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Cholesterol-loaded cyclodextrin inhibits premature acrosomal reactions in liquid-stored rabbit spermatozoa

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ABSTRACT

The effect of pretreatment of rabbit sperm cells with different concentrations of cholesterol-loaded cyclodextrin (CLC) on the occurrence of premature acrosome reactions during 72 h of liquid storage was investigated in three successive experiments. The aim of the first experiment was to establish a liquid storage model to facilitate premature acrosome reactions in rabbit sperm cells and, therefore, examined the relative effects of different dilution rates (1:5, 1:25 or 1:50) and storage temperatures (4 °C or 35 °C) on the occurrence of premature acrosome reactions. Increasing both dilution rate (from 1:5 to 1:25; $P<0.05$) and storage temperature (from 4 °C to 35 °C; $P<0.0001$) significantly enhanced the percentage of sperm cells that underwent premature acrosome reactions during storage. Therefore, a constant dilution rate of 1:25 and storage temperature of 35 °C was employed for the rest of the study.

The second experiment examined the effect of different CLC concentrations (0, 0.5, 1.0 and 3.0 mg per 120×10^6 spermatozoa) on the occurrence of premature acrosome reactions in sperm cells. CLC supplementation of the extender inhibited ($P<0.001$) premature acrosome reactions in sperm cells in a dose-dependent manner during 72 h of storage.

In the third experiment, the ability of CLC-pretreated sperm cells to undergo acrosome reactions induced by lysophosphatidylcholine (LPC) was evaluated following 72 h of storage. A considerable proportion of sperm cells pretreated with CLC (between 68.7 and 91.8%) underwent the acrosome reaction in response to LPC following 72 h of liquid storage. However, the ability of the sperm cells to undergo the acrosome reaction varied with regards to the dose levels of CLC pretreatment ($P<0.001$).

In conclusion, CLC supplementation prevents premature acrosome reactions in liquid-stored rabbit spermatozoa.

1. Introduction

Mammalian spermatozoa do not exhibit the ability to fertilise eggs immediately after ejaculation. They undergo a variety of modifications, including rearrangement of the membrane, alteration of motility pattern and change of metabolic activity, during the several hours they remain in the female genital tract. These physiological changes are collectively called capacitation (Yanagimachi, 1994). Capacitation is induced by the action of the fluid components of the female genital tract, including bicarbonate and calcium ions (Visconti et al., 1998). Consequently, spermatozoa become capable of undergoing the acrosome reaction and acrosomal exocytosis in response to physiological factors such as progesterone and zona pellucida glycoprotein (Kirkman-Brown et al., 2002; Wassarman, 2005). However,

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some sperm cells may undergo the acrosome reaction prematurely before coming into contact with the egg (Florman and Storey, 1982).

Correct timing of the acrosome reaction is an essential prerequisite for fertilisation in mammals. Premature or early acrosome reaction reduces the ability of sperm cells to penetrate the granulosa cell layer surrounding the oocyte and, consequently, to attach to the zona pellucida because of the progressive loss of the acrosomal enzymes (Bedford and Cross, 1998). Moreover, the state of "hyperactivation" that occurs with capacitation is characterised by a drastic change in sperm motility. This process also increases metabolism and energy use, which, in turn, may shorten the lifespan of sperm cells due to increased oxidative stress (Villegas et al., 2003).

It is well documented that cryopreservation affects the protein and lipid components of sperm membranes. Some of these molecular alterations lead to premature capacitation and, subsequently, premature acrosome reaction (Bailey et al., 2000). Kuroda et al. (2007) reported that premature capacitation is an important causal factor among subfertile Japanese black bulls used for artificial insemination. They suggested that prematurely capacitated spermatozoa underwent a spontaneous acrosome reaction due to an uncontrolled influx of calcium ions and, subsequently, exhibited significantly lower penetration rate into oocytes.

Likewise, Holt et al. (1997) stated that the higher responsiveness of boar spermatozoa to the capacitating agent A23187 is associated with lower litter size. They suggested that premature acrosome reactions may result in fewer spermatozoa being available to interact with the oocyte at the site of fertilisation after artificial insemination. Recent studies have revealed that premature acrosome reaction is also involved in the aetiology of the seasonal infertility in boars. Murase et al. (2007) reported that responsiveness of sperm acrosomes to capacitating agents (Ca^{2+} and A23187) varies with regards to the season with maximum sensitivity in the summer months.

In some wild ruminant species premature capacitation is regarded as one of the main obstacles to the large-scale use of frozen-thawed spermatozoa to preserve genetic biodiversity. Mejia et al. (2009) reported that in Barbary sheep (Ammotragus lervia) the proportion of prematurely capacitated spermatozoa in post-thaw samples was around 80% and this was an important factor that reduces fertility rates from artificial insemination.

