maintained the P: C ratio, which causes higher transmembrane mitochondrial potential in the spermatozoa as compared to untreated control group. Therefore, the sperm treated with CLC has higher motility, velocity and vanguard distance travelled by sperm.

Moreover, the CLC methodology could be very useful to minimize the cryocapacitation damages specifically in genetically high merit males as sperm of these male do not maintain the threshold SQPs after cryopreservation (poor freezable). In mithun, due to high individual variation in fertilizing capability as reported in other species (Presicce *et al*. 2003) and higher proportion of cryocapacitated sperm at thawing (Perumal 2014b), this CLC approach may permit to enhance the number of males to enroll as semen donors. Therefore, it would be required in future studies to assess the effect of the CLC supplementation in sperm from such males that do not freeze well or have poor freezability. Even though, the present study results are encouraging and assume that CLC-treated sperm will also exhibit a greater fertilizing capability *in-vivo*, *in-vivo* fertility trials need to be conducted to determine definitively the usefulness of this CLC treatment for mithun sperm cryopreservation.

In the present study, *in-vitro* SQPs and TAC were lowered and leakage of intra-cellular contents and sperm and nuclear abnormalities increased significantly in high cholesterol loaded group (Gr III vs Gr II), suggesting inhibitory action of cholesterol addition above the threshold/ optimum level (mg/120×10^6 spermatozoa) on these parameters and it may be due to changes of physical, physiological, osmotic pressure or osmolarity of the extender or may be due to aggregation effect of cholesterol and/or phospholipids may have adverse/harmful effects in sperm cryopreservation. This particular finding confirms with the study of Zahn *et al*. (2002) who observed that relatively very high concentrations of cholesterol in the sperm membranes interferes the normal physiological process of sperm. This may result alterations in plasma membrane fluidity, influx of Ca^{2+} subsequently affecting fusion capability of spermatozoa (Yeagle 1985). Very interestingly, it was reported that average cholesterol content of the sperm and its membrane, was maximum 2–3 times higher than the control sperm at CLC concentrations that provide maximum cryoprotection (Purdy and Graham 2004). However, if cholesterol level increase 4–5 times compared to control, it becomes deleterious to sperm survival, which may be due to alteration of plasma membrane fluidity and/or membrane function to the point of inhibition of normal membrane function. The optimum or threshold level of cholesterol in CLC varies for different species because of biochemical structure or composition of sperm membrane which inturn influence sensitivity to cold shock.

Incubation of the sperm in cholesterol before cryopreservation is a simple technology, but has significantly considerable benefits in cryoprotection of sperm. SQPs improved in dose dependent manner and optimum dose of CLC was 1 mg/120×10^6 sperm in mithun. However, the benefit of CLC needs to be confirmed by *in vivo* fertility trial.

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