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Cholesterol-loaded cyclodextrin pretreatment of ram spermatozoa protects structural integrity of plasma membrane during osmotic challenge and reduces their ability to undergo acrosome reaction in vitro

Ejaz Ahmad, Melih Aksoy*, İlker Serin, Niyazi Küçük, Ahmet Ceylan, Uğur Uçan

Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Adnan Menderes University, 09016 Aydin, Turkey

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ABSTRACT

The effect of cholesterol-loaded cyclodextrin (CLC) pretreatment on the structural integrity of ram sperm membranes during osmotic challenges and the ability of those spermatozoa, in the presence of cholesterol, to undergo acrosome reaction in vitro were examined in two successive experiments. In experiment 1, ejaculates ($n = 9$) were extended with Tris–citric acid–glucose (TCG) solution to a concentration of 400×10^6 spermatozoa/mL and then divided into two equal aliquots. The first aliquot was treated with CLC (3 mg/120×10^6 spermatozoa), and the second aliquot remained as control. The sperm samples were then incubated at 35 °C for 15 min in one of the five fructose solutions, adjusted to 20, 80, 290, 500, or 1500 mOsm/L. After osmotic challenges, the percent live sperm and the sperm plasma membrane integrity were estimated in both groups by a modified hypo-osmotic swelling test associated with supravital eosin staining. CLC pretreatment significantly increased the percentage of live, intact, and live-intact spermatozoa ($P < 0.05$) following exposure to different osmotic challenges. In experiment 2, ejaculates ($n = 8$) were first centrifuged twice to remove seminal plasma and extended with TCG to concentration of 200×10^6 spermatozoa/mL. After CLC supplementation, the ability of spermatozoa to undergo acrosome reaction was evaluated in the presence of an acrosome reaction inducing substance, lysophosphatidylcholine. The percentage of sperm that underwent acrosome reaction in CLC-supplemented group was lower ($P < 0.05$) compared to control. In conclusion, CLC pretreatment enhanced the structural integrity of ram sperm membranes in wide range of osmotic pressures, between 20 and 1500 mOsm/L. Furthermore, CLC pretreatment reduced the ability of ram spermatozoa to undergo acrosome reaction in vitro.

1. Introduction

Cryopreservation of spermatozoa is an important biotechnology for the widespread dissemination of selected male genetic material throughout the world. Despite continuous progress in semen-freezing technology, a considerable proportion of spermatozoa still undergo irreversible damage or dies during freezing. In order to

* Corresponding author. Tel.: +90 256 2470700x109; fax: +90 256 2470720.
E-mail addresses: aksoym@tnn.net, aksoymelih2003@yahoo.com (M. Aksoy).

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minimize this damage, new or modified freezing procedures have been described in different animal species (Chhillar et al., 2012; Emamverdi et al., 2013). Alternatively, technologies were developed to modify plasma membrane components of spermatozoa to improve their survival rate during the cryopreservation process (Purdy and Graham, 2004a).

The plasma membrane is responsible for the preservation of cellular homeostasis; thus, it has a vital role in sperm survival and preservation of the fertility potential in the female reproductive tract. The functional integrity of sperm membrane is crucial for capacitation, acrosome reaction, and successively, binding to the egg surface (Oura and Toshimori, 1990; Flesch and Gadella, 2000).

Cyclodextrins, cyclic heptasaccharides consisting of β-glucopyranose units, have a hydrophobic center and are soluble in water. Cholesterol-preloaded cyclodextrin (CLC) molecules have been used to incorporate cholesterol into the plasma membranes of spermatozoa (Purdy and Graham, 2004b). Elevated cryosurvival rates in stallion (Oliveira et al., 2010), bull (Purdy and Graham, 2004a), bison (Hussain et al., 2013), boar (Blanch et al., 2012), ram (Moce et al., 2010a), buck (Amidi et al., 2010) and mouse (Movassaghi et al., 2009) spermatozoa have been reported following CLC treatment. Although its mechanism of action is not elucidated, it is assumed that the protective impact of CLC treatment results from increased cholesterol contents in plasma membrane, which successively results in modulating membrane fluidity (Crockett, 1998) during freezing or broadened osmotic tolerance limits (Glazar et al., 2009; Aksoy et al., 2010) of spermatozoa.

The cholesterol:phospholipid ratio is regarded as the major factor determining the fluidity and stability of sperm plasma membrane. It is generally expected that animal species that have a low cholesterol:phospholipid ratio in the sperm plasma membrane are sensitive to cold shock and would derive the maximum benefit from CLC pretreatment. However, in contrast to expectations, spermatozoa of different animal species, including those with a low cholesterol:phospholipid ratio, may respond to CLC treatment differently (Moce et al., 2010b). In ram spermatozoa, although CLC treatment was reported to enhance cryotolerance and the osmotic limits where spermatozoa remained motile (Moce et al., 2010a); the impact of CLC pretreatment on the functional integrity of plasma membranes during osmotic challenges is not documented. Thus, the objective of the present study is to examine the impact of CLC pretreatment on the structural integrity of sperm plasma membranes during incubation in both hypo- and hyperosmotic conditions successively, and on the ability of treated spermatozoa to undergo acrosome reaction in vitro.

