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Protective effect of cholesterol loaded cyclodextrin on cryopreservation of buck spermatozoa

PRADEEP KUMAR[1], S K JINDAL[2], CHETNA GANGWAR[3], S D KHARCHE[4], JUHI PATHAK[5] and ANUJ KUMAR SINGH SIKARWAR[6]

ICAR-Central Institute for Research on Goats, Farah, Mathura, Uttar Pradesh 281 122 India

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Semen cryopreservation provides the opportunity for the extension of availability of sperm not only for research but also for artificial insemination (AI) and *in vitro* fertilization (IVF) irrespective of time and place (Gangwar *et al.* 2018). In spite of various advantages, there is a significant reduction in the fertilizing potential of the frozen–thawed sperm as compared to the fresh sperm, since the cryopreservation process induces lipid and protein rearrangements within the cell membranes when they are cooled from ambient to subzero temperature (Parks and Graham 1992, Salmon *et al.* 2017). Buck sperm contrasts from other species such as human and rabbit sperm that have high cholesterol to phospholipid ratio in the membrane, therefore, alleviating the membrane damage occurring during cryopreservation (Darin-Bennett and White 1977). Retrospective studies in the field indicated that the cholesterol/phospholipid ratio of the plasma membrane is a crucial determinant in plasma membrane fluidity and stability during the cryopreservation (Darin-Bennett and White 1977, Gangwar *et al.* 2014). Furthermore, cholesterol in the cell membrane is a critical modulator of membrane fluidity and permeability (Uçan *et al.* 2016). Incorporation of cholesterol into the membrane of cells can be readily achieved by the use of cyclodextrins (Salmon *et al*. 2017). Cyclodextrins are cyclic hepta saccharides consisting of (1–4) glucopyranose units, are water soluble but have a hydrophobic center (Purdy and Graham 2004), and can transport cholesterol in or out of membranes down a concentration gradient. A higher per cent of sperm motility was achieved when cholesterol was loaded with cyclodextrin (CLC) prior to cryopreservation as compared to unloaded sperm (Souza *et al*. 2016). However, this procedure has not yet been optimized for buck sperm, nor is it known how the cholesterol regulates the cryosurvival of buck sperm and the function of physiology of the membrane which formed the basis of the present study.

Methyl-β-cyclodextrin was loaded with cholesterol as described previously (Purdy and Graham 2004). 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.

Semen was collected by using an artificial vagina as per the standard method. A total of 32 ejaculates, six from each Sirohi buck (8×4 = 32) were collected during the period of February to April. The percentage of progressively motile sperm in each sample was determined by microscopic evaluation. 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 was done. After the initial semen evaluation, 4 equal fractions of the samples were made. The Gr 1 (control) was diluted with Tris, while Gr 2, 3 and 4 were treated with CLC solution at the rate of 1.0, 2.0 and 3.0 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.

Diluted semen was cooled to 4°C in 2 h and equilibrated for 4 h at 5°C, and extended semen was packaged in 0.25 ml straw with suction pump at 4°C in cold cabinet unit. The straws were frozen by manual freezing method and kept in LN_2 vapors for 10 min (–145°C) and 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, acrosomal integrity and plasma membrane integrity. Pre-freeze evaluation was done immediately after the equilibration period. Frozen

Present address: [1]M.V.Sc Scholar (drpradeepvet19@gmail.com), ICAR-Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh. [2,4]Principal Scientist (jindalmathura@gmail.com, Kharche62@gmail.com), [3]Scientist (chetnaom82@gmail.com), [5]Senior Research Fellow (juhi1690@gmail.com), [6]Research Associate (dranujkumar @rediffmail.com).

straws were thawed at 37°C for 45 sec after 24 h of freezing for post-thaw evaluation.

The progressive motility of the spermatozoa was subjectively assessed by 1 person throughout the study. For the motility assessments, aliquots (10 µl) of diluted semen samples were placed on a clean grease free slide and pre-warmed slide, covered with a glass cover slide (20 × 20 mm), and examined under high power phase objective lens (40×) equipped with an attached thermostatically controlled stage (37ºC). Per cent progressive motility was defined as the percentage of spermatozoa that had progressive motility, and was assessed randomly in at least 5 fields (Gangwar *et al*. 2015).

