Cryobiology 67 (2013) 124–131

Contents lists available at SciVerse ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/ycryo

Optimizing conditions for treating goat semen with cholesterol-loaded cyclodextrins prior to freezing to improve cryosurvival ☆

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ARTICLE INFO

Article history:
Received 8 October 2012
Accepted 3 June 2013
Available online 11 June 2013

Keywords:
Semen
Frozen–thawed sperm
Freezing
Goat
Caprine

ABSTRACT

The fertility of goat sperm is highly variable and new methods for improving sperm cryosurvival are needed. Cholesterol plays important roles in membrane fluidity, cold shock sensitivity and cryodamage, and treating sperm from cold-shock sensitive species with cholesterol-loaded cyclodextrins (CLC) prior to cryopreservation enhances sperm cryosurvival. The aim of this study was to develop a CLC-treatment to optimize goat sperm cryopreservation. A total of 45 ejaculates coming from eleven adult Murciano-Granadina bucks were used and three experiments were conducted to determine: (1) the optimal CLC concentration to treat goat sperm; (2) the optimal time to treat the sperm (before or after seminal plasma removal); and (3) optimal freezing diluent (either of two Tris-citrate diluents containing 2% or 20% egg yolk and 4% glycerol or a skim milk diluent with 7% glycerol) to cryopreserve goat sperm. Goat sperm cryosurvival rates were greatest when they were treated with 1 mg CLC/120 $\times$ 10^6 sperm prior to freezing. The benefit was also greatest if the sperm were treated with CLC after seminal plasma removal. Finally, CLC treatment improved sperm cryosurvival rates for sperm frozen in all three diluents, however, CLC treatment was most effective for sperm frozen in egg-yolk diluents. In conclusion, treating goat sperm, with CLC prior to cryopreservation, improved sperm cryosurvival rates. In addition, CLC treatment was effective for all freezing diluents tested, making this technology practical for the industry using current cryopreservation techniques. Nevertheless, additional studies should be conducted to determine how CLC might affect sperm functionality and fertilizing ability.

Introduction

The recent surge in centers specializing in providing reproductive technologies, including custom semen collection, cryopreservation and artificial insemination, for livestock species, necessitates developing cryopreservation protocols specific for each species. Semen cryopreservation is a valuable technique for goat production, since it permits collecting and storing semen outside of the breeding season, for use later, which can increase the number of females that can be inseminated with sperm from a specific male. In addition, semen can be transported to distant areas and can be used as insurance against injury or even death of a genetically valuable male. Therefore, cryopreserved sperm can economically impact the commercial livestock production for several species. In spite of the great advantages associated with using cryopreserved sperm, it is still not used extensively on commercial goat farms.

Cryopreservation can have detrimental effects on sperm quality [13,24,25,40,46], including a loss of sperm motility, induction of structural and functional changes in sperm, and sperm death. These detrimental effects can reduce the fertilizing ability of sperm, even though the cell may survive cryopreservation. Therefore, the kindling rate of fresh goat sperm is estimated to be approximately 12.1% higher than that observed for frozen–thawed sperm [26]. In addition, the fertility of frozen–thawed goat sperm ranges from 3% to 70% [18], and this disparity seen in the cryosurvival rates of goat semen is a significant reason that frozen–thawed goat semen is not widely used on commercial goat farms [5].

The cryosurvival rates of sperm, from the various species, can be very different. Some of the species differences observed can be

☆ *Statement of funding:* C. Konyali received a scholarship from CIHEAM (Zaragoza, Spain), C. Tomás was supported by a scholarship from Ministerio de Ciencia e Innovación (Ref. BES-2007-17063; Madrid, Spain) and E. Blanch was supported by a scholarship from Conselleria d'Agricultura, Pesca y Alimentació (CAPA, DOGV5324; Valencia, Spain). E. Mocé was supported by funds from INIA-CCAA and the European Social Fund (Ref. DR03-704) and is currently supported by funds from Ministerio de Ciencia e Innovación from Subprograma Ramón y Cajal (Ref. RYC-2010-06162; Madrid, Spain).

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http://dx.doi.org/10.1016/j.cryobiol.2013.06.001

attributed to differences in sperm membrane composition, which can affect cell survival during cooling [33]. Certainly some of the cell death that occurs during cooling is a result of membrane damage, caused by membrane component (lipid and protein) rearrangements that occur when the membrane undergoes a phase transition, from the fluid to the gel state [23]. The temperature range during which a membrane undergoes this phase transition depends upon a combination of membrane factors, including the membrane cholesterol/phospholipid ratio, the amount of nonbilayer-preferring lipids in the membrane, the degree of hydrocarbon chain saturation in the membrane lipids, and the protein/phospholipid ratio in the membrane [33].

Cholesterol plays an important role in membrane fluidity and membrane sensitivity to cold shock damage [14]. Cholesterol is also important in maintaining cell membrane structure, fluidity and function over the range of physiological temperatures [28]. Sperm plasma membrane lipids respond to temperature changes by undergoing a phase transition, from the fluid state to the gel state when cooled [25]; and it is this phase change during cooling, that has been implicated as one component of cooling-induced membrane damage [44]. When temperature decreases and a sperm membrane transitions from the liquid to the gel state (state where the membrane becomes more fragile) the presence of sterols can inhibit the phase transitions from occurring and minimize cooling-induced membrane damage [16,25]. Therefore, species whose sperm possess membranes with high cholesterol:phospholipid ratios (~1, such as human and rabbit sperm) are more resistant to cold shock damage than species whose sperm membranes have low cholesterol:phospholipid ratios (<0.5, such as horse, sheep and cattle sperm; [14,57]).