2. Materials and methods

2.1. Chemicals

All chemical used in this study were obtained from Sigma Chemical Company (St. Louis, MO, USA).

2.2. Animals

The study was carried out during January to April 2012. Three regular semen donor Kivircik rams were used. They were maintained at the experimental animal shed of the Department of Reproduction and AI, Adnan Menderes University, Aydin, Turkey, under optimum feeding and management conditions. Approval for use of the experimental animals was obtained from the ethical committee of Adnan Menderes University (Approval No. 124-HEK/2009/026) before the start of study.

2.3. Semen collection and evaluation

Semen was collected using an electroejaculator and a total of 17 ejaculates (6 or 5 ejaculates per ram) were collected throughout the study period. The volume of each ejaculate was recorded immediately after collection and diluted 10-folds (v/v) in TCG extender (Moce et al., 2010a). Sperm motility was assessed at 200× magnification using a phase contrast microscope (TMS Nikon, Tokyo, Japan) equipped with a heated stage adjusted to 37 °C. Sperm concentration was determined by using a Thoma counting chamber after dilution of a portion of semen in tap water (1:200, v/v). The percentage of morphologically abnormal spermatozoa in each ejaculate was determined on wet mount slides using two or three drops of semen diluted in Hancock's solution (Hancock, 1952) with use of a phase-contrast microscope at 1000× magnification. The ejaculates with a progressive motility higher than 60% and abnormal sperm rate lower than 20% were used in all replicates of both experiments.

2.4. CLC preparation

The CLC was prepared as described previously (Purdy and Graham, 2004a). Briefly, cyclodextrin solution (1 g methyl-β-cyclodextrin and 2 mL methanol) and cholesterol solution (200 mg cholesterol and 1 mL chloroform) were prepared in separate glass tubes. A 0.45 mL portion of the cholesterol solution was added to the cyclodextrin solution and mixed. A stream of nitrogen gas was then passed over the solution and the solvents were allowed to evaporate to obtain a white CLC powder. The CLC stock solution was prepared by dissolving 50 mg CLC powder in 1 mL Tris–citric acid–glucose (TCG) buffer (300 mM Tris base, 95 mM citric acid monohydrate, and 28 mM glucose, pH 7.0). The solution was held in a water bath at 39 °C for 10 min and then vortexed and sonicated for 30 min. Finally 3 mg/mL BSA was added to obtain a working CLC solution as reported earlier (Aksoy et al., 2010).

2.5. Experiment 1

This experiment was designed to investigate the effect of CLC pretreatment on the plasma membrane integrity of ram spermatozoa exposed to different osmotic challenges. The ejaculates ($n = 9$) were extended with TCG solution to make a final concentration of 400×10^6 spermatozoa/mL and then divided into two aliquots. The first aliquot was treated with CLC ($3\,mg/120 \times 10^6$ spermatozoa), whereas in the second aliquot, an equal volume of TCG was added to equalize the volume of both aliquots. The aliquots were then incubated at 35 °C for 15 min. The osmotic tolerance of spermatozoa from both aliquots was determined as described previously (Aksoy et al., 2010). Briefly a 25 μL sperm sample from each aliquot was added to 475 μL of one of the five fructose solutions, adjusted to 20, 80, 290, 500, or 1500 mOsm/L. They were incubated at 35 °C for 15 min. The hyperosmotic incubation samples (500 and 1500 mOsm/L), were returned to hypo-osmotic condition by resuspending the sperm pellet in 80 mOsm/L fructose solutions after centrifuging ($300 \times g$ for 5 min) and removing the supernatant. These samples were again incubated for 15 min at 35 °C. After osmotic challenges, the live sperm rate and the sperm plasma membrane integrity were estimated by using hypo-osmotic swelling test associated with supravital eosin staining (HE test) as described earlier (Ducci et al., 2002). Two slides from each osmotic challenge group were prepared, and total of 100 spermatozoa were counted from each slide to determine the percent live (unstained heads of spermatozoa with curled or straight tails), intact (curled tails with stained or unstained heads of spermatozoa), and live-intact (unstained heads of spermatozoa only with curled tails) spermatozoa at 400× magnification.

2.6. Experiment 2

This experiment was designed to determine the effect of CLC pretreatment on the ability of ram spermatozoa to undergo acrosome reaction after exposure to lysophosphatidylcholine (LPC), an acrosome reaction inducing agent. After initial evaluation, the ejaculates ($n = 8$) were diluted in 10 mL TCG and centrifuged twice at $800 \times g$ for 5 min to remove seminal