A method described by Bloom (1950) and Hancock (1951) was followed. Dead spermatozoa could be differentiated by their ability to get stained by Eosin dye. One small drop of semen sample (kept at 35°C) was mixed with 2 to 3 drops of Eosin-Nigrosin stain on a clean glass slide kept on a thermostatically warm stage (34–35°C). This mixture was kept for 2 min. A smear was then made from the mixture on a clean and grease free glass slide. It was dried in air and examined under the bright field 100× oil immersion objective lens. Around 200 sperms were assessed in 5 different fields of a slide. Sperms that were colourless (unstained) were classified as live and those that showed any pink colour were classified as dead.

Giemsa stain was used to assess the acrosomal integrity of frozen thawed buck spermatozoa as per Watson (1975). Diluted semen drop was kept on clean grease free slide and thin tongue shape smear was prepared. After air drying the smear, the slide was fixed in methanol for 15 min and then after washing the fixed slide was kept in working solution of Giemsa for 90 min. Excess stain was removed by gentle stream of water. It was dried in air and examined under the bright field 100× oil immersion objective lens. Around 200 sperms were assessed in 5 different fields of a slide.

Hypo-osmotic solution of 150 mOsmol/l was prepared (Sodium citrate 7.35 g, Fructose 13.51 g and Double distilled water up to 1000 ml). One ml of hypo-osmotic solution, having an osmotic strength of 150 mOsm/l was mixed with 0.1 ml of semen and incubated in a water bath at 37°C for 1 h. Following incubation, a drop of well mixed solution was taken on a clean dry glass slide and covered with a cover-slip. Sperm tail curling is recorded as an effect of swelling due to influx of water. A total of 200 spermatozoa were counted in different fields at 40× magnification (Jayendran *et al*. 1984).

Fresh or frozen thawed semen (1 ml) was taken and diluted in 5 ml of TALP medium. The diluted semen was centrifuged for 15 min at 1,800 rpm. The supernatant was discarded and the sperms were washed with equal volume of TALP medium. Incubation was done at 37°C in CO_2 incubator for 1 h in slanting position for the motile spermatozoa to swim up. The supernatant was collected and centrifuged. The sperm pellet was resuspended in TALP medium to a final concentration of 1×10^6 motile spermatozoa/ml. Similarly, oocytes were collected from slaughter goats ovaries and matured *in vitro* as described earlier (Kharche *et al.* 2011) with slight modification. After maturation, oocytes were denuded with 0.1% Hyaluronidase by repeated pipetting and washed with culture medium.

Ten microlitre droplet of 1×10^6 motile spermatozoa/ml was placed under mineral oil in 35 mm multi well petridishes. Then 1 oocyte was added to each droplet (at least 20 oocytes were used to estimate the sperm zona pellucida binding of each samples).The gametes were incubated at 38.5°C in a CO_2 incubator for 4 h. After incubation, the oocyte sperm complexes were rinsed 5 times with culture media, using a narrow bore Pasteur pipette to remove loosely attached spermatozoa. The sperm oocyte complexes were fixed with 2.5% glutaraldehyde for 10 min, washed with PBS, stained with 0.1 µg/ml 4,6-diamino-2-phenylindol- (DAPI) in PBS or 1 mg/ml Hochest 33342 dye (Sigma) for 10 min and washed with PBS. The sperm oocyte complexes were placed on slides slightly compressed with cover slip and sealed. The number of spermatozoa bound to the zona pellucida of the oocytes was counted under fluorescence microscope.

The one-way ANOVA assumption was evaluated using Tukey' s test for normality for all data distribution at fresh, equilibration and post-thaw stages of cryopreservation. Data were subjected to ANOVA, using the Post hoc procedure from SPSS package, version 20. Data were presented as mean±SEM, and a 5% probability was considered significant.

The difference in the progressive motility among all the 4 groups was significant ($P<0.05$), being highest in Gr 2 followed by Gr 3. The viable spermatozoa differed significantly ($P<0.05$) among all the 4 groups, being highest in Gr 2 followed by Gr 3 (Table 1, Fig. 1). The acrosomal integrity demonstrated a significant difference ($P<0.05$) among all the 4 groups, being highest in Gr 2 followed by Gr 3 (Fig. 2). The HOS response differed significantly ($P<0.05$) among all the 4 groups, being highest in Gr 2 followed by Gr 3 (Fig. 3).The zona binding assay was significantly different ($P<0.05$) among all the 4 groups, being highest in Gr 2 followed by Gr 3 (Table 2).

