EFFECT OF CHOLESTEROL- LOADED CYCLODEXTRIN SUPPLEMENTATION ON THERMORESISTANCE OF CRYOPRESERVED MALABARI BUCK SPERMATOZOA*

Sunita Behera[1], Hiron M. Harshan[2], Lekshmi Bhai K.[3], K. N. Aravinda Ghosh[4], Metilda Joseph[5] and M. Mini[6]
Department of Animal Reproduction,
Gynaecology and Obstetrics,
College of Veterinary and Animal Sciences,
Mannuthy-680 651, Thrissur, Kerala.

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Abstract

A study was carried out to evaluate thermo resistance of thawed Malabari buck spermatozoa frozen in Tris based extenders with varying cholesterol levels. Thirty two ejaculates from two adult Malabari bucks were used for the study. Cholesterol was supplemented in the extender for semen freezing as Cholesterol-loaded-cyclodextrin (CLC) with methyl-β-cyclodextrin as carrier. The semen was treated with 1 mg or 2 mg of CLC/ 120 million spermatozoa before cryopreservation as per standard protocols. It was observed that the CLC treatment of spermatozoa resulted in improved thermo resistance up to 2 h of incubation.

Key words: M*alabari buck, spermatozoa, cholesterol-loaded-cyclodextrin, cryopreservation, thermo resistance*

Sperm damage occurring during cryopreservation is multifactorial and induces partially irreversible damage to sperm membranes, which decreases the quality of sperm after freezing and thawing (Moore *et al.,* 2005). It has been suggested that the sperm plasma membrane is the primary site of damage induced by cryopreservation and is one of the main reason for reduced motility and fertility of spermatozoa after cryopreservation (Chakrabarty *et al.,* 2007). Cholesterol is an important component in the regulation of membrane fluidity, aiding in the stabilization of the membrane (Muller *et al.,* 2008). Cold shock inflicted during cryopreservation induces plasma membrane lipid phase transitions, which can be reduced by increasing the proportion of cholesterol within the plasma membrane (Drobnis *et al.,* 1993). Additionally, cryopreservation induces cholesterol depletion from plasma membrane, which in turn causes membrane destabilization (Bailey *et al.,* 2008).

Adding cholesterol-loaded cyclodextrin (CLC) as carriers of cholesterol, to extenders of mammalian spermatozoa increased the stability and rigidity of plasma membrane (Moore *et al.,* 2005). Several authors have reported increased cryosurvival rates when boar, bull, buck, ram and stallion spermatozoa were supplemented with cholesterol. Hence a study on the freezability and post thaw thermo-resistance of Malabari buck semen supplemented with CLC was

* *Part of MVSc thesis submitted by the first author to the Kerala Veterinary and Animal Sciences University*
[1]*Assistant Professor, Dept. of Animal Reproduction, Aravali Veterinary College, Rajasthan*
[2&3] *Assistant Professors*
[4] *Professor and Head*
[5] *Associate Professor*
[6] *Professor and Head, Dept. of Veterinary Microbiology*

carried out.

Material and methods

Cholesterol-loaded cyclodextrin was prepared as described by Purdy and Graham (2004). In brief, 500 mg of methyl-β-cyclodextrin was dissolved in 1.0 ml of methanol in a glass test tube. In another test tube, 200 mg cholesterol was dissolved in 1.0 ml of chloroform. An aliquot of 0.225 ml of cholesterol solution was transferred to methyl-β-cyclodextrin solution and stirred to make a clear solution. The prepared solution was then poured into a glass Petri dish. The solvents were removed under a stream of nitrogen gas. The resulting crystals were kept at room temperature for another 24 h for drying and stored in a glass vial at room temperature until use. The working solution of CLC was prepared by adding 40 mg of CLC to 1.0 ml of tris-citric acid-glucose (TCG) buffer and the solution mixed using a vortex shaker.

