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Protective Efficacy of Cholesterol-Loaded Cyclodextrin (CLC) Against Sperm Morphological Abnormalities in Marwari Stallions

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ABSTRACT

The present study aimed to examine the protective potential of cholesterol loaded cyclodextrin (CLC) at varying concentrations *against sperm morphological abnormalities of* cooled and frozen thawed semen of Marwari stallions. A total of forty eight ejaculates (six stallions and eight ejaculates per stallion) were promptly processed following semen collection and subjected to gross assessment of semen quality attributes. The assessment of sperm morphologies in fresh equine semen was conducted using Eosin and Nigrosine staining technique. The semen samples from individual Marwari stallions were evenly divided into five fractions and incubated in a water bath at 37°C for 15 min after dilution with primary extender to obtain the spermatozoa concentration at 120×10^6 sperm/ml. During incubation, the fractions of every semen sample were exposed to different concentrations of CLC as 0, 1.0, 1.5, 2.0 and 3.0 mg/ml of extender (C, T_1, T_2, T_3, and T_4, respectively). All the 5 aliquots were cooled and subsequently subjected to cryopreservation. Each cooled and frozen- thawed (at 37°C for 30 sec) semen samples were analyzed to assess the morphologies of the spermatozoa. Based on the findings, it was concluded that none of the CLC treatment could render any significant protection against the sperm morphological abnormalities at either pre-freeze and/or post-thaw stage of semen samples from Marwari stallions.

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Introduction

The Marwari breed (*Equus ferus caballus*) is a majestic breed of horse from the Marwar region of Rajasthan, India. Artificial insemination (AI) has proved its potential for the propagation and conservation of animals using frozen semen worldwide. The utilization of AI has witnessed a substantial surge in its application within horse breeding over the past few decades (Moran et al., 1992). Nevertheless, certain studbooks have imposed restrictions on the registration of foals produced by AI with frozen semen, especially in the thoroughbreds (Allen, 2005). The primary constraints in the production of frozen semen from stallions encompass seasonal factors, breed variations, and individual variations within a particular breed in terms of semen quality and the ability to undergo successful cryopreservation. A notable decline in mare fertility with less than 30% conception rate per cycle is observed while using cryopreserved semen. This decrease in fertility is particularly pronounced when the semen originates from sources characterized as "bad freezers" (Allen, 2005). There needs to be a universally accepted protocol for the cryopreservation of equine spermatozoa (Sieme et al., 2004). Hence, it is crucial to ascertain the most effective techniques for optimizing the production of frozen semen through appropriate processing for cryopreservation and implementing suitable seasonal management practices. This will ensure the prolonged preservation of the fertilizing potential of a stallion's sperm.

During cryopreservation, the decrease in temperature causes alteration in sperm membrane structure of the fluid state to the gel state, which leads to lipid loss and rearrangements of lipids/ protein within the membrane. Mainly, phospholipids composition and cholesterol/phospholipids ratio in the sperm membrane is affected, which makes it sensitive to temperature reduction. This in turn leads to compromised membranes, which becomes permeable to water and ions, ultimately leading to abnormal motility and spermatozoa die prematurely. Including cholesterol in the semen extender exerts control over the structural integrity of sperm membranes by interacting with the hydrocarbon chains of phospholipids. This interaction renders more stability to the membrane at temperatures below the phase transition point (Darin-Bennett and White, 1977; Quinn, 1989). Few studies have reported that semen quality can be improved by minimizing sperm cryoinjury during cooling and freezing process which may be done by the addition of cholesterol in the semen extender (Navratil et al., 2003; Belmonte et al., 2005; Moore et al., 2005).

