Animal Reproduction Science 118 (2010) 236–247

Contents lists available at ScienceDirect

Animal Reproduction Science

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

Treating ram sperm with cholesterol-loaded cyclodextrins improves cryosurvival☆

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ARTICLE INFO

Article history:
Received 5 August 2008
Received in revised form 18 May 2009
Accepted 18 June 2009
Available online 26 June 2009

Keywords:
Cryopreservation
Semen
Sheep
Freezing
Sperm analysis

ABSTRACT

Acceptable fertility using cryopreserved ram sperm is currently only achieved using laparoscopic intrauterine insemination. Improving the cryosurvival of ram sperm may permit greater fertility rates using more practical techniques. This study was conducted to determine if treating ram sperm with six different cyclodextrins pre-loaded with cholesterol (CLC), prior to cryopreservation increases sperm cryosurvival and if this technology can be used with neat semen. Subsequent experiments evaluated how adding CLC to sperm affected sperm cholesterol content, sperm osmotic tolerance limits, sperm post-thaw survival after incubation and the capacity of sperm to bind to zona pellucidae of cattle and sheep oocytes. Sperm treated with 2-hydroxypropyl-β-cyclodextrin prior to cryopreservation exhibited greater percentages of motile sperm (62%) compared to the control (no CLC treatment) samples (43%, $P < 0.05$), after thawing. In addition, samples treated with methyl-β-cyclodextrin exhibited percentages of motile and viable sperm similar to samples treated with 2-hydroxypropyl-β-cyclodextrin. Other CLC-treated samples were similar to the control. The CLC concentration that optimized sperm cryosurvival was 2 mg CLC/120 × 10^6 sperm for both methyl-β- and 2-hydroxypropyl-β-cyclodextrin when added to neat semen prior to cryopreservation. Addition of 2 mg CLC not only maintained greater percentages of motile sperm compared to the control samples, but maintained greater percentages of motile sperm during a 3 h incubation after thawing. In addition, 2-hydroxypropyl-β-cyclodextrin pre-loaded with cholesterol maintained greater percentages of viable sperm (33%), than control sperm (18%; $P < 0.05$). Treating ram sperm with CLC increased the sperm cholesterol content > 1.9-fold and although some cholesterol was lost from the sperm during cooling and cryopreservation, the cholesterol content remained greater in CLC-treated sperm after cooling and after thawing than in control sperm ($P < 0.05$). In addition, CLC-treated sperm maintained greater percentages of motile sperm through a wide range of osmotic solutions (150 and 425 mOsm) while control sperm lost motility in solutions outside a more narrow range (270 to 370 mOsm). Greater numbers of CLC-treated sperm bound to zona pellucida than control sperm ($P < 0.05$), although number of sperm binding cattle and sheep oocytes, was similar ($P > 0.05$). In conclusion, treating ram sperm with CLC

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0378-4320/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.anireprosci.2009.06.013

increases sperm cryosurvival rates and sperm longevity after thawing. It also increases the cholesterol content, osmotic tolerance, and zona-binding capabilities of sperm. Finally, CLCs can be added to neat semen, making this technology feasible for practical application using current cryopreservation techniques for ram semen.

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1. Introduction

Artificial insemination is the main technology for rapidly dispersing valuable genetics of livestock. The method is simple, successful and economical for establishing genes in a population compared to embryo transfer or natural mating (Vishwanath, 2003). The ability to cryopreserve sperm plays an important role in artificial insemination of livestock and for conservation of endangered species, as cryopreserved sperm can be stored for long periods of time, and can be sent worldwide facilitating the exchange of genetic material between distant animal populations.