Cholesterol is the major sterol in the ejaculated sperm of most animal species and is involved in the capacitation process. The cholesterol content of sperm gradually decreases when they are exposed to the capacitating media (Go and Wolf, 1985). Adding cholesterol to the medium has been shown to prevent the acrosome reaction in sperm cells (Zarintash and Cross, 1996).

Cyclodextrins are cyclical oligomers of six to eight glucose molecules that can solubilise hydrophobic molecules, such as cholesterol, because of their hydrophobic interior. Recently, the use of cholesterol preloaded methyl-cyclodextrin was proposed to facilitate cholesterol transfer into the plasma membranes of bull spermatozoa (Purdy and Graham, 2004b).

The objective of the present study was to evaluate the effect of different dose levels of cholesterol-loaded cyclodextrin (CLC), as a cholesterol provider to sperm membranes, to inhibit premature acrosome reactions in rabbit spermatozoa during liquid storage.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Merck (Darmstadt, Germany). Bovine Serum Albumin (BSA), cholesterol, chloroform, methyl-β-cyclodextrin and lysophosphatidylcholine (LPC) were obtained from Sigma Chemical Company (St. Louis, MO, USA).

2.2. Cholesterol-loaded cyclodextrin

The CLC was prepared as previously described by Purdy and Graham (2004a), however, the solution was sonicated in addition to being vortexed. Briefly, 1 g methyl-β-cyclodextrin was dissolved in 2 ml methanol and the cholesterol solution was prepared by dissolving 200 mg cholesterol in 1 ml chloroform in a separate tube. A 0.45 ml aliquot of the cholesterol solution was added to the cyclodextrin solution and mixed. The solution was then placed under a stream of nitrogen gas to evaporate the solvents and obtain a white CLC powder. The CLC was stored in a glass container at room temperature. A stock solution was prepared by dissolving 50 mg CLC powder in 1 ml tris–citric acid–glucose (TCG) extender containing 313.8 mM tris, 103.1 mM citric acid and 33.3 mM glucose (Roca et al., 2000). The TCG extender used for CLC preparation also contained 5 mg/ml BSA. The solution was placed in a water bath at 37 °C for 5 min, then vortexed and sonicated to obtain a working CLC solution.

2.3. Animals and semen collection

Approval for the animal experiments in this study was obtained from the Local Ethical Committee. Four New Zealand white rabbit bucks between the ages of two and four years were used as sperm donors. The bucks were housed in individual cages, with water and food provided *ad libitum* and no special lighting regimen was employed throughout the study. Semen samples were collected by artificial vagina and gel plugs in semen were removed immediately after collection.

2.4. Semen examination

Using a phase-contrast microscope with a stage heated to 37 °C at 400× magnification, individual sperm motility was assessed after semen samples were diluted by 10–12-fold (v/v) in TCG extender supplemented with 1000 IU/ml penicillin and 1 mg/ml streptomycin (Roca et al., 2000). Motility estimations of each sample were performed in three different fields by the same person throughout the study. A mean value was averaged from three successive estimations and used as the final motility score.

Sperm concentration per milliliter of semen was determined haemocytometrically as described by Evans and Maxwell (1987) using a Thoma counting chamber after dilution to 1:200 (v/v) in tap water.

The ejaculates with a progressive motility lower than 70% were discarded and were not used in experiments.

2.5. Experiment 1

The first experiment was conducted to establish an experimental liquid storage model to facilitate premature acrosome reactions (to examine the influence of CLC) in rabbit spermatozoa. The effects of three different dilution rates (1:5, 1:25 or 1:50) and two storage temperatures (4 °C or 35 °C) on the occurrence of premature acrosome reactions during 72-h of storage were examined.

The semen samples were divided into three equal aliquots and were further diluted with TCG extender to either 1:5, 1:25 or 1:50 in 10 ml conical tubes at 35 °C. The extended samples were incubated at either 4 °C, after cooling gradually from 35 °C to 4 °C for 2–2.5 h in a water bath, or directly at 35 °C for 72 h. The percentage of sperm cells that underwent premature acrosome reactions was determined at 24-h intervals throughout the storage period.

To identify acrosome-reacted cells, spermatozoa were stained using a Coomassie Blue G-250 staining procedure as described by Larson and Miller (1999). Briefly, sperm cells that had been incubated in TCG extender were fixed with 4% paraformaldehyde solution (110 mM Na_2HPO_4, 2.5 mM NaH_2PO_4, 4% paraformaldehyde, pH 7.4) for 10 min at 24 °C. Sperm were centrifuged and washed twice using 1.5 ml of 100 mM ammonium acetate (pH 9.0). The final sperm pellet was resuspended in 10–30 μl of 100 mM ammonium acetate, and 6–8 μl of the sperm suspension was smeared on to glass microscope slides using another glass slide and then air dried. Sperm on the slides were incubated in freshly made Coomassie stain (0.22% Coomassie Blue G-250, 50% methanol, 10% glacial acetic acid, 40% water) for 40 min at room temperature. The slides were then washed thoroughly with distilled water to remove any excess stain and air dried. Stained spermatozoa were examined under bright field microscopy at 1000× magnification. A minimum of 10 fields per slide and a total of two slides (a total of 400 spermatozoa) per experiment were observed.