Cyclodextrins represent a group of cyclic oligosaccharides derived from starch through enzymatic breakdown. These molecules exhibit an outer hydrophilic surface and an inner hydrophobic core, enabling the encapsulation of hydrophobic substances, such as cholesterol. Furthermore, when cyclodextrins are preloaded with cholesterol, they are capable of incorporating cholesterol into cellular membranes (Navratil et al., 2003). Cholesterol-loaded cyclodextrins (CLCs) have been used effectively to add cholesterol to sperm membrane, before cryopreservation of semen. Effect of CLCs has been investigated in different species, including Stallions (Moore et al., 2005; Amorim et al., 2009; Cox et al., 2013; Hartwig et al., 2014; Moraes et al., 2015), Donkey (Alvarez et al., 2005, 2006; Oliveira et al., 2014; Kumar et al., 2020), Bull (Purdy and Graham, 2004; Moce and Graham, 2006; Boscarato et al., 2016; Yadav et al., 2017), Buffalo bull (Lone et al., 2016, Rajoria et al., 2016), Ram (Moce et al., 2010; Zahid et al., 2015; Ucan et al., 2016), buck (Konyali et al., 2013; Behera et al. 2015; Salmon et al., 2016), Boar (Blanch et al. 2012; Tomas et al. 2014; Lee et al., 2015), Camel (Crichton et al., 2015), Chicken (Partyka et al., 2016) and Dog (Kiso et al. 2012; Khan et al., 2017; Inanc et al. 2018). Considering the context as mentioned earlier, the present study aimed to investigate the beneficial impact of CLC in semen extenders on reducing sperm abnormalities and thereby improving semen quality in Marwari stallions.

Materials and Methods

Experimental animals- Six healthy Marwari stallions between 4 to 7 years of age, maintained in iso-managerial conditions at Equine Production Campus, ICAR-National Research Centre on Equines, Bikaner were used in the study.

Research materials- The following chemicals employed in the study are provided along with the respective sources from where they were acquired: Cholesterol (Hi-Media), Eosin Y (Sigma-Aldrich), Methyl-β-cyclodextrin (Sigma-Aldrich), Nigrosine (Hi-Media), and the rest of all others were procured from established standard commercial suppliers.

CLC preparation- Cyclodextrins were prepared as per the procedure described by (Purdy and Graham, 2004). At the outset, 200 mg of cholesterol was taken in a glass tube and dissolved in 1 ml of chloroform. Simultaneously, 1 gm of methyl-β-cyclodextrin was dissolved in another glass tube containing 2 ml of methanol. Subsequently, a 0.45 ml aliquot of cholesterol solution was decanted into the cyclodextrin solution, followed by thorough stirring until the resultant solution achieved visual clarity. This solution was transferred to a glass petri dish, and the solvents were evaporated using nitrogen gas vapors. The resulting crys-

tals thus obtained were left to dry further for an additional period of 24 h. These crystals were subsequently extracted from the dish and stored at room temperature in a glass container. To prepare a CLC stock solution, 50 mg of the CLC was mixed with 1 ml of primary extender at 37°C, and vortexed for 30 sec. As required on the days of semen collection, the working solutions with diverse concentrations of CLCs were freshly prepared by diluting the stock solution with the primary extender.

Semen collection and processing- Procedures for semen collection and processing including cryopreservation were followed as described by Talluri et al. (2016). Semen was collected twice a week in the early morning hours as per the standard method using an artificial vagina of Colorado model. A total of forty-eight ejaculates were collected from six Marwari stallions, where each stallion contributed eight ejaculates. Following every collection, the semen sample immediately underwent macroscopic or gross evaluation to assess its color and consistency. The semen was filtered into a warm, graduated measuring bottle to get gel free semen. Different volumes of total semen, gel and gel free semen were noted down. Other evaluations of fresh semen like pH using a digital pH meter (ERMA INC, Japan) and sperm concentration (Neubars chamber) were performed as previously described by Kumar et al. (2019a). Following the fresh stage evaluation, the semen sample was divided into five equal fractions: C, T1, T2, T3, and T4.