Since sperm that contain high cholesterol:phospholipid ratios survive cooling more efficiently than sperm with low cholesterol:phospholipid ratios, it may be reasonable to expect that enriching the cholesterol content of sperm membranes having low cholesterol levels, prior to cooling and freezing might reduce cell damage caused during cryopreservation. Indeed, when the cholesterol content of stallion, ram and bull sperm is increased, by treating these cells with cholesterol-loaded cyclodextrins (CLC), the cryosurvival rates and quality of the frozen–thawed sperm is increased (for a review, see [34]). Therefore, CLC-treatment prior to cryopreservation may be able to minimize the cryoinjury of goat sperm, after cryopreservation, as well. The aim of this study was to optimize a protocol for treating goat sperm with cholesterol-loaded cyclodextrins (CLC), and three experiments were conducted to determine: (1) the optimal CLC concentration to treat goat sperm; (2) when during processing CLC treatment best improves sperm quality after thawing; and (3) if CLC treatment can improve sperm quality when sperm are cryopreserved in different freezing diluents, commonly used to freeze goat sperm.

Materials and methods

Materials and preparation of diluents

All chemicals were reagent grade and purchased from Sigma–Aldrich (Madrid, Spain), except for Propidium iodide (PI) and SYBR-14, which were purchased from Invitrogen (Barcelona, Spain).

The basic solution to dilute sperm and to make the egg-yolk freezing diluents was a Tris-citrate diluent (250 mM of Tris[hydroxymethyl]aminomethane, 83 mM of citric acid anhydrous and 69 mM of D(+) glucose; 300 mOsm, pH = 7; [35]).

Sperm were frozen in three different freezing diluents (two containing egg yolk and one skim milk-based), that are routinely used to cryopreserve goat semen. For each diluent, two fractions were used; the first contained no glycerol and was used to dilute the sperm at room temperature and cool the sperm to 5 °C. The second contained glycerol at 2-fold the final desired concentration and was added to the sperm (1:1; vol:vol) after the sperm had reached 5 °C.

The egg-yolk–Tris diluents were made by adding egg yolk from fresh eggs (20% or 2%, vol:vol; depending on the diluent) to the Tris diluent. For the glycerolated fractions, 8% (vol:vol) glycerol was added. The diluents were clarified by centrifuging at 12,000 × *g* for 20 min at 5 °C. The supernatant was recovered and filtered sequentially through 5, 3 and 1.2 μm filters.

The Skim milk-based diluent was made by adding 10 g of dried skim milk (Skimmed Milk Powder; Central Lechera Asturiana, Oviedo, Spain) and 0.2 g of D(+) glucose to ultrapure water (final volume of 100 mL), and heating the diluent in a water-bath at 95 °C for 10 min. The glycerolated fraction contained 14% (vol:vol) glycerol.

Preparation of cholesterol-loaded cyclodextrins

Methyl-β-cyclodextrin was preloaded with cholesterol according to the protocol developed by Purdy and Graham [45].

A working solution of CLC was prepared by adding 50 mg of CLC to 1 mL of Tris diluent [45] and mixing the solution using a vortex mixer. Aliquots (0.5 mL) of this working solution were stored frozen in eppendorf tubes until use.

In addition, a working solution of 50 mg methyl-β-cyclodextrin/mL (not loaded with cholesterol) in Tris diluent was also made.

Animals

Eleven adult Murciano-Granadina goats (2–7 years of age), housed in the Centro de Tecnología Animal, Instituto Valenciano de Investigaciones Agrarias (CITA-IVIA; Segorbe, Castellón, Spain), were used. The goats were housed in a common pen and were fed hay *ad libitum* and a daily complement of 1 kg concentrated feed (crude protein 16.5%, crude oils and fat 4.5%) per male. Fresh water was provided *ad libitum*. Animal housing, care and protocols for semen collection were approved by the Animal Care and Use Committee of CITA-IVIA and fulfilled the European regulations for the care and use of animals for scientific purposes (EC Directive 2010/63/EU).

Semen collection

Ejaculates were collected using an artificial vagina, as described by Silvestre et al. [52], early in the morning. Two ejaculates were collected per week from each male on two separate days. The ejaculates were placed in a 37 °C water bath until processing.

Semen volume was measured using the calibrated collection tube and the sperm concentration was determined, using a spectrophotometer calibrated for goat sperm after diluting the semen 1:400 (v:v) with a sodium chloride solution (0.9%; w:v). A separate semen aliquot was diluted to 15×10^6 sperm/mL with Tris buffered diluent supplemented with bovine serum albumin (6 mg/mL; Tris-BSA) and incubated for 10 min at 38 °C for motility analysis. The ejaculates used had an average volume of 1.04 mL, a sperm concentration of 3.735×10^9 sperm/mL, and contained 88% total motile and 54% progressively motile sperm.

Freezing protocol

When required, semen was washed twice by centrifugation to remove the seminal plasma. For washing, ejaculates were cooled to 22 °C, diluted 1:10 (vol:vol) with Tris diluent and centrifuged at 500 × *g* for 15 min. After centrifugation, the supernatant was re-