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Cholesterol Loaded Cyclodextrin Increases cryopreservability of Marwari Stallion (*Equus ferus caballus*) Spermatozoa by Increasing Cholesterol to Phospholipid Ratio

Pramod Kumar[1]*, J.S. Mehta[1], S.K. Ravi[2], Talluri T Rao[3], G.N. Purohit[1] and Ashok Kumar Chaudhary[1]

[1]Department of Veterinary Gynaecology and Obstetrics, College of Veterinary and Animal Science, RAJUVAS, Bikaner, Rajasthan, INDIA

[2]Division of Animal Reproduction, ICAR- Central Island Agricultural Research Institute (CIARI), Port Blair, INDIA

[3]Division of Animal Reproduction, EPC-NRCE, Bikaner, Rajasthan, INDIA

**Corresponding author: P Kumar; Email: dhaterwal.pramod@gmail.com*

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ABSTRACT

The study was conducted to investigate the effects of different levels of cholesterol loaded cyclodextrin (CLC) addition on cooled and frozen-thawed spermatozoa of Marwari stallion. A total of 48 ejaculates were collected from six adult Marwari stallions (8 ejaculates from each stallion) aged between 4 to 7 years. Immediately after collection semen sample was macroscopically evaluated and filtered into a warm, graduated measuring bottle to get gel free semen. The level of cholesterol (C) and phospholipid (P) in fresh spermatozoa were measured using ELISA. The semen sample was divided in to five equal aliquots (T_0, T_1, T_2, T_3 and T_4). Primary extender containing different concentrations of CLC was added to each aliquot (0, 1, 1.5, 2 and 3 mg/ml CLC in T_0, T_1, T_2, T_3 and T_4, respectively). All the aliquots were incubated for 15 minutes at 37°C for incorporation of CLC in sperm plasma membrane and then cryopreserved. Level of C and P in spermatozoa was also evaluated at pre-freeze and post-thaw stages. The mean C, P and C: P ratio in fresh sperm was 15.36±0.47 µg/100 × 10^6 sperm cells, 46.21±1.27 µg/100 × 10^6 sperm cells and 0.33±0.071, respectively. The mean C content and C: P ratio were significantly higher ($P<0.05$) in group T_3 as compared to other groups at both pre-freeze and post-thaw stages. It was concluded that addition of CLC may be helpful in increasing cryopservability of stallion spermatozoa.

Keywords: Marwari stallion, cholesterol loaded cyclodextrin (CLC), cholesterol, phospholipid, cholesterol: phospholipid (C: P) ratio

The successful use of cryopreserved sperm largely depends on cryosurvival rates, which show large variation among species and individuals of the same species (Vidament *et al*., 2009; Wu *et al*., 2015). The major limitations of stallion frozen semen production are seasonal influence, variation between the breeds and between individuals within the breed in semen quality and freezability. Sperm sensitivity to cold shock damage is determined by membrane phospholipid composition as well as the membrane cholesterol: phospholipid (C: P) ratio (Holt, 2000). Sperm possessing high C: P ratio (human and rabbit sperm) are more resistant to the cold shock damage than sperm which have low C: P ratio (stallions, rams and boars) (Pamornsakda *et al*., 2011). The integrity of the plasma membrane is important for the spermatozoa to with stand harmful effects of the cryopreservation process. Capacitation can be reduced by adding cholesterol or cholesterol analogs to the medium (Oliveira *et al*., 2010) and can be stimulated by cholesterol acceptors such as β-cyclodextrins (Serin *et al*., 2011).

Stallions have low C: P ratio, which makes the stallion sperm more cryo-susceptible. Therefore, it seems to be important to increase cholesterol content in the semen to alter the C: P ratio of membrane for making sperm more resistance to cold shock. Researches in the past have shown that sperm treated with cholesterol loaded cyclodextrin (CLC) before

freezing in stallion (Pamornsakda *et al*., 2011; Hartwig *et al*., 2014), bull (Moraes *et al*., 2010), boar (Tomas *et al*., 2011) and ram (Farshad *et al*., 2011) exhibited greater cryosurvival rates. Rajoria *et al*. (2014) concluded that addition of CLC may be helpful in increasing freezability of buffalo spermatozoa by increasing the C/P ratio of spermatozoa and plays an important role in maintaining membrane architecture of spermatozoa.

