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

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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 $\times$ 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.

1. Introduction

Artificial insemination with cooled semen is a key biotechnique for current equine reproductive programs. With

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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 metil-β-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 metil-β-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), Itapuã (Private stud farm, Arandú, SP, Brazil) and LUB (Private breeding center, Cesário Lange, SP, Brazil), remained stabled, 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 50×10^6 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 carboxifluorescein diacetate–positive and IP-negative cells intact.