Use of cholesterol-loaded cyclodextrin: An alternative for bad cooler stallions

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ARTICLE INFO

Article history:
Received 1 August 2013
Received in revised form 3 September 2013
Accepted 1 October 2013

Keywords:
Equine semen
Cooling
Cholesterol
Fertility

ABSTRACT

During the cooling process, sperm may suffer irreversible damage that compromises the fertility rate. Incorporating cholesterol-loaded cyclodextrin (CLC) represents a strategy to increase sperm resistance at low temperatures; however, high levels of cholesterol in the cell membrane can interfere with sperm capacitation. The goals of this study were to determine the CLC concentration and cooling temperature that produce optimal kinetic parameters and viability of sperm from stallions identified as bad coolers (BCs) and good coolers (GCs), as well as the effect of adding CLC on the occurrence of the acrosome reaction (ACR) and on the fertility rate of cooled sperm. In experiment 1, each ejaculate was divided into four groups: Control and treated with 1 (CLC-1), 1.5 (CLC-1.5), or 2 mg (CLC-2) of CLC/120 × 10^6 sperm and cooled for 48 hours at 5 °C. In experiment 2, each ejaculate was divided into four groups: Control and CLC-1.5 cooled at 15 °C or 5 °C for 24 hours. For experiment 3, GC and BC stallions were used, and the ejaculates were divided into control and CLC-1.5 cooled at 5 °C for 48 hours. According to experiment, the sperm kinetics (SK) and plasma membrane integrity (PMI) were analyzed before and after 24 and 48 hours of cooling. In experiment 4, the ejaculates were divided into four groups: Control and CLC-1.5 maintained at room temperature or cooled at 5 °C for 24 hours. Each group was incubated with ionophore calcium at 37 °C for 3 hours. The incidence of ACR was analyzed before and after 1, 2, and 3 hours of incubation. For experiment 5, two cycles of 10 mares for a GC stallion and two cycles of 25 for a BC stallion were used. The inseminations were performed with control and CLC-1.5 groups cooled at 5 °C for 24 hours. According to results, all groups treated with CLC exhibited higher PMI compared with controls, and CLC-1.5 and CLC-2 exhibited the best SK results. The cooling temperature of 5 °C was superior to 15 °C when the sperm was treated with CLC. The GC and BC stallions benefited from the CLC-1.5 treatment, but the BCs were more evident, which presented greatly increased PMI and SK. There was a delay in capacitation of at least 3 hours for the fresh sperm and at least 1 hour for cooled sperm supplemented with CLC-1.5. After adding CLC-1.5, the fertility of BC stallion significantly increased, but that of the GC was not altered. Thus, incorporating CLC is an effective technique to cool equine semen, although it is indicated mainly for BC stallions.

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1. Introduction

Artificial insemination with cooled semen is a key biotechnique for current equine reproductive programs. With
cooling at 5 °C, it is possible to reduce cell metabolism and increase the viability time of the sperm after ejaculation, allowing inseminations to be performed after 24 to 48 hours of storage [1]. However, between 8 °C and 19 °C, the sperm suffers a phase transition of the phospholipids in the cell membrane, which go from a liquid to a gel state [2]. This change causes a decrease in membrane fluidity and a higher predisposition to ruptures, as well as alterations of the original lipid and protein organization, which compromises cell functioning [3–5].

The cholesterol/phospholipid ratio of the plasma membrane is the main factor for maintaining membrane fluidity and stability during cooling [6,7]. High concentrations of cholesterol lead to a reduction of the temperature at which this phase transition occurs, maintaining the fluid state at lower temperatures and consequently reducing the damage to the plasma membrane structure [3]. Sperm from species that have high cholesterol content in the membrane, such as rabbits and dogs, display a greater resistance to the cryopreservation process [6]. Conversely, equines have only 36% cholesterol in the plasma membrane and are considered more susceptible to cryodamage [4]. In addition, there are differences in the lipid composition of the membrane between individuals from the same species, which may explain the variations in sperm resistance to the cooling and freezing processes. Thus, some stallions are classified as bad coolers (BC), because they exhibit a sudden drop in fertility after processing, cooling, and transporting of semen, a situation that limits their use in reproduction and causes significant economic losses [8,9].