Although several cryopreservation protocols and freezing diluents have been developed to cryopreserve ram sperm (reviewed by Salamon and Maxwell, 2000), only about half of the sperm survive the process due to cryodamage and the surviving sperm exhibit reduced fertilizing capacity (Curry and Watson, 1994). Cell cryo-damage occurs due to intracellular ice formation (Mazur, 1984), membrane alterations induced by phase transitions that occur when membranes are cooled (Amann and Pickett, 1987; Hammerstedt et al., 1990; Watson, 2000; Medeiros et al., 2002), and osmotic stresses due to cryoprotectant addition and removal, as well as the cryopreservation process itself (Curry and Watson, 1994; Gilmore et al., 1995; Holt, 2000; Guthrie et al., 2002; Morris et al., 2007). Recent evidence indicates that due to the limited free water in a sperm, intracellular ice formation does not induce significant cryodamage (Morris, 2006; Morris et al., 2007). Therefore, focusing on methods to improve membrane function at low temperatures may be key to improving the cryosurvival of sperm.

As sperm are cooled the membrane phase transition occurs, lipids aggregate into micro-domains, which alter membrane function and induce membrane gaps between the gel and remaining fluid membrane domains (Amann, 1999). Cholesterol controls membrane structure by interacting with the phospholipid hydrocarbon chains (Darin-Bennett and White, 1977), and at temperatures below the phase transition, forces the chains apart, making the membrane more stable (Quinn, 1989).

Cyclodextrins are cyclic oligosaccharides which possess an external hydrophilic face and an internal hydrophobic core (Christian et al., 1997). These molecules have a high affinity for sterols *in vitro*, and if they are pre-loaded with cholesterol can insert cholesterol into cell membranes (Navratil et al., 2003). Several authors have reported increased cryosurvival rates when stallion (Combes et al., 2000; Moore et al., 2005), bull (Purdy and Graham, 2004), donkey (Alvarez et al., 2006), pig (Galantino-Homer et al., 2006), and ram (Morrier et al., 2004) sperm were treated with methyl-β-cyclodextrin pre-loaded with cholesterol, prior to cryopreservation.

In an effort to learn more about how added cholesterol affects sperm, studies were conducted to determine if treating ram sperm with different cyclodextrins pre-loaded with cholesterol (CLC), prior to cryopreservation, could increase sperm cryosurvival rates, and its influence on ram sperm quality and physiology. These studies were conducted in order to develop a cryopreservation technique to improve ram sperm cryosurvival and that could be used commercially with neat ram semen.

2. Materials and methods

2.1. Materials

All chemicals were reagent grade and purchased from Sigma–Aldrich, St. Louis, MO, USA; except for SYBR-14 and propidium iodide (PI) which were purchased from Invitrogen, Eugene, OR, USA.

2.2. Cyclodextrin preparation

Six different cyclodextrins (α-cyclodextrin, α-cyclodextrin hydrate, β-cyclodextrin, β-cyclodextrin hydrate, 2-hydroxypropyl-β-cyclodextrin or methyl-β-cyclodextrin) were pre-loaded with cholesterol as described by Purdy and Graham (2004). Briefly, a 0.45 mL aliquot of cholesterol dissolved in chloroform (200 mg cholesterol per 1 mL chloroform) was added to 2 mL of methanol containing 1 g cyclodextrin and each mixture stirred until the combined solution was clear. The solvents were removed, the resulting crystals allowed to dry for 24 h, and the crystals were stored at 22 °C until use. A working solution of each CLC was prepared by adding 50 mg of CLC to 1 mL of Tris diluent (Purdy and Graham, 2004) at 37 °C and mixing the solution using a vortex mixer for 30 s. For the negative control, methyl-β-cyclodextrin (not pre-loaded with cholesterol) was prepared similarly, but no cholesterol solution was added to the cyclodextrin solution.

2.3. Semen collection

Semen was collected by electro-ejaculation September through March, as described by Evans and Maxwell (1987), from adult rams of Southdown and Rambouillet breeds housed at Colorado State University, Fort Collins, CO, USA. Rams were fed a diet providing 100% of their nutritional needs, and provided water *ad libitum*. All animal care and semen collection procedures were approved by the Animal Care and Use Committee of Colorado State University.

After collection, semen was diluted 1:1 with Tris diluent (300 mM tris[hydroxymethyl]aminomethane, 95 mM citric acid monohydrate, 28 mM D-(+)-Glucose, pH 7.0) and transported to the laboratory in an insulated Styrofoam box and used within 45 min of collection.