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Effect of cholesterol-loaded-cyclodextrin on sperm viability and acrosome reaction in boar semen cryopreservation

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ABSTRACT

This study was undertaken to examine the effect of cholesterol-loaded-cyclodextrin (CLC) on boar sperm viability and spermatozoa cryosurvival during boar semen cryopreservation, and methyl-β-cyclodextrin (MBCD) was treated for comparing with CLC. Boar semen treated with CLC and MBCD before freezing process to monitor the effect on survival and capacitation status by flow cytometry with appropriate fluorescent probes. Sperm viability was higher in 1.5 mg CLC-treated sperm ($76.9 \pm 1.01\%$, $P < 0.05$) than un-treated and MBCD-treated sperm before cryopreservation ($58.7 \pm 1.31\%$ and $60.3 \pm 0.31\%$, respectively). For CTC patterns, F-pattern was higher in CLC treated sperm than MBCD-treated sperm, for B-pattern was higher in CLC-treated sperm than fresh sperm ($P < 0.05$). For AR pattern (an acrosome-reacted sperm) was lower in CLC-treated sperm than MBCD-treated sperm ($P < 0.05$). Moreover, we examined *in vitro* development of porcine oocytes after *in vitro* fertilization using CLC-treated frozen–thawed semen, in which CLC treatment prior to freezing and thawing increased the development of oocytes to blastocyst stage *in vitro*. In conclusion, CLC could protect the viability of spermatozoa from cryodamage prior to cryopreservation in boar semen.

1. Introduction

Semen cryopreservation extends the availability of sperm for research, artificial insemination (AI), and *in vitro* fertilization (IVF) irrespective of time or location. This technique provides the accessibility to sperm, despite of time and location, and permitted the successful development of AI and IVF techniques (Bailey et al., 2008). However, frozen–thawed sperm do not possess the same fertilizing potential as fresh sperm, since the cooling process induces lipid and protein rearrangements within the cell membranes when they are cooled from 22 to 1 °C (Parks and Graham, 1992). This variation was induced by the membrane changing from the fluid to the gel-state at low temperature. Thermotropic phase transitions are considered to be one of the mains reasons for reduced viability and fertility of sperm during the cryopreservation. Especially, boar semen is extremely vulnerable to 'cold shock' by cooling during the cryopreservation compared to other species. Sperm sensitivity to cold shock damage is determined primarily by phospholipid constitution and the cholesterol to phospholipid ratio in membrane (Holt, 2000). The cholesterol to phospholipid ratio of the sperm membrane is

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widely considered to be a major determinant in membrane fluidity and stability during cryopreservation. Boar sperm differ from those species that have a high cholesterol to phospholipid ratio in the membrane, such as human and rabbit sperm do not experience this membrane damage when cooled (Darin-Bennett and White, 1977). Membrane damage is considered to be one of the major reasons for cell death (Watson, 1981). Membrane destabilization occurs when the membrane undergoes the phase transition, from the fluid phase to the gel phase, as temperature is decreased. During this phase transition, phospholipids are lost from the plasma membrane leading to increased membrane permeability, membrane disruption, and cell death (Darin-Bennett et al., 1973; Watson, 1981). If the temperature at which the membrane lipid phase transition occurs could be lowered, or if the membrane phase transition could be eliminated completely, membranes would remain fluid at low temperatures and the damage that occur to membranes may be reduced. Previous studies indicated that the cholesterol/phospholipid ratio of the plasma membrane is a major determinant in plasma membrane fluidity and stability during the cryopreservation (Darin-Bennett and White, 1977; Watson, 1981).

Cholesterol, a major structural constituent of the membrane, plays an important role as a regulator of membrane function (Yeagle, 1985). Furthermore, cholesterol in the cell membrane has been shown to be an important determinant of membrane fluidity (Hartel et al., 1998) and permeability (McGrath, 1988). Also, cholesterol reduces the phase transition temperature of membrane, and maintains it in a fluid state at reduced temperature thereby reducing the membrane damage that occurs at low temperatures (Amann and Pickett, 1987).