With the goal of increasing sperm resistance at low temperatures, some researchers have increased the cholesterol content of the equine sperm cell membrane before the cooling process by adding cholesterol-loaded cyclodextrin (CLC) [10–13]. Cyclodextrin is an oligosaccharide with a hydrophilic outer surface and a hydrophobic inner surface, so it can dissolve, carry, and increase the solubility of hydrophobic compounds, such as cholesterol, in aqueous solutions [14].

Despite the powerful cryoprotective action of CLC for maintaining sperm quality, high levels of cholesterol in the membrane interfere with the physiological process of sperm capacitation and acrosome reaction (ACR) [10,15]. Additionally, fertility tests performed to date with frozen semen demonstrate that there is no benefit or even a negative effect of using CLC [10,16]. In contrast with the freezing process, where the optimal CLC concentration seems to be established, a great variation of CLC doses are still used in the cooling of equine semen [12,13,17].

The goals of this study were to determine the CLC concentration and cooling temperature that lead to the optimal kinetic parameters and viability of sperm from stallions identified as BC and good coolers (GCs), as well as the effect of adding CLC on the occurrence of ACR and the fertility rate of cooled sperm.

2. Material and methods

All reagents used in this study were purchased from Sigma-Aldrich, St. Louis, MO.

2.1. Preparation of CLC

The CLC used in this experiment was prepared as described by Purdy and Graham [18]; first adding 1 g of m-cyclodextrin to 2 mL of methanol, and 200 mg of cholesterol was added to 1 mL chloroform. The chloroform/cholesterol solution (450 μL) was then added to the m-cyclodextrin/methanol solution and mixed until a clear solution was obtained. The solvents were removed by incubation at 50 °C for 24 hours and the remaining crystals were stored at room temperature. Before use 50 mg of CLC solution was added to 1 mL of tyrosine albumin lactate pyruvate media, and incubated in a water bath at 37 °C until use.

2.2. Animals and sample processing

The experiment was conducted at the School of Veterinary Medicine and Animal Science of the São Paulo State University of Botucatu city (FMVZ-UNESP/Botucatu), located in São Paulo state at 22°53′09″ south latitude and 48°26′42″ west longitude. The work was carried out during the breeding season, from between October 2011 to April 2012. In this study, 30 stallions of the Mangalarga Marchador, Quarter Horse, and Brazilian Jumping Horses breeds and 35 cross-bred mares with ages ranging from 4 to 18 years were used. The stallions were located at Department of Animal Reproduction and Veterinary Radiology, FMVZ, UNESP/Botucatu, Ogar (Private stud farm, Lins, SP, Brazil), Itapuá (Private stud farm, Arandú, SP, Brazil) and LUB (Private breeding center, Cesário Lange, SP, Brazil), remained stalled, fed with hay and concentrate, and given water ad libitum. The mares were at the FMVZ-UNESP/Botucatu and fed similar to the stallions, but were kept loose in paddocks.

After collecting the ejaculate with an artificial vagina, the semen was filtered, diluted at a 1:1 ratio, and evaluated for sperm motility and concentration in a Neubauer chamber. Immediately after the initial evaluations, the samples were adjusted to a concentration of 50 × 10⁶ sperm/mL with a Botu-Sêmen diluent (Botupharma, Botucatu, SP, Brazil).

2.3. Sperm analyses

2.3.1. Sperm kinetics

Five fields per sample were selected for the evaluation of sperm kinetic (SK) by CASA (HTM-IVOS 12, Hamilton Thorne Research, Beverly, MA) were evaluated the parameters of total motility (TM [%]), progressive motility (PM [%]), average path velocity (VAP [μm/s]), straight-line velocity (μm/s), curvilinear velocity (μm/s), and percentage of rapid sperm (RAP [%]). The CASA setup used in this experiment is described in Table 1.

