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Novel technical strategies to optimize cryopreservation of goat semen using cholesterol-loaded cyclodextrin

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ABSTRACT

Artificial insemination is well-established in dairy cattle, with sires housed in commercial studs for processing. In some species, however, sires located on-farm are used for artificial insemination by shipping their semen to an off-site laboratory for processing within 24 h of collection. To expedite semen transport from the farm to laboratory, protocols must be uncomplicated. For goat semen, an obstacle is the seminal plasma, which must be removed because it contains proteins that impede sperm quality. Our objective is to develop a simple strategy to transiently store goat semen for 24 h prior to freezing. Cholesterol-loaded cyclodextrin (CLC) has been demonstrated to improve sperm tolerance to cryopreservation. Therefore, we hypothesized that CLC improves goat sperm resistance to seminal plasma damage, over 24 h prior to cryopreservation. We first evaluated the ability of CLC to protect goat sperm against seminal plasma damage by treating fresh semen with or without seminal plasma prior to cryopreservation. Second, fresh goat semen with seminal plasma was extended in skim milk-based extender ± CLC and held for 24 h at 5 °C prior to freezing. Our results indicate that CLC treatment improves goat sperm resistance to seminal plasma-mediated injury and protects sperm quality over 24 h prior to freezing ($P < 0.05$). Although the in vivo fertility of semen must first be assessed, it is possible that protocols for goat semen cryopreservation can be simplified by including CLC and eliminating seminal plasma removal. Processing and distribution of goat semen for AI would thereby be facilitated.

1. Introduction

Since the first report of successful semen cryopreservation in the bovine [31], these techniques have continued to improve, providing a better understanding of semen composition and techniques to improve sperm cryoresistance [5,18,22]. During cryopreservation, sperm membranes are exposed to mechanical, thermal and chemical stresses [9,13], which principally affect the sperm membrane. Due to its thermostability, exogenous cholesterol incorporated in sperm using cyclodextrin has been reported to reinforce the membrane to improve bull [24,33], stallion [26,29], ram [25,27] and goat [17,40] sperm cryoresistance.

Seminal plasma is an important component of semen, which has been shown to improve sperm motility [42], membrane integrity [6,23,43] and resistance to cold shock [4,34] and oxidative stress [36,37] in a variety of mammals. Some seminal plasma proteins, however, have been demonstrated to be detrimental for sperm storage in the bull [2,12], ram [6,14], goat [18,44], stallion [7,23] and boar [7]. For example, Binder of SPerm (BSP) proteins destabilize bull sperm membranes by removing cholesterol and phospholipids [7,21]. In goat sperm, another source of damage is a glycoprotein lipase (Busgp60) from the bulbourethral gland [1,19,30]. Busgp60 hydrolyzes triolein and triglycerides from milk-based and egg yolk-based semen extenders into free fatty acids, which inhibit motility and damage the sperm membranes [16,19,39]. Therefore, most semen handling protocols for goat semen recommend removing seminal plasma to enhance sperm function [35]. Goat sperm membranes could be reinforced using cholesterol-loaded

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cyclodextrins (CLC) to reduce damage induced by prolonged contact with seminal plasma.

Such a strategy would enable on-farm collection and subsequent semen shipping to a central processing laboratory with expertise in semen cryopreservation. Such an approach is common in countries without an established caprine industry, including the US and Canada. A similar tactic is also often used for stallion semen cryopreservation [3]. Indeed, it is more practical to transport semen rather than the animal itself and diluted goat semen can be held at 5 °C for up to 24 h prior to cryopreservation without detrimental effects on sperm physiology [15]. In addition, new cryopreservation technologies, such as treating buck sperm with CLC show promise in increasing sperm cryosurvival [17,40].

This study was conducted to test the hypothesis that exogenous cholesterol via CLC improves goat sperm resistance to seminal plasma and cryopreservation damage. We further hypothesized that CLC maintains the quality of fresh sperm during temporary storage prior to freezing. In this study, we demonstrate that goat sperm treated with CLC and diluted in skim milk-based extender are more resistant to seminal plasma and cryopreservation damage compared to untreated control. Additionally, we show that fresh semen can be preserved for 24 h at 5 °C in skim milk-based extender prior to cryopreservation with acceptable sperm quality after thawing.

2. Material and methods

2.1. Preparation of the skim milk extender

Unless specified otherwise, all chemicals, including the CLC (47 mg cholesterol in 1 g methyl β-cyclodextrin), were purchased from Sigma Chemical Co. (St. Louis, MO). The skim milk-based extender contained 25 g commercial skim milk powder and 0.25 g D-glucose mixed with 250 mL Milli-Q water, and was heated at 95 °C for 10 min. After 30 min of stirring at room temperature, 0.03 mg/mL penicillin G sodium salt and 0.05 mg/mL streptomycin sulfate were added. The extender was split in two fractions, one of which was glycerolated by the addition of glycerol to a concentration of 14% (v:v).