Cholesterol is a hydrophobic molecule and is not soluble in aqueous semen diluents. Cyclodextrin have been used to insert or remove cholesterol from synthetic and cell membranes. Cyclodextrins are cyclic oligosaccharides obtained by the enzymatic degradation of starch, and they possess an external hydrophilic face and an internal hydrophobic core (Dobziuk, 2006) that can encapsulate hydrophobic compounds such as cholesterol.

Cholesterol content of sperm membranes can be modified using CLC (Purdy *et al*., 2004a). Since cholesterol efflux from the sperm membranes plays an important role in sperm capacitation, it is possible that increasing sperm cholesterol content, using CLC technology, may reduce premature sperm capacitation thereby increasing the lifespan of a cryopreserved sperm cell, in addition increasing the number of sperm that survive cryopreservation. Cholesterol also decreases the capacitation like changes (cryocapacitation) that occurs when sperms are frozen. CLC have been used in several species like bull, ram, stallion, boar and donkey's semen cryopreservation (Spizziri *et al*., 2010). The objective of this study was aimed to assess the effects of levels of CLC addition on cooled and frozen thawed spermatozoa of Marwari stallion.

MATERIALS AND METHODS

Experimental animals

Six apparently healthy Marwari horses aged between 4 and 7 years maintained at Equine Production Campus, ICAR-National Research Centre on Equines, Bikaner under uniform conditions of feeding and management were utilized for the present study.

CLC preparation

CLC was prepared as described by (Purdy and Graham, 2004a). In brief, 200 mg of cholesterol (Hi-media) was dissolved in 1 ml of chloroform (Merck) in a glass tube. In second glass tube, 1 gm of methyl-β-cyclodextrin (Sigma- Aldrich) was dissolved in 2 ml of methanol (Sigma- Aldrich). Then, 0.45 ml aliquot of the cholesterol solution was added to the cyclodextrin solution, and the mixture was stirred until the combined solution appears clear. After which, the mixture was poured into a glass petri dish and solvents were removed using a stream of nitrogen gas. The resulting crystals were allowed to dry for an additional 24 h. The crystals were removed from the dish and stored in a glass container at room temperature. A stock solution of CLC was prepared by adding 50 mg of CLC to 1 ml of primary extender at 37°C by mixing the solution using a vortex mixer for 30s. Working solutions of different concentration of CLCs were prepared from the stock solution (dilution with primary extender) on the day of semen collection.

Collection of semen and its processing

Semen was collected from six stallions by using an artificial vagina (Colorado model) as per the standard method. Semen collection was done during breeding season at early morning hours twice a week. A total of 48 ejaculates, eight from each stallion were collected. Immediately after collection, the semen samples were subjected to macroscopic or gross evaluation (for colour and consistency). The semen was filtered into a warm, graduated measuring bottle to get gel free semen. Total volume of semen, gel volume and gel free semen volume were recorded immediately after semen collection. Other fresh semen stage evaluations like pH (using digital pH meter) and sperm concentration (using Neubauer chamber) were performed. The percentage of progressively motile sperm in each sample was determined by Computer Assisted semen analyser (CASA) (HTB CEROS II, Version 1.3, Hamilton Thorne Research, Beverly, MA, USA). Livability of spermatozoa was doing by using eosin-nigrosin stained smears of semen sample under microscope at a magnification of 100 X (Nikon Instech Co. Ltd., Kanagawa, Japan). The level of C and P was measured in fresh spermatozoa by ELISA kits. After fresh semen evaluation, the semen samples were divided in five equal fractions (T_0, T_1, T_2, T_3 and T_4). All the five aliquots were incubated for 15 minutes in water bath at 37°C after addition of primary extender without CLC in T_0 (Control); and primary extender with 1, 1.5, 2 and 3mg/ml CLC in

T_1, T_2, T_3 and T_4, respectively to obtain 120 × 10^6 sperm/ml as final spermatozoa concentration. The aliquots were centrifuged in 50 ml centrifuge tube (300 X g for 5 min for 3 min) at 10°C to get sperm pellet. The supernatant i.e. seminal plasma was removed and each sperm pellet was then extended with secondary semen extender to make the final concentration 150 × 10^6 sperm/ml. French medium straws of 0.5 ml capacity were filled with extended semen of T_0, T_1, T_2, T_3 and T_4 by automatic straw filling and sealing machine and kept at 4°C for 2 hrs equilibration in cooling cabinet. 3 straws were taken out from each group and thawed at 37°C for 30 seconds for the estimation of progressive motility, livability, C and P in spermatozoa at this stage (pre-freeze). After equilibration, the straws were laid horizontally onto a wired net and lowered into a styrofoam box containing two inch level of liquid nitrogen for 10-12 minutes before plunging in to liquid nitrogen. After 24 hrs of its storage, straws from each group were thawed at 37°C for 30 sec for post-thaw progressive motility, livability, C and P estimation in spermatozoa.