Each of the five aliquots were diluted with a primary extender having varying concentrations of CLC as 0, 1, 1.5, 2, and 3 mg/ml CLC, respectively in C, T1, T2, T3, and T4 fractions. These diluted semen fractions having a final spermatozoa concentration of 120×10^6 sperm/ml, were subjected to 15-minute incubation in a water bath at 37°C. The aliquots in 50 ml centrifuge tubes were centrifuged at 300 X g for 5 min at 10°C to obtain a sperm pellet. The supernatant, consisting of the seminal plasma, was separated and removed. Each sperm pellet was subsequently diluted with a secondary semen extender to achieve a final concentration of 150×10^6 sperm/ml. The extended semen samples (C, T1, T2, T3, and T4) were filled into 0.5 ml French medium straws using an automatic filling and sealing machine. The filled straws were placed in a cooling cabinet for 2 hours of equilibration at 4°C. A Styrofoam box fixed with wired net at 5 inches above the bottom was filled with liquid nitrogen up to a depth of two inches. Next to equilibration, the straws were positioned horizontally on this wired net (3 inches above the level of liquid nitrogen) and kept submerged in the nitrogen fumes for 10-12 min before being fully plunged into liquid nitrogen. After 24 h of storage, straws from each group were thawed at 37°C for 30 sec for post-thaw assessment.

Evaluation of sperm morphology

Sperm morphology was evaluated in fresh semen, pre-freeze, and frozen-thawed semen through making Eosin-Nigrosin stained smears of the semen sample and observed microscopically under 1000X magnification with oil immersion (Nikon Instech Co. Ltd., Kanagawa, Japan).

The following steps were followed to prepare Eosin-Nigrosin stain for the assessment of live-dead sperm count and sperm morphology:

1. A 3% sodium citrate solution was prepared by dissolving 3 gm of sodium citrate (dehydrate) in 100 ml distilled water.
2. Eosin B (1 gm) and Nigrosin (5 gm) stain powders were taken and dissolved in 100 ml of the 3% sodium citrate (dehydrate) solution.
3. The pH of this staining solution was adjusted to 7.0 by pouring some drops of 0.1 M NaH_2PO_4 and then the mixture was filtered. This staining solution was stored at room temperature for further use.

The semen smear was prepared by placing a drop of semen on clean, grease free glass slide corner which was added with a drop of Eosin-Nigrosin stain and mixed well by agitating the slide and then the stained semen drop was spread with another glass slide. At least 100 spermatozoa were observed from each sample for assessing various sperm abnormalities like detached head, abnormal head, abnormal mid piece, bent or coiled tail, proximal or distal cytoplasmic droplet etc.

Statistical analysis

The recorded data were subjected to statistical analysis using the SPSS/PC computer software (version 20.0) following the standard procedures outlined by Snedecor and Cochran (1994). Analysis of variance (ANOVA) or F-test was employed for the statistical analysis. The significant difference ($P < 0.05$) was observed among the stallions for the total sperm abnormalities and results are presented as mean±SE.

Results

Sperm morphological abnormalities in fresh semen

The sperm morphological abnormality was evaluated using eosin-Nigrosine stain under microscope and indicated in Fig. 1. The mean percentage of spermatozoa with abnormal head in fresh semen of each Marwari stallion

was 1.61±0.12, 1.66±0.12, 2.09±0.09, 1.77±0.13, 1.65±0.11 and 2±0.07%, respectively, whereas the overall mean percentage of sperm abnormalities was 1.8±0.08%. Significant individual variations (P<0.05) were observed among stallions for the percentage of head abnormalities in their spermatozoa. Similarly, the mean percentages of spermatozoa with the abnormal mid piece in fresh semen of different stallions also differed significantly (P<0.05) and recorded as 2.25±0.09, 2.83±0.06, 3.14±0.12, 2.91±0.08, 2.22±0.09 and 3.04±0.09%, respectively, with an overall mean as 2.73±0.09%. The sperm tail abnormalities in fresh semen of individual Marwari stallions also varied significantly (P<0.05) and found to be 2.33±0.2, 2.63±0.1, 3.22±0.13, 2.78±0.13, 2.16±0.07 and 2.9±0.16%, respectively, with an overall mean as 2.67±0.1%. The mean percentage of total sperm abnormalities in fresh semen of individual stallion was 6.19±0.34, 7.12±0.13, 8.44±0.21, 7.46±0.15, 6.03±0.13 and 7.93±0.19%, respectively, with an overall mean as 7.19±0.15% (Table 1).