2.3.2. Plasma membrane integrity

The plasma membrane integrity (PMI [%]) was evaluated by epifluorescence microscopy (Leica Microsystems, DMLB, Germany) based on the association of the fluorescent probes propidium iodide (IP) and 6-carboxyfluorescein diacetate [19]. We considered carboxyfluorescein diacetate–positive and IP-negative cells intact.
2.3.3. ACR rate

The ACR (%) was evaluated by flow cytometry, through the association of the fluorescent probes Hoechst 33342, IP, and fluorescein isothiocyanate-conjugated *Pisum sativum*, as described by Freitas-Dell’Aqua [20]. Fluorescein isothiocyanate-conjugated *Pisum sativum*–positive cells (regardless of IP staining) were considered to have undergone acrosomal reaction. For this purpose, we used the BD LSR Fortessa (Becton Dickinson, Mountain View, CA) device equipped with the following lasers: Blue, 488 nm, 100 mW; red, 640 nm, 40 mW; and purple, 405 nm. The data were evaluated by the software BD FACSDiva, v6.1 (Becton Dickinson).

2.3.4. Experiment 1: Evaluation of different CLC concentrations for cooling equine spermatozoa

Two ejaculates of 24 stallions were used. After collection, the semen was processed as described in section 2.2 and divided into four aliquots as described below:

- Control: Botu-Sêmen
- CLC-1: Botu-Sêmen + 1 mg CLC/120 × 10^6 sperm
- CLC-1.5: Botu-Sêmen + 1.5 mg CLC/120 × 10^6 sperm
- CLC-2: Botu-Sêmen + 2 mg CLC/120 × 10^6 sperm

Before being cooled at 5 °C, the samples were incubated for 15 minutes at room temperature (22 °C), and the sperm parameters were evaluated according to sections 2.3.1 and 2.3.2 at different times: Up to 2 hours after dilution with Botu-Sêmen (T0) and after 24 (T24) and 48 hours (T48) of cooling at 5 °C. By the time T24 samples remained in passive cooling system for transporting equine semen Botu-Flex (Botupharma) model, after which the samples were transferred to a commercial refrigerator (Minitub do Brazil, Porto Alegre, RS, Brazil) until T48.

2.3.5. Experiment 2: Effect of the cooling temperature of equine sperm supplemented or not with CLC

Two ejaculates from each of 12 stallions were used. After processing the ejaculate as in experiment 1, the semen was divided into two groups: Control and CLC-1.5. Next, the samples were stored at 15 °C or 5 °C in the Botu-Flex (Botupharma). The sperm characteristics evaluated in this experiment were TM, PM, RAP, and PMI, at T0 and T24.

2.3.6. Experiment 3: Influence of adding CLC on the quality of cooled semen from stallions classified as BC and GC

Two ejaculates of each of nine stallions classified as GCs and nine classified as BCs were used. The classification of stallions as BCs and GCs was adapted from Brinsko, et al. [8], and in this experiment was determined according to the decrease in TM percentage after 24 hours of cooling at 5 °C compared with T0. The animals considered BCs exhibited a 30% or greater decrease and GCs exhibited a 20% or less decrease of TM. The ejaculates were processed according to previous experiments and divided into two aliquots: Control and CLC-1.5 cooled at 5 °C for 48 hours. The analyses of sperm characteristics were performed according to section 2.3.1 and 2.3.2 at T0, T24, and T48.

2.3.7. Experiment 4: Influence of adding CLC on the occurrence of ACR in fresh and cooled equine sperm

Two ejaculates from each of five stallions were used. The semen was processed as above, and the samples were divided into the following groups: Fresh sperm or cooled at 5 °C for 24 hours and supplemented (CLC 1.5) or not (control) with CLC. To induce sperm capacitation and ACR, four 5-ml aliquots from each ejaculate (previously diluted) were incubated at 37 °C with 10 μmol/L of ionophore calcium (A23187). We analyzed the ACR in the fresh sperm and after cooling at four time points: Before and after 1, 2, and 3 hours of incubation with A23187.