Cholesterol and phospholipid estimation in spermatozoa

Washing of spermatozoa

Washing of spermatozoa was necessitated for the estimation of cholesterol and phospholipid levels of spermatozoa. Fresh, pre-freeze and frozen thawed spermatozoa were washed using percoll density gradient (Moore *et al*., 2005; Pamornsakda *et al*., 2011) to remove dead cells, debris and egg yolk particles as described below.

One ml layer of 45% percoll (v/v, Sigma Aldrich, USA) was taken in a disposable centrifuge tube and then one ml fresh or pre-freeze (T_1, T_2, T_3, T_4 and T_0) or frozen thawed semen aliquots (T_1, T_2, T_3, T_4 and T_0) were gently layered on the top of percoll column and then test tubes were centrifuged at 600 g for 25 minutes.

After centrifugation, the pellets were washed once again with PBS and resuspended in PBS to make desired concentration of sperm using Neubauer counting chamber depending upon experiments.

An aliquot of 0.5 ml (in duplicate) was taken in cryovial and stored at -20°C until used for estimation of cholesterol and phospholipid levels of spermatozoa.

Cholesterol and phospholipid assay

A total of 100 million washed spermatozoa were taken in a 10 ml vial. The sperm pellet was extracted with 20 volume of chloroform: Methanol (1:1, V/V) solution and vertexed for 20 sec. Thereafter, it was centrifuged at 800 g for 5 min. The spermatozoa were evaporated to dryness under Nitrogen gas and again resuspend in PBS. Total cholesterol in semen samples was quantitatively estimated using Horse total cholesterol (TC) ELISA Kit (Bioassay Technology Laboratory, Shanghai, China) while phospholipid in the lipid extract of spermatozoa was quantitatively estimated using Bovine Phospholipid (PL) ELISA Kit (Bioassay Technology Laboratory, Shanghai, China).

STATISTICAL ANALYSIS

Data obtained were analysed statistically by one way or two way ANOVA using the SPSS/PC computer program (version 20.0), based on the standard procedures outlined by Snedecor and Cochran (1994).

RESULTS AND DISCUSSION

The colour of the Marwari stallion's semen was graded as milky white or creamy white and the consistency of Marwari stallion's semen was variably thin in the present study. Similar observations were made by Arifiantini *et al.* (2013), Tejpal (2015), Soni (2016), Kumar (2017) and Kumar (2018). Any deviantion in colour of the ejaculate may indicate the contamination of semen i.e. presence of admixtures, such as blood, urine or purulent material (Brinsko *et al*., 2011).

The total ejaculate volume, gel volume and gel free semen volume recorded in the present study were 57.84±4.00ml (ranged between 39.25±3.59 to 79±10.29ml), 10.73±0.41ml (7.5±1.56 to 13.25±4.83ml) and 46.5±3.23ml (30.38±3.01 to 62.25±5.98 ml), respectively. In other previous records, semen volume was reported to vary from 20 to 160 ml (Pal and Legha, 2009) and 30 to 225 ml (Pal *et al*., 2009). Similar to Soni (2016), stallions were found to be significant source of variation for total semen volume in the present study. In the contrary, Kumar (2017) and Rabindra (2017) found non- significant variation for total semen volume among stallions. The gel volume of Marwari stallion in the present study was in resemblance

with the findings of Soni (2016), Kumar (2017), Rabindra (2017) and Kumar (2018). Non-significant difference was found in gel volume among the stallions. The values of gel free volume were in accordance with the previous studies by Soni (2016), Kumar (2017), Rabindra (2017) and Talluri *et al*. (2017). The individual variation in semen volume may be due to various factors viz., breed, age, season, teasing time, frequency of semen collection, work load etc. (Gamal *et al*., 2016).