Cholesterol can be incorporated readily into or extracted from the membrane of cells using cyclodextrins (Purdy and Graham, 2004; Zeng and Terada, 2001). Cyclodextrins are cyclic heptasaccharides consisting of β (1–4) glucopyranose units, are water soluble but have a hydrophobic center (Purdy and Graham, 2004), and can transport cholesterol in or out of membranes down a concentration gradient (Klein et al., 1995). When cholesterol is loaded into bull sperm membrane prior to cryopreservation with cholesterol-loaded cyclodextrin (CLC), a higher percent sperm motility and membrane were recovered after thawing compare to un-loaded sperm (Purdy and Graham, 2004). However, this procedure has not yet been optimized for boar sperm, nor is it known how the cholesterol regulates the cryosurvival of boar sperm and the function of physiology of the membrane.

Additionally, we also used methyl-β-cyclodextrin (MBCD) for this study, since MBCD induces the capacitation and efflux of human sperm (Chiu et al., 2005). Thus, understanding of the regulation of the cholesterol and the protection of sperm membrane and viability are important to the overall understanding cryosurvival during sperm cryopreservation. In this study, we evaluated the effect of cholesterol modification on the cryosurvival of boar sperm during a conventional semen freezing procedure. Further, using cyclodextrin, we investigated *in vitro* development of porcine oocytes after *in vitro* fertilization using CLC-treated frozen–thawed semen.

2. Materials and methods

2.1. Preparation of CLC

We prepared cholesterol-loaded-cyclodextrins (CLC) such as method of Purdy and Graham (2004). Briefly, 1 g methyl-β-cyclodextrin (Sigma, St. Louis, MO, USA) was dissolved in 2 mL methanol. 200 mg cholesterol (Sigma) was dissolved in 1 mL chloroform in a separate glass tube. Then a 0.45 mL CLC solution was added to the cyclodextrin solution, and mixed and stirred. The solvents removed using a stream of N_2 gas. Finally, the resulting crystals were stored in a glass container at 22 °C, after dry. The working solution was prepared by adding 100 mg of CLC to 1 mL m-Modena B (Lee et al., 2005) at 37 °C.

2.2. Semen collection and treatment with CLC

Semen was collected from six miniature pigs (PWG Company, Seoul, Korea) by gloved-hand technique and filtered through cotton gauze to remove the gel particles. All procedures that involved the use of animals were approved by the Kangwon National University Institutional Animal Care and Use Committee (KIACUC-09-0139). The ejaculated sperm were transported to the laboratory at 25 °C within 1 h. The ejaculated sperm were diluted with same volume of extender (m-Modena B). After maintenance at room temperature for 10 min, semen was diluted to 1.2×10^8 sperm in 1 mL of m-Modena B, and incubated with different levels of CLC (0, 0.75, 1.5, 3.0, and 6.0 mg/mL) and 1.5 mg/mL MBCD (negative control) for 15 min at 25 °C. The control group (0 mg/mL) was considered to be a positive control. The incubated semen samples were later centrifuged ($400 \times g$, 10 min) to remove CLC and MBCD from sperm before freezing.

2.3. Semen freezing and thawing

Semen treated with CLC and MBCD were processed using the straw freezing procedure described with minor modifications (Kim et al., 2006). The incubated semen was re-suspended with first lactose-egg yolk (LEY) extender (80 mL of 11% lactose and 20 mL egg yolk) to provide 5×10^8 sperm/mL and later centrifuged ($400 \times g$, 10 min, 22 °C) to remove CLC and MBCD from sperm, and were cooled to 5 °C for 3 h. After cooling the semen were diluted 2:1 (v:v) with second LEY extender (LEY extender with 1.5% Orvus Es Paste and 9% glycerol). The sperm were packaged into 0.5 mL straws cooled to −120 °C for 10 min before being plunged into liquid nitrogen for storage using static nitrogen vapor. Frozen-sperm was thawed in a water bath at 50 °C for 10 s.

2.4. Cholesterol incorporation in sperm membrane

Semen was extended to 1.2×10^8 sperm/mL in m-Modena B and incubated with 0.75 and 1.5 mg CLC/1.2×10^8 sperm for 15 min at 25 °C. In this experiment, the cyclodextrin has been pre-loaded with cholesterol in which the cholesterol was labeled with the fluorescent molecule 22-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)