2.3.8. Experiment 5: Fertility of cooled semen from stallions classified as BC and GC treated or not with CLC

A BC and a GC stallion (classified according to the criteria described in experiment 3), with cooled semen pregnancy rates below 50% and above 70% (respectively), were used. Two cycles of 25 mares were used for the fertility test of the BC stallion, and two cycles of 10 mares for the GC stallion. The semen was initially diluted as described in section 2.2 and processed according to the group to be used for artificial insemination (AI): Control and CLC-1.5 cooled at 5 °C for 24 hours. The AIs were randomly performed in a cross-over design with the GC and BC stallions.

To perform the AIs, the mares were monitored daily until a 35-mm-diameter follicle was verified in one of the ovaries. At that time, ovulation was induced with 1 mg (im) of deslorelin acetate (Botupharma), and the semen was collected and processed as previously described. Twenty-four hours after ovulation induction, the mares were inseminated with 1 × 10^6 motile sperm/mL. The cycle was discarded in every mare that did not exhibit ovulation 24 hours after the AI, and the next cycle was used. Ultrasound (Pie Medical Falco 100, Nutricell, Campinas, SP, Brazil) examination was performed 14 days after the detection of ovulation to diagnose pregnancy and was interrupted with an application of 5 mg (im) of dinoprostone tromethamine (Lutalyse, Nutrivet, Jaguariúna, SP, Brazil.)

2.4. Statistical analyses

A robust (permutation-based) ANOVA was performed to analyze the laboratory sperm characteristics of the groups studied, with repeated measures in every path (considered significant when P < 0.05). When significant differences were
detected between groups regarding the effects of treatment or the interaction between time and treatment, a post hoc analysis was performed by comparing the confidence intervals obtained by the bootstrap method between each pair of groups, and the significance level was adjusted according to the number of comparisons (P < 0.0125). A paired t-test was used for the ACR analyses, considering P < 0.05 as significant. For the fertility test, Fisher’s exact test was used, and P < 0.05 was considered significant. The analyses were performed using R software, version 2.15.3 [21], with the lmPerm package for permutation-based ANOVA [22].

3. Results

3.1. Experiment 1

At T24 and T48, the groups CLC-1.5 and CLC-2 exhibited TM, PM, and RAP values higher than those of CLC-1 and control, and CLC-1 displayed significantly greater values than the control group (Table 2). As for the parameters average path velocity, straight-line velocity, curvilinear velocity, and PMI at T24 and T48, all groups supplemented with CLC exhibited significantly greater values than the control group (Tables 2 and 3).

3.2. Experiment 2

Regarding the effects of cooling temperature on groups supplemented or not with CLC, the parameters TM, PM, RAP, and PMI in the control group were similar between the two temperatures (5 °C and 15 °C). However, when the samples were supplemented with CLC-1.5, TM and PM at 5 °C were higher than at 15 °C. In contrast, RAP and PMI were similar at both temperatures. The comparison of CLC supplementation with nonsupplementation at 5 °C showed that every parameter was significantly higher in the supplemented group. In contrast, at 15 °C, only TM and RAP were higher in the CLC group than the control group (Table 4).

3.4. Experiment 4

We next analyzed the influence of CLC supplementation on the ACR rate. The control group had a higher ACR rate.
than CLC-1.5 in fresh semen and after 24 hours at 5 °C. After the 2-hour time point, the control group exhibited a higher ACR rate than the CLC-1.5 group at every other time point (Table 8).

3.5. Experiment 5

Regarding the fertility rate obtained with semen cooled at 5 °C for 24 hours, supplemented or not with CLC, we observed no difference in the conception rate for GC stallion. Conversely, the BC stallion exhibited a significantly higher fertility rate in samples supplemented with CLC compared with the control group (Table 9).

4. Discussion

The cooling process slows cell catabolism and microbial growth, ensuring the increase in sperm longevity and allowing the use of the semen with satisfactory fertility results 24 to 48 hours after collection [1,23]. Despite the countless benefits offered, cooling equine semen promotes irreversible damage, which is harmful to the performance of sperm function [3–5]. An option to increase sperm resistance at low temperatures is modifying the lipid content of the plasma membrane by incorporating CLC [11].