2.2. Animals and semen collection

Four healthy Alpine bucks, aged between two and four years were housed individually at the "Centre de Recherche en Sciences Animales de Deschambault" (CRSAD; Deschambault, QC, Canada). Four females in estrus were used to enhance male libido for semen collection. Estrus was induced by an injection of 0.2 mL (USP 1 mg/mL) estradiol cypionate two days before semen collection. Animals were subjected to an alternating photoperiod of six weeks of long days (16 h of light and 8 h of dark; 16 L/8 D) and six weeks of short days (8 L/16 D) as described by Delgadillo et al. [11]. Each buck was fed hay and water ad libitum and a daily complement of 500 g concentrate.

All research was carried out with the approval of the institutional animal care committee according to the guidelines of the Canadian Council on Animal Care [38].

2.3. Experiment 1: do CLC and seminal plasma affect the function of cryopreserved goat sperm?

Semen was collected by the same technician using an artificial vagina (37 °C) fitted with a calibrated, prewarmed (37 °C) Falcon tube, to evaluate ejaculate volume. Immediately after collection, ejaculates were placed in a 37 °C water bath to avoid temperature shock. Sperm concentrations were determined using a spectrophotometer precalibrated for goat sperm after diluting the semen 1:400 (v:v) with sodium citrate solution (0.9%; w:v). For Experiment 1, a single ejaculate from each of 4 different bucks ($n = 4$) was divided in two aliquots. One aliquot, referred to as the "Washed" treatment, was immediately (within 5 min of collection) diluted to 500×10^6 sperm/mL in a prewarmed (37 °C) "washing buffer" (10.54 mM glucose, 130 mM NaCl, 5.1 mM KCl, 9.86 mM Na_2HPO_4, 1.33 mM $MgSO_4$, 0.95 mM $CaCl_2$, 1.47 mM KH_2PO_4, pH 7.4, 290 mOsm) and centrifuged twice to remove the seminal plasma ($500 \times g$ for 15 min), discarding the supernatants and resuspending in the same buffer after each centrifugation. During this time, the second aliquot, referred to as the "SP" treatment, was held in a water bath at 37 °C and not centrifuged thereby keeping the seminal plasma with the sperm. Both the Washed and SP aliquots were then diluted to 1×10^9 sperm/mL in the skim milk-based extender fraction without glycerol and treated ±3 mg/mL CLC (corresponding to 141 μg/mL cholesterol). The four treatments were thereby identified: Washed (W), Washed with addition of CLC (W + CLC), Wnwashed (SP) and Wnwashed with addition of CLC (SP + CLC).

2.4. Semen freezing

The four semen treatments, all diluted in the skim milk-based extender without glycerol, weretransported to the laboratory in a cooler that progressively decreases the temperature to 5 °C in 1 h. Manipulating in a 5 °C cold room, the extended semen was further diluted 1:1 with the precooled, glycerolated skim milk-based extender fraction, which was added in thirds every 10 min (7% final glycerol concentration). The sperm were equilibrated for 90 min prior to packaging into 0.25 mL straws (IMV Technologies, l'Aigle, France) to a final concentration of 125×10^6 sperm/straw. The straws were frozen in liquid nitrogen vapor using a floating freezing rack (Minitube, Hauptstrasse 41, Germany) 3 cm above the liquid nitrogen as per the freezing curve provided by the manufacturer (from 5 to 0 °C for 3 min, 0 to −150 °C for 3 min and −150 to −196 °C for 14 min). The straws were stored in liquid nitrogen until thawing in 37 °C water for 30 s prior to use.

2.5. Sperm quality analyses

Sperm were subjected to motility and viability analyses before freezing and at 0 h, 2 h and 4 h after thawing. Prior to analysis, sperm samples were diluted to 50×10^6 sperm/mL with warmed PBS (calcium- and magnesium-free; Sigma) and incubated for 15 min in a 5% CO_2 incubator at 38.5 °C (Sanyo North America Corporation, San Diego, CA).

Sperm motility was assessed on a 10 μL sample placed on a counting chamber (MicroCell™, Vitrolife, California, USA), prewarmed to 37 °C on a thermal plate connected to the microscope. The percentages of motile sperm were determined by Computer-Assisted Sperm Analysis (CASA; Ceros Analyzer, with software version 12-f; Hamilton Thorne Research, Beverly, MA) adjusted to operate 30 video frames per second (60 Hz) with minimum particle size of 7 μm and minimum contrast at 60. Sperm were defined as non-motile when the average path velocity (VAP) was lower than 21.9 μm/s. Sperm were considered progressively motile when VAP >75 μm/s and straightness index (STR) > 80%. A minimum of 200 sperm from 5 different fields was assessed. The sperm motility parameters were determined using a 10× negative phase contrast objective transmitted through a monochrome Olympus U-CMAD3 video camera (Olympus Corporation, Tokyo, Japan).

The proportions of live sperm (defined as sperm with an intact plasma membrane) and sperm with intact acrosomes were evaluated using the EasyCyte Plus flow cytometer (IMV Technologies,