The seminal pH of Marwari stallions in the present study ranged from 7.3±0.05 to 7.45±0.03 with an overall mean of 7.38±0.02. The observations in present study were in accordance with the previous observations made by Talluri *et al*. 2012, Tejpal (2015), Soni (2016) and Talluri *et al*. (2017) in Indian breed horses. Several factors e.g. season of year; frequency of ejaculation, sperm concentration can influence the pH of semen.

The range of sperm concentration in Marwari stallion recorded in the present study was 97.75±8.25 to 232.38±26.91 million/ml with an overall mean of 180.91±10.73 million/ml. Significant difference was found in sperm concentration among the stallions ($P<0.05$) in the present study similar to previous studies by Soni (2016), Kumar (2017) and Rabindra (2017).

In the present study, progressive sperm motility observed in freshly ejaculated semen of Marwari stallions using CASA ranged from 72.7±2.70 to 80.15±2.01% with an overall mean of 76.62±0.09%. Significant difference ($P<0.05$) was found among the stallions for the values**.** The results were consistent with the previous observations made by Ravi *et al.* (2013) who found progressive sperm motility in gel free stallion semen as 77.00±1.51%.

In the present study, the sperm viability of Marwari stallions ranged from 80.88±0.31 to 86.21±1.42% with an overall mean 84.07±0.39%. Significant difference ($P<0.05$) was found among the stallions for the values**.** Arifiantini *et al.* (2013) found 83.30±18.4% viable sperms in freshly ejaculated stallion semen, in accordence to the present study. Kumar *et al*. (2011), Talluri *et al*. (2012) and Tejpal (2015) observed slightly lower viability in freshly ejaculated stallion semen as 74.17±1.61%, 76.78±0.08% and 78.36±2.16%, respectively.

The mean C content in fresh spermatozoa of Marwari stallions ranged from 13.64±1.25 to 16.60±1.58 with an overall mean of 15.36±0.47µg/100 × 10^6 sperm cells. Non-significant difference was found among the stallions for the values similar to Pal *et al*. (2009) and Kumar (2018). The mean P content in fresh spermatozoa of Marwari stallions ranged from 43.27±2.65 to 49.74±4.54 with an overall mean of 46.21±1.27 µg/100×10^6 sperm cells. Non-significant difference was found among the stallions for the values. The mean C: P ratio in fresh spermatozoa of Marwari stallions ranged from 0.31±0.013 to 0.34±0.077 with an overall mean of 0.33±0.071. Non-significant difference was found among the stallions for the values too.

Sperm sensitivity to cold shock damage is determined by membrane P composition as well as the membrane C: P ratio (Holt, 2000) and sperm possessing high C: P ratio (rabbit and human sperm) are more resistant to the cold shock damage than sperm from boars, stallions, rams and bulls, which have low cholesterol: phospholipid ratios (Pamornsakda *et al*., 2011). There is an active participation of sperm plasma membrane in the process of capacitation, mainly through loss of cholesterol (Oliveira *et al*., 2010).

Initial Progressive Motility, Livability, C, P content and C: P Ratio of Spermatozoa at Prefreeze and Post-thaw Stages

The mean value of pre-freeze individual sperm motility of Marwari stallions spermatozoa was significantly ($P<0.05$) higher in Group T_3 (75.08±0.40%) as compared to other four groups. Similarly, at post-thaw stage significant difference was seen ($P<0.05$) in the progressive motility among the groups. Group T_3 (46.63±0.60%) showed highest post thaw progressive motility followed by group T_4, T_2, T_1 and T_0.

Increase in sperm motility was also observed in CLC treated cryopreserved spermatozoa in stallions (Cox *et al*., 2013; Crespilho *et al*., 2013; Murphy *et al*., 2014; Papa *et al*., 2014 and Moraes *et al*., 2015); in donkey (Oliveira *et al*., 2010; Cox *et al*., 2013 and Oliveira *et al*., 2014); in bull (Amorim *et al*., 2009; Amidi *et al*., 2010 and Yadav *et al*., 2017); in buffalo bull (Rajoria *et al*., 2014 and Lone *et al*. 2016); in ram (Moce *et al*., 2010; Motamedi-Mojdehi *et al*., 2014; Zahid *et al*., 2015, Ucan *et al*., 2016 and Salmon *et al*., 2017); in buck (Farshad *et al*., 2011; Behera *et al*., 2015; Salmon *et al*., 2016 and Souza *et al*., 2016); in boar (Tomas *et al*., 2014); in camel (Crichton *et al*.,

2015); in dog (Khan *et al*., 2017) and in chicken (Partyka *et al*., 2016).