Cholesterol helps in maintaining a fluid state of membrane by reduction of the transition temperature of membranes, thereby alleviating the damage sustained by membranes at low temperatures (Glazar *et al*. 2009). The possible mechanism behind increased progressive motility of spermatozoa following cryopreservation after incorporation of cholesterol in sperm membrane can be attributed to protection of mitochondria (Purdy and Graham 2004). In the present study, 10.12% improvement in the sperm viability was observed after treatment with 1 mg CLC, which was higher than the values (6.37%) after treatment with 2 mg CLC. Higher viability at post-thaw stage after CLC treatment may be due to stabilization of plasma membrane of spermatozoa and hence reduction in sperm loss during cryopreservation was witnessed.

CLC-treated sperm exhibited higher acrosomal and plasma membrane integrity. The possible explanation may

Table 1. Effect of CLC on various parameters of spermatozoa at pre-freeze and post-thaw stage (Mean±SE, n=32)

Group	Stage	Progressive motility	Viability	Acrosomal integrity	Plasma membrane integrity
I	Pre-freeze	69.79±0.47^{ab}	75.91±0.56^{a}	76.66±0.51^{b}	65.66±0.41^{b}
	Post-thaw	41.87±0.50^{a}	54.92±0.28^{a}	69.95±0.25^{a}	53.66±0.23^{a}
II	Pre-freeze	73.95±0.42^{c}	80.66±0.51^{c}	81.79±0.21^{d}	70.04±0.22^{d}
	Post-thaw	53.54±0.47^{d}	65.54±0.46^{d}	79.87±0.23^{d}	62.79±0.21^{d}
III	Pre-freeze	70.83±0.49^{b}	77.95±0.34^{b}	78.54±0.67^{c}	68.83±0.24^{c}
	Post-thaw	48.95±0.60^{c}	61.29±0.25^{c}	76.79±0.41^{c}	60.66±0.44^{c}
IV	Pre-freeze	69.85±0.87^{ab}	76.58±0.76^{ab}	75.92±0.65^{b}	66.17±0.33^{b}
	Post-thaw	43.33±0.57^{b}	58.20±0.22^{b}	73.62±0.41^{b}	56.83±0.36^{b}

Means bearing different superscripts in a column differ significantly (P<0.05) between groups.

Table 2. Effect of CLC on number of buck spermatozoa bound to zona pellucida at post-thaw stage (Mean±SE, n=32)

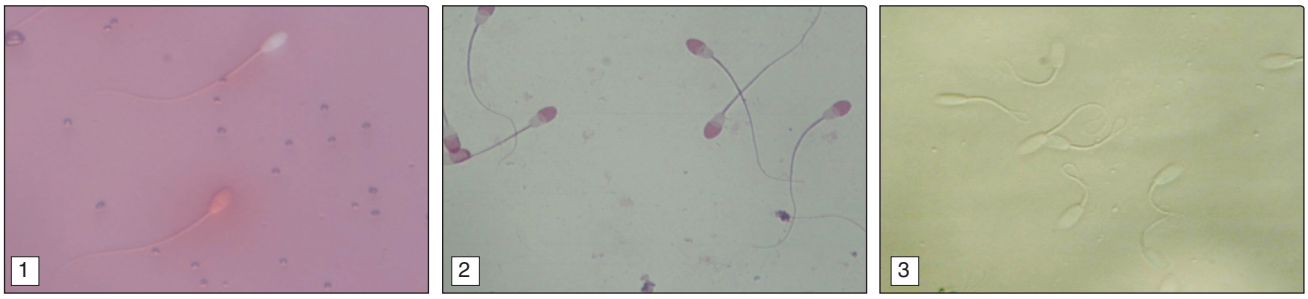
Stage	Group I	Group II	Group III	Group IV
Post thaw	58.12±1.27^{a}	89.38±0.64^{d}	82.67±1.74^{c}	74.62±2.11^{b}

Means bearing different superscripts in a row differ significantly (P<0.05).

be that CLC treated sperm had high cholesterol content in the membrane (between 1.93 and 2.7 folds) than control sperm in bulls, stallions and rams (Moce *et al*. 2010). In accordance to our findings, Farshad *et al*. (2011) reported that treating goat sperm with CLC (0.75 mg CLC/120×10^6 sperm) prior to cryopreservation increases the percentages of motile sperm after thawing. Therefore an inference can be made that, with an increase in cholesterol content in the plasma membrane, crystallization of membrane hydrocarbon chains at low temperature is inhibited, thereby diminishing the phase transition disruption (Andrabi 2009). On the other hand, CLC-treated sperm exhibited wider osmotic tolerance limits than control sperm in bulls, stallion, rams and bucks (Glazar *et al*. 2009, Moce *et al*. 2010, Salmon *et al*. 2016, Souza *et al*. 2016). This could provide resistance to increase post-thaw survival of sperm.