Aiming to discover the optimal method for cooling equine sperm, in experiment 1 the concentrations of 1, 1.5, and 2 mg of CLC/120 × 10⁶ sperm were tested. Confirming the findings of Kirk, et al. [17], Torres, et al. [12], and Crespilho, et al. [13], regarding the dose tested, supplementing with CLC promoted the improvement of SK and PML after cooling. However, in contrast with Torres, et al. [12] and Crespilho, et al. [13], who used 2.5 and 3 mg of CLC/120 × 10⁶ sperm, respectively, in the current study, the best results were obtained with the doses of 1.5 and 2 mg of CLC/120 × 10⁶ sperm, concentrations very close to those commonly used when freezing equine semen [10,11,16]. High levels of cholesterol in the plasma membrane can interfere with the sperm capacitation process and with the occurrence of ACR [10,15,16]. Therefore, CLC-1.5 was preferred over the CLC-2 treatment in the remaining experiments of this study because it was the lowest concentration associated with the best results.

Sperm cooling at 15 °C is commonly used in equine reproduction for transportation over short distances, and also as an alternative for BC stallions for inseminations 24 hours after collection [9]. However, the studies that have analyzed the use of CLC in equine sperm cooling have used only the temperature of 5 °C [12,13,17]. In experiment 2, 5 °C was compared with 15 °C regarding sperm characteristics in the presence or absence of CLC. Despite the beneficial action of 1.5 mg CLC/120 × 10⁶ sperm, cooled at 15 °C, compared with the control group, it was clear that 5 °C was the best temperature to cool equine sperm that had been supplemented with CLC. These results most likely occurred owing to the better preservation of the energy reserves of the sperm at 5 °C, because every decrease of 10 °C promotes a decrease of 50% of sperm metabolic demand [24]. In other words, sperm stored at 5 °C and 15 °C retained approximately 10% and 20% of their original metabolic activity, respectively. This is most likely the reason why the CLC-1.5 group stored at 5 °C reached higher TM and PM values after 24 hours compared with the CLC-1.5 group refrigerated at 15 °C (Table 4).

The main factor influencing the quality and longevity of cooled equine sperm is the stallion itself [1]. According to
Brinsko, et al. [8], stallions can be classified as BCs and GCs according to the sperm’s resistance at low temperatures. This huge variability in semen quality happens mainly because of the way the stallions are selected, which is based mainly on their performance in sporting competitions and genealogy. Thus, the reproductive characteristics are largely ignored [25].

In that context, the effects of CLC on cooling GC and BC sperm was evaluated and compared in experiment 3. The results showed a beneficial effect of CLC on sperm quality in both groups of stallions. However, confirming the observations of Kirk, et al. [17] for cooled semen and Moore, et al. [11] for frozen semen, the BC stallions were helped the most by the CLC supplementation. After 24 hours of refrigeration at 5 °C, the nonsupplemented BCs exhibited a mean decrease of 46%, 57%, and 24% in TM, PM, and PMI, respectively. In contrast, the CLC-1.5 group showed a mean decrease of only 11%, 12%, and 3% in TM, PM, and PMI, respectively, which were still superior to those of the control group of GCs, which exhibited a mean decrease of 15%, 25%, and 12% for the same parameters.

Although the lipid arrangement of the sperm membrane was not evaluated in this study, the plasma membrane composition is intimately related to the susceptibility of the sperm to cooling and freezing. The cholesterol/phospholipid ratio is a determinant to the maintenance of the fluidity and stability of the membrane at low temperatures [6,7]. Therefore, the results of this study suggest that the cholesterol/phospholipid ratio of the membrane of BC stallions is lower than that of the GCs. This characteristic would explain the notoriously superior effect of CLC on sperm cooling in BCs, which would be favored by the supply of this cholesterol in the membrane.

Cholesterol efflux from the plasma membrane is a vital event to trigger the sperm capacitation process [26]. Therefore, despite the known cryoprotective effect of CLC on cells, high cholesterol/phospholipid ratios modify the fertilization capacity of the equine sperm [15]. Additionally, Zahn, et al. [10] found that incorporating CLC into the plasma membrane was harmful to the fertility of cryopreserved equine semen, with a rate of 25% versus 75% pregnancies per cycle for the sperm supplemented or not with CLC, respectively.