Percent decrease in motility observed in post-thaw semen from fresh values was 48.26, 45.80, 44.19, 39.14 and 42.59%, respectively in C, T_1, T_2, T_3 and T_4 groups in stallions indicating lowest damage in group T_3.

The present study suggests that better pre-freeze and post–thaw motility of stallion spermatozoa was obtained when 2 mg CLC/120 million spermatozoa was added to the cells. When the level of CLC was increased beyond 2 mg, it significantly reduced sperm motility and hence, had detrimental effects. These observations were in agreement with other findings, which showed that freezing of sperm with a higher concentration of CLC decreased the rate of motility (Purdy and Graham, 2004a and Rajoria *et al*., 2016).

The mean per cent viable spermatozoa of Marwari stallions observed at pre-freeze stage showed significant difference ($P<0.05$) among different groups with higher values in Group T_3 (79.11±0.46%) as compared to other four groups. Similarly, at post-thaw stage there was significant difference ($P<0.05$) in the viability among the groups with Group T_3 (69.43±0.5%) showing the highest post thaw viability followed by group T_4, T_2, T_1 and C.

The present study suggests that increased viability of stallion's spermatozoa is obtained when 2 mg CLC/120 million spermatozoa were added to the cells. When the threshold level of CLC was increased beyond 2 mg it significantly ($P<0.05$) reduced the spermatozoa viability and hence had detrimental effects. These observations were in agreement with findings on buck spermatozoa (Farshad *et al*., 2011) and buffalo bull spermatozoa (Rajoria *et al*. 2016) which showed that freezing of sperm at a higher concentration of CLC decreased the viability and quality of spermatozoa after freezing and thawing.

The mean C, P contents (μg/100 × 10^6 spermatozoa) and C: P ratios of Group T_0, T_1, T_2, T_3 and T_4 at pre-freeze and post-thaw stages are given in table 1 and 2, respectively. The mean C content was significantly higher ($P<0.05$) in group T_3 (treated with 2.0 mg CLC/120 million spermatozoa) and significantly ($P<0.05$) lowest in group T_0 as compared to other groups at pre-freeze stage in stallions (Table 1). At post-thaw stage, it was also significantly ($P<0.05$) higher in treatment groups compared to control group (Table 2). An appreciable reduction in cholesterol content of stallion spermatozoa at pre-freeze and post-thaw stages in control group as compare of fresh stage might be due to cold shock and freeze thaw which leads to cholesterol efflux. Cholesterol efflux leads to changes in membrane architecture and fluidity that gives rise to the capacitation of the frozen sperm cells.

No significant difference was observed in the P content among different groups at the pre-freeze and post-thawed stages. Present study indicated that pre-freeze and post-thaw values of phospholipid content of spermatozoa were higher than the fresh spermatozoa. This may have happened because phospholipid present in egg yolk dilutor (contain 80% phospholipid) used in the study, incorporated into the sperm membrane thereby increasing the phospholipid content of spermatozoa. This result indicated active sperm lipid metabolism might be responsible for the increase in lipid content (Cerolini *et al.,* 2001).

Table 1: Initial Progressive Motility, Livability, C, P contents and C: P ratio in pre-freeze spermatozoa of Marwari stallions treated with different concentration of CLC

Groups	Initial Progressive Motility (%)	Livability (%)	C content	P content	C: P ratio
T_0	70.56^{A}± 0.32	74.20^{A}±0.36	13.27^{A}±0.42	56.77±1.45	0.24^{A}±0.002
T_1	71.60^{B}±0.31	75.38^{AB}±0.39	17.56^{B}±0.32	57.08±1.41	0.31^{B}±0.01
T_2	72.77^{C}± 0.32	76.27^{AB}± 0.37	24.16^{C}±0.35	56.98±1.39	0.43^{C}±0.01
T_3	75.08^{D}±0.40	79.11^{C}± 0.46	30.20^{D}±0.55	57.02±1.38	0.54^{D}±0.01
T_4	73.39^{C}±0.40	77.20^{C}± 0.39	22.56^{C}±0.38	57.03±1.37	0.40^{C}±0.01

Note: Mean values with different superscripts between treatment groups differ significantly ($P<0.05$). (Mean ± SE)

Group T_0, T_1, T_2, T_3, T_4 contain 0, 1, 1.5, 2 and 3 mg CLC/120×10^6 sperm, respectively.