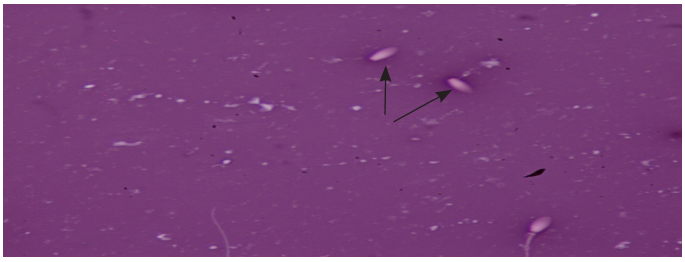

Fig. 1. Microphotograph showing sperm morphological abnormality (Decapitated head) (Eosine-Nigrosine, 1000X)

Table 1. Percentage of morphological abnormalities (Mean±SE) in sperm head, mid-piece, tail, and total sperm abnormalities in fresh semen of Marwari stallions

Marwari stallions	Head (%)	Mid-piece (%)	Tail (%)	Total (%)
Overall	1.80±0.08	2.73±0.09	2.67±0.10	7.19±0.15

Sperm morphological abnormalities in cooled semen

The mean percentage of sperm abnormalities in head, mid piece, and tail in the semen of Marwari stallions exposed to varying concentrations of CLC during cryopreservation represented in Table 2. The overall mean percentage for abnormal head were 2.24±0.07, 2.01±0.07, 2.06±0.07, 2.02±0.06, and 1.99±0.07 % in the C, T1, T2, T3, and T4 groups, respectively. No significant differences were found between the groups. Similarly, the mean percentages of sperm with abnormal mid piece were 3.02±0.08, 2.92±0.08, 3.01±0.08, 3.03±0.08, and 3.31±0.07 % in the respective groups, with no significant differences between them. For abnormal tail, the mean percentage was 3.00±0.06, 3.24±0.07, 3.03±0.05, 3.08±0.05, and 3.08±0.06 % in the C, T1, T2, T3, and T4 groups, respectively. Again, there were no significant differences among the groups. The overall mean values for all abnormalities combined were 8.28±0.16%, 8.10±0.15%, 8.02±0.14%, 8.08±0.12%, and 8.25±0.13% of the respective groups, with no significant differences (Table 2).

Groups C, T1, T2, T3, and T4 were exposed with CLC at concentrations of 0, 1, 1.5, 2, and 3 mg/mL, respectively, in the semen samples.

Sperm morphological abnormalities in post thaw semen

The mean values of sperm abnormalities in the head region of the semen samples from individual Marwari stallion subjected to different concentrations of CLC treatments is presented in Table 3. The overall mean percentages were 2.64±0.07, 2.7±0.07, 2.75±0.07, 2.67±0.07, and 2.66±0.07 % of group C, T1, T2, T3, and T4, respectively. No significant differences were found among the groups. Similarly, the mean values of sperm abnormalities in the mid-piece region were examined individually for semen ejaculates from Marwari stallions exposed to various concentrations of CLC (Table 3). The overall mean percentages were 4.98±0.09, 4.9±0.08, 4.93±0.07, 4.83±0.06, and 4.91±0.08 % in the group C, T1, T2, T3, and T4, respectively. No significant difference was observed between the groups. Furthermore, the mean values of sperm abnormalities in the tail region were analysed for each semen sample of Marwari stallions exposed to different CLC concentrations (Table 3). The overall mean percentages were 5.18±0.08, 5.05±0.05, 4.99±0.07, 4.91±0.06, and 5.03±0.07 % in the group C, T1, T2, T3, and T4, respectively, with non-significant differences between the groups.

Additionally, the mean values of total sperm abnormalities were evaluated for each semen sample from Marwari stallions subjected to different CLC treatment concentrations (Table 3). The overall mean percentages are 12.79±0.09, 12.64±0.11, 12.65±0.08, 12.4±0.08, and 12.59±0.1% of the respective groups. No significant differences were observed among the groups.

Table 4 displays the percentage changes in sperm abnormalities from the fresh stage to the post-thaw stages, with the minimum changes observed in the T3 group, followed by T4, T1, T2, and the control group.

Table 2. Percentage of morphological abnormalities (Mean±SE) in sperm head, mid-piece, and tail regions in the pre-freeze semen samples of Marwari stallions.