The optimal level of CLC for cryopreservation of buck sperm was 1.0 mg CLC per 1.2 × 10^8 sperm, which was similar to that reported for bull, stallion, buck and ram sperm (Purdy and Graham 2004, Moore *et al*. 2005, Konyali *et al*. 2013, Ucan *et al.* 2016). However, deleterious effects on sperm cell survival were witnessed when semen samples were exposed to CLC levels more than 3.0 mg per 1.2 × 10^8 sperm. Probably, it may occur by affecting membrane fluidity and membrane function which leads to sperm death during the freezing as cholesterol plays an vital role in stabilizing sperm membranes. A loss in cholesterol had been reported, especially during the cooling and cryopreservation process, the membranes undergo a phase transition, which ultimately leads to rearrangement of membrane lipids and proteins (Parks and Graham 1992), which consequentially results in loss of membrane-selective permeability, culminating into premature capacitation of sperm. This premature capacitation ultimately causes the reduction in fertilizing ability and lifespan of the sperm and this might be the possible reason behind the higher number of CLC treated sperm bound to the zona in zona binding assay. In our results, sperm cells treated with CLC indicated higher percentage of acrosomal integrity which might be due to cholesterol incorporation in the acrosomal membrane (Lee *et al.* 2015). This might be due to effective protection of the sperm acrosomal membrane from cryodamage. Moreover, it has been stated that cholesterol directly alters the physical properties of lipid bilayer and membrane response to the degenerative processes including lipid peroxidation in different biological systems (Salmon *et al*. 2017).

In conclusion, CLC effectively imbibes cholesterol into the sperm membrane of buck spermatozoa leading to effective reduction of cryodamage experienced during cryopreservation. Therefore, addition of CLC to buck sperm before cryopreservation could improve post-thaw sperm



Figs 1–3. **1**. Viability of Buck sperm with Eosin/Nigrosin stain (40×); sperm having pink colour are dead (a) and sperm having no colour are live (b). **2**. Acrosomal integrity of Buck sperm with Giemsa stain (40×); sperm showing pink acrosomal cap have intact acrosome (a) and sperm showing damaged acrosome have no cap (b). **3**. Plasma membrane integrity of Buck sperm with HOST (40×); sperm showing curled tail have active plasma membrane (a) and sperm showing straight tail have damaged plasma membrane (b).

survival. Maximum beneficial effect on semen cryopreservation was observed at dose rate of 1.0 mg CLC/ 120×10^6 spermatozoa. Addition of CLC significantly improved semen freezability as revealed by higher progressive motility, livability, acrosomal integrity, HOS reaction and plasma membrane integrity.

SUMMARY

The present study was conducted to evaluate the protective effect of cholesterol loaded cyclodextrin on buck semen quality during cryopreservation. Semen samples (n=32) after the evaluation of initial motility and viability, were divided into 4 equal fractions. Gr 1 was used as control and diluted with Tris, while Gr 2, 3 and 4 were subjected to CLC treatment at the rate of 1.0, 2.0 and 3.0 mg/ml respectively to obtain 120×10^6 sperm/ml as final spermatozoa concentration. Control and all the 3 treatment groups were incubated for action of CLC, followed by freezing. Semen samples from different groups were evaluated for progressive motility, viability, acrosomal integrity and plasma membrane integrity after dilution as well as at post-thaw stage. Zona binding assay was also performed for different groups. Findings of the present study revealed that there was significant increase in progressive motility (53.54±0.47), viability (65.54±0.46), acrosomal integrity (79.87±0.23) and plasma membrane integrity (62.79±0.21) in 1.0 mg CLC/ 120×10^6 spermatozoa group (Gr 2). Similarly, findings of zona binding assay revealed that there was significant increase in the number of sperm bound to the zona in 1 mg CLC treated group as compared to control group (89.38 ± 0.64 vs 58.12 ± 1.27).

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REFERENCES

Andrabi S M H. 2009. Factors affecting the quality of cryopreserved buffalo (*Bubalus bubalis*) bull spermatozoa. *Reproduction in Domestic Animals* **44**: 552–69.

Bloom E. 1950. A one minute live-dead sperm stain by means of eosin-nigrosin. *Fertility and Sterility* **1**: 176–77.