Table 2: Initial Progressive Motility, Livability, C, P contents and C: P ratio in Post-thawed Spermatozoa of Marwari Stallions treated with different concentration of CLC

Groups	Initial Progressive Motility (%)	Livability (%)	C content	P content	C: P ratio
T_0	39.64^{A}±0.37	63.62^{A}±0.52	9.17^{A}±0.16	55.23±0.95	0.17^{A}±0.002
T_1	41.53^{B}±0.36	64.96^{AB}±0.46	11.77^{B}±0.18	55.42±0.91	0.22^{B}±0.002
T_2	42.76^{C}±0.43	66.53^{ABC}±0.43	17.89^{D}±0.20	55.66±0.91	0.34^{D}±0.003
T_3	46.63^{E}±0.60	69.43^{C}±0.50	23.70^{E}±0.20	55.94±0.89	0.43^{E}±0.005
T_4	43.99^{D}±0.63	67.84^{BC}±0.52	15.96^{C}±0.30	55.82±0.90	0.29^{C}±0.003

Note: Mean values with different superscripts between treatment groups differ significantly (P<0.05). (Mean ± SE).

Group T_0, T_1, T_2, T_3, T_4 contain 0, 1, 1.5, 2 and 3 mg CLC/120×10^6 sperm, respectively.

The C: P ratio of spermatozoa differed significantly (P<0.05) among all the groups at pre-freeze and post-thaw stages in the stallions (Table 1 & 2). Group T_3 showed significantly (P<0.05) higher C: P ratio in spermatozoa as compared to all the other four groups at pre-freeze and post-thaw stages in the stallions.

Treatment with CLC in Group T_3 increased C content by 2.28 times at pre-freeze and 2.58 times more at post-thaw stage. However, P content increased by 1.23 times at pre-freeze and 1.21 times at post-thaw stage in comparison to a fresh stage. The results indicated that cholesterol content in spermatozoa increases as the amount of CLC increases in the media. Similar findings for cholesterol content of spermatozoa treated with different amount of CLC were reported by Purdy and Graham (2004b) in bull; Kumar (2012) and Rajoria *et al*. (2014) in buffalo bull; Moore *et al.* (2005) in stallion; Moce *et al.* (2010) and Salmon *et al*. (2017) in ram; Salmon *et al*. (2016) in buck and Kiso *et al*. (2012) in elephant. There was a linear relationship between cholesterol incorporation in the membrane and amount of CLC added to the media (Purdy and Graham, 2004b). The amount of cholesterol that incorporated into the membranes of the sperm cells increased in polynomial fashion and incorporated into all sperm membranes in stallion (Moore *et al.,* 2005 and Pamornsakda *et al.,* 2011).When 1.5 mg or more CLC was added, the amount of cholesterol in the sperm was higher than control sperm in bull and stallion (Purdy and Graham, 2004a and Moore *et al.,* 2005).

The percentage change in C: P ratio from fresh stage to post thaw stage in the Marwari stallions was 48.48, 33.33, 3.03, 30.30 and 12.12%, respectively, in T_0, T_1, T_2, T_3 and T_4 group in the present study (Fig. 1). Comparing the C: P ratio at fresh stage and post-thaw stage between group T_0 and T_3; in the control group a reduction in C: P of about 48.48% was noticed in comparison to about 30.30% increase in group T_3 (treated with 2 mg CLC/120 × 10^6 spermatozoa). This clearly indicates that CLC treatment may maintain the C: P ratio unlike fresh stage and play important role in maintaining membrane architecture of spermatozoa. Hence, addition of CLC may helpful in increasing freezability of stallion spermatozoa by increasing the C: P ratio of spermatozoa.

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CONCLUSION

Addition of CLC improved the cryosurvival rate of stallion spermatozoa. CLC at the dose rate of 2 mg /120 million spermatozoa had the maximum beneficial effect on stallion spermatozoa cryosurvivability.

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