Marwari stallion	**Head (%)**					**Mid-piece (%)**					**Tail (%)**				
Groups	**C**	**T1**	**T2**	**T3**	**T4**	**C**	**T1**	**T2**	**T3**	**T4**	**C**	**T1**	**T2**	**T3**	**T4**
	2.24± 0.07	2.01± 0.07	2.06± 0.07	2.02± 0.06	1.99± 0.07	3.02± 0.08	2.92± 0.08	3.01± 0.08	3.03± 0.08	3.31± 0.07	3± 0.06	3.24± 0.07	3.03± 0.05	3.08± 0.05	3.08± 0.06

Table 3. Percentage of morphological abnormalities (Mean±SE) of sperm in the head, mid-piece, and tail regions in post-thaw semen samples of Marwari Stallions underwent various CLC treatments during cryopreservation

Marwari stallion→		**Mohit**	**Karan**	**Ritik**	**Roshan**	**Sultan**	**Kazzak**	**Overall**
Groups								
C	Head (%)	2.41±0.09	2.46±0.09	3.52±0.10	2.5±0.09	2.51±0.10	2.50±0.09	2.64±0.07
	Mid-piece (%)	4.94±0.17	5.07±0.17	4.23±0.19	5.16±0.16	5.04±0.18	5.41±0.19	4.98±0.09
	Tail (%)	4.87±0.15	5.17±0.20	5.23^{c}±0.20	5.19±0.21	5.15±0.20	5.44±0.21	5.18±0.08
	Total (%)	12.22±0.22	12.71±0.18	12.98±0.18	12.85±0.19	12.59±0.19	13.35±0.11	12.79±0.09
T1	Head (%)	2.42±0.13	2.51±0.11	3.62±0.08	2.58±0.09	2.44±0.13	2.58±0.09	2.7±0.07
	Mid-piece (%)	4.92±0.18	5.04±0.13	4.09±0.13	5.06±0.14	4.95±0.17	5.31±0.16	4.9±0.08
	Tail (%)	4.78±0.15	5.06±0.08	5.08^{bc}±0.09	5.08±0.09	4.93±0.15	5.33±0.14	5.05±0.05
	Total (%)	12.13±0.26	12.61±0.21	12.78±0.23	12.73±0.25	12.32±0.30	13.23±0.18	12.64±0.11
T2	Head (%)	2.36±0.09	2.57±0.08	3.65±0.07	2.64±0.08	2.51±0.10	2.72±0.11	2.75±0.07
	Mid-piece (%)	4.90±0.16	5.01±0.18	4.42±0.17	5.06±0.17	4.96±0.16	5.18±0.16	4.93±0.07
	Tail (%)	4.92±0.14	5.04±0.16	4.68^{abc}±0.28	5.04±0.16	5.04±0.16	5.2±0.13	4.99±0.07
	Total (%)	12.18±0.19	12.63±0.20	12.75±0.16	12.74±0.18	12.51±0.25	13.1±0.16	12.65±0.08
T3	Head (%)	2.42±0.08	2.41±0.08	3.53±0.05	2.53±0.05	2.41±0.08	2.69±0.12	2.67±0.07
	Mid-piece (%)	4.77±0.15	4.86±0.15	4.49±0.14	4.86±0.15	4.86±0.15	5.11±0.14	4.83±0.06
	Tail (%)	4.87±0.15	4.97±0.11	4.48^{a}±0.13	4.97±0.11	4.91±0.15	5.22±0.17	4.91±0.06
	Total (%)	12.07±0.17	12.25±0.12	12.49±0.13	12.36±0.09	12.18±0.16	13.02±0.28	12.4±0.08
T4	Head (%)	2.37±0.07	2.52±0.07	3.50±0.06	2.5±0.06	2.45±0.08	2.62±0.15	2.66±0.07
	Mid-piece (%)	4.80±0.18	4.98±0.22	4.55±0.16	4.95±0.20	4.85±0.17	5.32±0.14	4.91±0.08
	Tail (%)	4.95±0.15	5.16±0.13	4.63^{ab}±0.21	5.13±0.15	5.03±0.12	5.25±0.17	5.03±0.07
	Total (%)	12.12±0.24	12.66±0.25	12.67±0.18	12.57±0.23	12.33±0.24	13.2±0.23	12.59±0.10

Note: Mean values with distinct superscripts exhibit statistically significant differences ($P<0.05$) among treatment groups, where Group C, T_1, T_2, T_3, T_4 were exposed with CLC at concentrations of 0, 1, 1.5, 2, and 3 mg/mL, respectively, in the semen samples during cryopreservation.

