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Cholesterol-loaded cyclodextrin improves ram sperm cryoresistance in skim milk-extender

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ABSTRACT

Cholesterol-loaded cyclodextrin (CLC) is known to improve ram sperm cryosurvival. This study expands on previous research to: (1) determine the mechanism by which CLC improves ram sperm cryosurvival and (2) compare the efficiency of a novel, skim milk-based extender containing CLC to a traditional egg yolk-based extender. Hypothesis #1 was that CLC enhances membrane cholesterol content to increase the resistance of ram sperm to cold and osmotic stress, thereby improving cryosurvival. We first assessed the ability of fresh sperm treated with CLC to withstand cold shock. Second, fresh sperm were treated with CLC to evaluate their tolerance to osmotic stress. Third, to confirm that cholesterol is incorporated into the sperm using CLC, we quantified sperm cholesterol. To test Hypothesis #2 that CLC is most effective in a medium without competing cholesterol, we compared sperm cryosurvival and fertility in skim milk-based extender containing CLC versus in a traditional egg yolk-based freezing extender without CLC. Our data confirmed that CLC treatment improves ram sperm cold shock and osmotic stress resistance, and augments sperm cholesterol content. Semen in skim milk-based extender containing CLC prior to freezing, had more motile sperm with intact acrosomes after thawing compared to semen in egg yolk-based extender. In contrast, sperm plasma membrane integrity and *in vivo* fertility of the semen cryopreserved in the skim milk-based extender with CLC did not differ from semen that was cryopreserved in egg yolk-based extender. Further research is warranted to combine CLC with other cryoprotection strategies or to modify the insemination protocol.

1. Introduction

Semen cryopreservation is a critical technology that enables long term storage and worldwide distribution of valuable livestock genetics. Combined with artificial insemination, semen cryopreservation is the most profitable method for establishing genes of interest in a population compared to natural mating or embryo trans-

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fer (Vishwanath, 2003). The freezing process, however, involves major temperature and osmotic variations and the formation of ice crystals, which together induce membrane damage and reduce sperm fertilizing capacity after thawing (Hammerstedt et al., 1990; Curry and Watson, 1994). As the sperm are cooled, membrane phospholipids undergo phase transitions (Crowe et al., 1999), transforming from a liquid crystalline phase that is characterized by high rotational and lateral mobility of the lipids, to a crystal gel phase in which lipid mobility is restricted. Furthermore, plasma membrane dysfunction is likely the major source of sperm cryodamage because the limited free water in sperm prevents intracellular ice crystal formation (Morris, 2006; Morris et al., 2007; Kim et al., 2011; Oldenhof et al., 2013). Therefore, increasing membrane resistance at low temperatures may be the key to improve sperm cryosurvival. Several cryopreservation protocols and freezing diluents have been developed to cryopreserve ram sperm (Salamon and Maxwell, 2000), only insufficient sperm numbers survive the process due to the sensitivity of the membrane to cryodamage and the surviving sperm exhibit reduced fertilizing capacity (Curry and Watson, 1994).

Cholesterol controls membrane structure and maintains membrane fluidity at low temperatures, thereby reducing sperm membrane sensitivity to chilling injuries (White, 1993). Sperm membrane cholesterol/phospholipid ratio is known to influence sperm resistance to cold shock. Human sperm, which have a high cholesterol/phospholipid ratio close to 0.99, do not appear to undergo an abrupt lipid phase transition during cold shock and are thus quite resistant to cooling (Drobnis et al., 1993). In contrast, ram sperm, which have a 0.37 cholesterol/phospholipid ratio (Davis, 1981; Rana et al., 1991), are notoriously sensitive to cooling (Salamon and Maxwell, 2000). Being insoluble in aqueous media, including many semen extenders, exogenous cholesterol can be incorporated into sperm membranes using cyclodextrins, which are cyclic oligosaccharides that possess an external hydrophilic face and an internal hydrophobic core (Christian et al., 1997). Cyclodextrins have a high affinity for cholesterol *in vitro*, and have been used to manipulate cell membrane cholesterol (Navratil et al., 2003; Purdy and Graham, 2004b). Early studies suggested that cold tolerance improves in boar sperm due to cholesterol removal using 2-hydroxypropyl-beta-cyclodextrin (Zeng and Terada, 2000, 2001). In contrast, numerous recent studies have reported increased sperm survival when bull (Purdy and Graham, 2004b; Moce and Graham, 2006), goat (Konyali et al., 2013), stallion (Combes et al., 2000; Moore et al., 2005; Murphy et al., 2014), boar (Tomas et al., 2013; Tomas et al., 2014) and ram (Morrier et al., 2004; Motamedi-Mojdehi et al., 2014) semen was treated with cholesterol-loaded cyclodextrin (CLC) prior to cryopreservation.

Here, we expand on these previous studies to test two hypotheses. Hypothesis #1 states that CLC augments the cholesterol content of ram sperm to improve their tolerance to cold temperatures and osmotic stress as mechanisms for increasing sperm cryoresistance. To test Hypothesis #2 that CLC is most effective in a medium without competing cholesterol, we compared sperm cryosurvival and fertility in skim milk-based extender containing CLC versus in a traditional egg yolk-based freezing extender without CLC, Triladyl®. Although CLC treatment improved post-thaw sperm quality only in the skim milk-based extender, *in vivo* fertility was not improved by freezing semen in the skim milk-based extender supplemented with CLC compared to semen in egg yolk-based extender, and alternative hypotheses to explain this result are discussed.

2. Materials and methods

2.1. Chemicals

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). Chloroform was obtained from VWR International (West Chester, PA), methanol from Fisher Scientific (Fair Lawn, NJ), acetic anhydride from Caledon Laboratories (Georgetown, ON, Canada) and Filipin III from Cayman Chemical (Ann Arbor, MI).

2.2. Preparation of extenders

The skim milk-based semen extender used for cryopreservation was prepared by combining 25 g commercial skim milk powder and 0.25 g D-glucose in 250 ml Milli-Q water. After heating at 95 °C for 10 min, the solution was stirred gently at room temperature for 30 min, and 0.03 mg/ml penicillin G sodium salt and 0.05 mg/ml streptomycin sulfate were added. Because the semen is first diluted in extender without glycerol, then cooled and further diluted with extender containing glycerol, the extender was split in two fractions, one of which was glycerolated by the addition of glycerol to a concentration of 14% (v:v).

Triladyl® (Minitube, Germany), a commercial cryopreservation extender, is frequently used for sheep insemination. Triladyl® contains Tris, citric acid, fructose, glycerol, water and antibiotics (tylosin, gentamincin, spectinomycin and lincomycin), all in proprietary concentrations. It was supplemented with 20% fresh egg yolk according to the manufacturer's instructions. Semen prepared with Triladyl® is also diluted in two steps, however, both dilutions are conducted with the complete, glycerolized extender.

2.3. Animals

Five healthy Dorset rams, between two and five years old and of proven fertility, were housed together at the "Centre d'expertise en production ovine du Québec" (CEPOQ; La Pocatière, QC, Canada). The rams were fed hay and water *ad libitum* and received 500 g/d/head of a commercial concentrate. Before starting the study, all protocols were authorized by Université Laval's animal care committee according to the guidelines of the Canadian Council on Animal Care (Rowsell, 1991).