Regarding the possibility of using CLC for the cooling of equine sperm, no reports have evaluated the influence of CLC on in vivo fertility. The treatment with CLC in experiment 5 did not hinder the fertility of the GC stallion, in contrast with what was found by Zahn, et al. [10]. However, a striking result was observed for the BC stallion, in which supplementing with CLC resulted in an increase of 42% in fertility rate compared with the control group, reaching a final rate of 76% conceptions per cycle, similar to the results observed in this experiment for the GC.

Aiming to synchronize the presence of viable sperm in the oviduct with the moment of ovulation, AIs with frozen equine semen are normally performed after ovulation, within a maximum period of 6 hours [27,28]. That differs from the protocol used in this experiment, in which the AIs were performed with cooled semen 12 to 24 hours before ovulation. By adding CLC, the sperm membrane acquires great stability, modifying its response to different capacitation inducers, or even generating the need for a longer period of stimulation to trigger the ACR [10,16,29]. In this sense, in experiment 4, the cooled sperm from CLC-1.5 group exhibited ACR rates similar to those observed in the control group only after 2 hours of incubation in a capacitation medium. Therefore, the fertility results obtained in this study suggest that there was an adequate availability of time in female reproductive tract, which allowed the sperm treated with CLC to undergo the capacitation in time for fertilization.

### Table 7

<table>
<thead>
<tr>
<th>Stallion</th>
<th>Time (h)</th>
<th>Control</th>
<th>CLC-1.5</th>
<th>Control</th>
<th>CLC-1.5</th>
</tr>
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<tbody>
<tr>
<td>T0</td>
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<td>T24</td>
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<td>T48</td>
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| Abbreviations: BC, bad cooler; C group, semen diluted in Botu-Sêmên; CLC, cholesterol-loaded cyclodextrin; CLC-1.5 group, semen diluted in Botu-Sêmên + 1.5 mg CLC/120 × 10⁶ sperm. ab Different letters in the same line denote significant differences (P < 0.0125).

### Table 8

Mean values (%) and standard error for ACR-positive of fresh and cooled equine sperm at 5 °C for 24 hours before (Pre-Ca) and after 1, 2, or 3 hours of incubation in ionophore calcium (Ca) at 37 °C.

<table>
<thead>
<tr>
<th>Time-point</th>
<th>ACR fresh</th>
<th>ACR cooled</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>CLC 1.5</td>
</tr>
<tr>
<td>Pre-Ca</td>
<td>30 ± 2.2a</td>
<td>30 ± 2.2a</td>
</tr>
<tr>
<td>Ca – 24 h</td>
<td>55 ± 8.4a</td>
<td>44 ± 5.6a</td>
</tr>
<tr>
<td>Ca – 48 h</td>
<td>77 ± 4.9a</td>
<td>57 ± 6.0a</td>
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<tr>
<td>Ca – 72 h</td>
<td>88 ± 3.8b</td>
<td>69 ± 6.6b</td>
</tr>
</tbody>
</table>

Abbreviations: ACR, acrosome reaction; C group, semen diluted in Botu-Sêmên; CLC, cholesterol-loaded cyclodextrin; CLC-1.5 group, semen diluted in Botu-Sêmên + 1.5 mg CLC/120 × 10⁶ sperm.

ab Different letters in the same line (within each cooling group) denote significant differences (P < 0.05).

### 4.1 Conclusion

Supplementing with CLC exerts a cryoprotective effect on equine sperm from BC and GC stallions during cooling for 24 and 48 hours. A concentration of 1.5 mg of CLC/120 × 10⁶ sperm and the temperature of 5 °C must be used to obtain the best results with equine sperm cooling. The
addition of CLC causes an in vitro delay of at least 1 hour in the capacitation of cooled sperm, which did not affect fertility with reproductive management adopted, as well as can provide an increase in fertility rates of BC stallions.

Acknowledgments

The authors thank São Paulo Research Foundation - FAPESP for financial support and the Botupharma for providing the semen extenders.

References