A total of 32 ejaculates from two adult Malabari bucks maintained at the Artificial Insemination centre, Mannuthy were used for the study. Semen was collected using artificial vagina and immediately after collection, the preliminary evaluation was done. Spermatozoa concentration was determined by haemocytometer. Ejaculates having "++++" mass activity, more than 80% of progressive motile spermatozoa, and density of "DDDD" were used for the study. For each cycle of experiment, four ejaculates, consisting of two consecutive ejaculates collected at 10 min interval from each buck, were pooled after preliminary evaluation. The pooled ejaculates were split into three, with two treatment groups

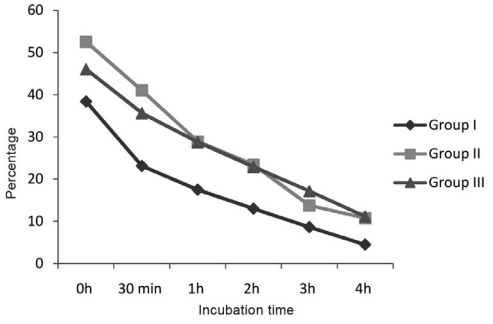

Fig.1. Effect of post-thaw incubation at 37°C on progressive motility of Malabari buck spermatozoa frozen in extenders with varying level of cholesterol (n=8) Group I: control; Group II: supplemented with CLC @ 1 mg/ 120´10^6 spermatozoa; Group III: supplemented with CLC @ 2 mg/ 120´10^6 spermatozoa

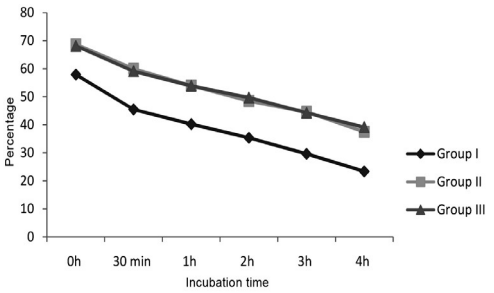

Fig.2. Effect of post-thaw incubation at 37°C on viability of Malabari buck spermatozoa frozen in extenders with varying level of cholesterol (n=8) Group I: control; Group II: supplemented with CLC @ 1 mg/ 120´10^6 spermatozoa; Group III: supplemented with CLC @ 2 mg/ 120´10^6 spermatozoa

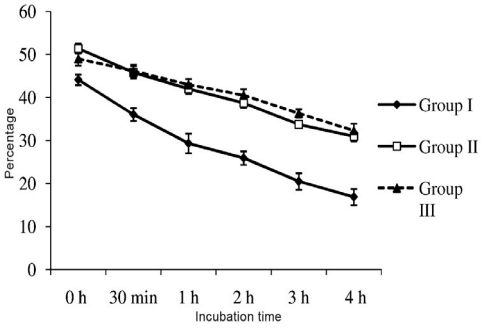

Fig. 3. Effect of post-thaw incubation at 37°C on HOS responseof Malabari buck spermatozoa frozen in extenders with varying level of cholesterol (n=8) Group I: control; Group II: supplemented with CLC @ 1 mg/ 120´10^6 spermatozoa; Group III: supplemented with CLC @ 2 mg/ 120´10^6 spermatozoa

and one control. Semen samples of the treatment groups were treated with either 1 mg or 2 mg of CLC/ 120 million buck spermatozoa in Tris-citric acid- glucose (TCG) buffer (group I and group II, respectively) and incubated for 15 min and processed for manual freezing using liquid nitrogen. The control group was frozen after extension in TCG buffer. Resistance of post thaw spermatozoa to incubation was assessed by incubating at 37ºC and examining progressive motility, viability and functional membrane integrity of spermatozoa of each group at 0 h, 30 min, 1 h, 2 h, 3 h and 4 h intervals as described below.