Table 4. Comparative effects of various CLC treatments on physico-morphological seminal attributes in fresh, pre-freeze, and post-thaw stages of Marwari stallions (Mean±SE)

Semen State	**Total sperm abnormalities (%)**				
	C	**T_1**	**T_2**	**T_3**	**T_4**
Fresh	7.19±0.15	7.19±0.15	7.19±0.15	7.19±0.15	7.19±0.15
Pre-freeze	8.28±0.16	8.1±0.15	8.02±0.14	8.08±0.12	8.25±0.13
Post-Thaw	12.79±0.09	12.64±0.11	12.65±0.08	12.4±0.08	12.59±0.1
% Changes from fresh to post-thaw	77.88%	75.79%	75.93%	72.46%	75.1%

Discussion

Stallions exhibiting high fertility often demonstrate a percentage of normal sperm exceeding 60% while maintaining a low occurrence of abnormalities (<5%), specifically in the acrosome and mid-piece regions (Samper et al., 2007). Morphological defects especially head and tail defects, which hinder the progressive motility of spermatozoa, can impede their ability to reach and fertilize the oocyte (Pereira et al., 2017). The process of cryopreservation exposes spermatozoa to both intracellular and extracellular stresses, which can lead to damage of their plasma membranes and subsequent cell death. Loss of membrane integrity occurs when the sperm plasmalemma undergoes a transition from a liquid phase to a gel state. Spermatozoa in certain species like stallion, bull and ram, which possess lower cholesterol to phospholipid ratio in their semen, are more vulnerable to cold shock (Darin-Bennett and White, 1977). Cholesterol plays a crucial role in regulating membrane fluidity, which becomes particularly important during the cryopreservation of sperm cells, as destabilization of the plasmalemma can lead to intracellular ice formation and cell death. Previous studies have indicated that the incubation of sperm cells with CLC prior to cryopreservation results in a higher post-thaw percentage of normal and motile spermatozoa compared to control groups (Purdy and Graham, 2004). In the present study, though the sperm morphological abnormalities reduced in the treatment groups compared to control but there was no significant difference among the groups of pre-freeze and post-thaw semen samples from Marwari stallions.

The research findings of our study align with previous studies conducted by Inanc et al. (2018) and Kumar et al. (2019b), where they observed no significant differences in the percentage of abnormal spermatozoa when ram and Poitou jack semen were treated with CLC. Conversely, Alvarez et al. (2006) observed a significant decrease ($P < 0.05$) in the percentage of abnormalities in stallion spermatozoa, while Rajoria et al. (2016) reported a noteworthy reduction in morphological abnormalities in buffalo bull spermatozoa treated with CLC compared to control groups. These findings are consistent with other investigations on frozen-thawed semen in horses (Zahn et al., 2002; Moore et al., 2005) and cattle (Moce and Graham, 2006; Gemeda et al., 2022). Besides, few other studies have also demonstrated an improvement in sperm quality in terms of reduced morphological abnormalities in frozen-thawed equine (Combes et al., 2000; Alvarez et al., 2006) and bovine (Purdy and Graham, 2004) semen.

In our study, the methodology for incorporating cholesterol was adopted following the protocols used for bulls (Purdy and Graham, 2004) and horses (Moore et al., 2005) semen. Discrepancies observed between our study and previous investigations in cattle (Purdy and Graham, 2004) may be attributed to inherent species variations in terms of size, shape, lipid composition (Parks et al., 1981; Parks and Lynch, 1992), and the permeability characteristics of sperm membranes (Guthrie et al., 2002).

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Conflict of interest

None of the authors have any conflict of interest to declare.

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