Darin-Bennett A and White I. 1977. Influence of the cholesterol content of mammalian spermatozoa on susceptibility to cold-shock. *Cryobiology* **14**: 466–70.

Farshad A, Amidi F, Khor A K and Rashidi A. 2011. Effect of CLC in presence and absence of egg yolk during freezing step on quality of Markhoz buck's spermatozoa. *Asian Australasian Journal of Animal Science* **24**(2): 181–89.

Gangwar C, Kharche S D, Ranjan R, Kumar S, Goel A K and Jindal S K. 2015. Effect of vitamin C supplementation on freezability of Barbari buck semen. *Small Ruminant Research* **129**: 104–07.

Gangwar C, Ranjan R, Kumar Satish, Karche S D, Goel A K, Ramachandran N and Jindal S K. 2014. Use of chelating agent for optimum post thaw quality of buck Semen. *Indian Journal of Animal Sciences* **84**(8): 839–41.

Gangwar C, Saxena A, Patel Akhil, Singh S P, Yadav Sarvajeet, Kumar Ravindra and Singh Vijay. 2018. Effect of reduced glutathione supplementation on cryopreservation induced sperm cryoinjuries in Murrah bull semen. *Animal Reproduction Science* **192**: 171–78.

Glazar A I, Mullen S F, Liu J, Benson J D, Critser J K, Squires E L and Graham J K. 2009. Osmotic tolerance limits and membrane permeability characteristics of stallion spermatozoa treated with cholesterol. *Cryobiology* **59**: 201–06.

Hancock J L. 1951. A staining technique for the study of temperature shock in semen. *Nature* **169**: 323–26.

Jayendran R S, Vander-Ven H, Perez-Pelaez M, Crabo B G and Zanevld L J D. 1984. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characters. *Journal of Reproduction and Fertility* **70**: 219–28.

Kharche S D, Goel A K, Jindal S K, Goel P and Jha B K. 2011. Birth of twin kids following transfer of *in-vitro* produced goat embryos. *Indian Journal of Animal Sciences* **81**: 1132–34.

Konyali C, Tomas C, Blanch E, Gomez E A, Graham J K and Moce E. 2013. Optimizing conditions for treating goat semen with cholesterol-loaded cyclodextrins prior to freezing to improve cryosurvival. *Cryobiology* **67**: 124–31.

Lee Y S, Lee S, Lee S H, Yang B K and Park C K. 2015. Effect of cholesterol-loaded-cyclodextrin on sperm viability and acrosome reaction in boar semen cryopreservation. *Animal Reproduction Science* **159**: 124–30.

Moce E, Purdy P H and Graham J K. 2010. Treating ram sperm with cholesterol-loaded cyclodextrins improves cryosurvival. *Animal Reproduction Science* **118**(2–4): 236–47.

Moore A I, Squires E L and Graham J K. 2005. Adding cholesterol to the stallion sperm plasma membrane improves cryosurvival. *Cryobiology* **51**: 241–49.

Parks J and Graham J. 1992. Effects of cryopreservation procedures on sperm membranes. *Theriogenology* **38**: 209–22.

Purdy P and Graham J. 2004. Effect of cholesterol-loaded cyclodextrin on the cryosurvival of bull sperm. *Cryobiology* **48**: 36–45.

Salmon V M, Leclerc P and Bailey J L. 2017. Novel technical strategies to optimize cryopreservation of goat semen using cholesterol-loaded cyclodextrin. *Cryobiology* **74**: 19–24.

Salmon V M, Leclerc P and Bailey J L. 2016. Cholesterol-loaded cyclodextrin increases the cholesterol content of goat sperm to improve cold and osmotic resistance and maintain sperm function after cryopreservation. *Biology of Reproduction* **94**: 85.

Souza W L, Moraes E A, Costa J M S and James K. 2016. Cholesterol-loaded cyclodextrin in fresh goat sperm improves cryosurvival rates. *Brazilian Journal of Veterinary Science* **23**(1–2): 93–98.

Uçan U, Küçük N, Ahmad E, Naseer Z, Aksoy M, Serin Ý and Ceylan A. 2016. Effect of different sugars supplemented to the extender in combination with cholesterol-loaded cyclodextrin (CLC) on post-thaw quality of ram spermatozoa. *Small Ruminant Research* **136**: 243–46.

Watson P F. 1975. Use of Giemsa stain to detect changes in acrosomes of frozen ram spermatozoa. *Veterinary Record* **97**: 12–15.