Percentage of progressively motile spermatozoa was estimated by examining under a microscope (400X). Viability of spermatozoa was assessed by eosin-nigrosin staining technique (Campbell *et al*., 1953). Functional membrane integrity of spermatozoa was assessed by hypo-osmotic swelling (HOS) test as per Jeyendran *et al.* (1984). Data observed

were transformed using Arcsine transformation and was analyzed by one-way analysis of variance (Snedecor and Cochran, 1994).

Results and Discussion

Semen treated with 1mg of CLC / 120 $\times 10^6$ spermatozoa prior to cryopreservation maintained a significantly higher ($p<0.05$) percentage of progressive motile spermatozoa immediately after thawing and at 30 min of thawing than other groups. Spermatozoa of cholesterol-loaded-cyclodextrin treated groups maintained a higher percentage of progressive motility up to 2 hours of thawing (Fig. 1).Similar results were reported by Konyali (2009) in buck semen up to 150 min of incubation and Pamornsakda *et al.* (2011) in equine epididymal spermatozoa up to four hour of incubation.

The percentage of viable spermatozoa were observed to be significantly higher ($p<0.05$) in groups treated with 1 mg or 2 mg of CLC/ 120 × 10^6 spermatozoa than the group without CLC treatment (Fig. 2). The results obtained are comparable to the observations of Pamornsakda *et al.* (2011) in equine epididymal spermatozoa. Increasing the cholesterol content in spermatozoa to be cryopreserved was stated to have a positive impact on post thaw sperm longevity (Cross, 1998). The maintenance of higher progressive sperm motility and viability during incubation had been reported to have a direct correlation with the survivability of the spermatozoa in the female reproductive tract, with prolongation of viability enhancing the chances of fertilization (Dorado *et al.*, 2007).

Functional membrane integrity was tested using HOS test. Though no significant difference was observed in the percentage of HOS positive spermatozoa of all the groups (Fig. 3) immediately after thawing, the spermatozoa of CLC treated groups maintained higher ($p<0.05$) percentage of HOS positive spermatozoa after 30 min of incubation to 4h of incubation, when compared to spermatozoa of control group.The results are in accordance with the findings of Moce *et al.* (2010) who reported that treatment with CLC increases cryosurvival of ram spermatozoa by increasing the osmotic tolerance.

Cold shock resulted in plasma membrane undergoing lipid phase transitions during the cooling process and was inversely correlated with the proportion of cholesterol within the plasma membrane (Drobnis *et al.,*1993). Addition of sufficient levels of liposome containing cholesterol to sperm plasma membrane, when plasma membrane is exposed to low temperatures, increases its cryo-resistance and as a result it does not undergo a transition phase (Wilhelm *et al.,*1996).

Cholesterol can be incorporated to all compartments of plasma membrane by adding sufficient level of cholersterol to biological membranes, which prevents premature capacitation at low temperature during phase transition, thus increasing cryosurvival of sperms. CLC treated spermatozoa, which started with greater membrane cholesterol content prior to cooling, lost only a small amount of cholesterol during cryopreservation and had greater amount of cholesterol after thawing than control group spermatozoa, which prevented premature capacitation and increased the longevity of cryopreserved sperms (Purdy and Graham, 2004).

In the present study, treatment of spermatozoa with CLC was found to bring about an increased HOS response than the control group. When sperms are diluted in a hypotonic solution, water moves into the cell at a faster rate, causing increase in the cell volume, which may cause membrane damage and even cell lysis (Guthrie *et al.,* 2002). Spermatozoa treated with cholesterol were able to withstand hypotonic condition better than the control group spermatozoa, indicating that the cholesterol treatment may reduce some amount of membrane damage occurring due to aniso-osmotic condition (Moraes *et al.,* 2010).

Thus, in the present study, it was observed that Malabari buck spermatozoa treated with CLC had better post thaw thermo-resistance, thus better chances of being fertile than untreated spermatozoa.

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