2.6. Experiment 2

The second experiment was designed according to the results obtained in the first experiment. The main objective was to evaluate the effect of CLC supplementation of the TCG extender on the ability of sperm cells to undergo premature acrosome reactions during 72 h of liquid storage. Fresh semen samples were diluted to 1:25 in 10 ml conical tubes in TCG extender at 35 °C and were further divided into four equal aliquots. The aliquots were supplemented with CLC at a dose level of either 0 (control), 0.5, 1.0 or 3.0 mg per 120×10^6 spermatozoa and were incubated for 72 h in a water bath adjusted to 35 °C. The percentage of the sperm cells that underwent premature acrosome reactions was determined by 24-h intervals throughout the storage period by using Coomassie Blue G-250 staining procedure as described before.

2.7. Experiment 3

Experiment 3 was performed according to the results obtained in the experiments 1 and 2. The objective of the experiment was to evaluate the ability of sperm cells to undergo acrosome reactions after treatment with a capacitating agent LPC and after exposure to different CLC concentrations in the liquid storage media before 72 h of storage at 35 °C.

The ejaculates were diluted to 1:25 in 10 ml conical tubes in TCG extender at 35 °C and were further divided into four equal aliquots. The aliquots were supplemented with CLC at a dose level of either 0 (control), 0.5, 1.0 or 3.0 mg per 120×10^6 spermatozoa and were incubated for 72 h at 35 °C. After the storage period, the aliquots were supplemented with LPC and incubated for 60 min at 35 °C. LPC from a 1 mg/ml stock in TCG solution was mixed into the sample to make a final concentration of 100 μg/ml. To identify cells that had undergone the acrosome reaction after exposure to LPC, spermatozoa were stained using the Coomassie Blue G-250 staining procedure as stated above.

2.8. Statistical analyses

Each experiment was replicated at least seven times. The mean values were calculated simply by averaging the results obtained in the replicated experiments and were presented together with the standard errors throughout the study. In the first and second experiments, the data were initially analysed by a two-way analysis of variance (ANOVA) to reveal potential interactions between the variables (dilution rate and storage temperature in the first experiment and CLC dose level and duration of storage in the second experiment) and the differences between the mean values of the main effects. The percentages of sperm cells that had undergone acrosome reactions in the control and treatment groups were compared by using one-way ANOVA in all three experiments. The statistical differences were regarded as significant when P values were less than 0.05.

3. Results

3.1. Experiment 1

A significant interaction was found between the storage temperature and dilution rate on the occurrence of premature acrosome reactions in sperm cells during the storage period ($P < 0.0001$). Storage temperature ($P < 0.0001$) of the ejaculate and the dilution rate ($P < 0.05$) both significantly affected the percentage of sperm cells prematurely undergoing acrosome reactions. Elevation of the storage temperature from 4 °C to 35 °C resulted in a drastic increase in the percentage of premature acrosome-reacted sperm cells in the extended storage periods of up to 72 h (Fig. 1). This increase was more prominent at high dilution rates ($P < 0.05$) such as 1:25 and 1:50.

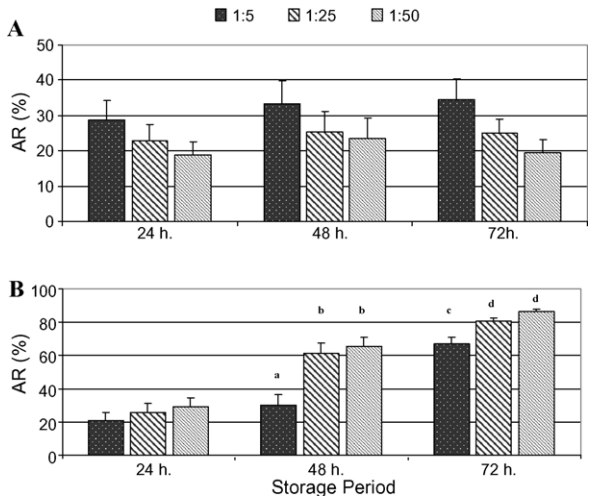

Fig. 1. Effect of dilution rate, 1:5, 1:25 or 1:50, and storage temperature: (A) 4 °C and (B) 35 °C on the occurrence of premature acrosome reactions in sperm cells during 72 h of storage (a, b: $P < 0.01$ and c, d: $P < 0.001$).

Based on these results, a constant dilution rate of 1:25 and storage temperature of 35 °C were chosen as the experimental storage conditions that best facilitate premature acrosome reactions of rabbit spermatozoa for the second experiment.

3.2. Experiment 2

CLC supplementation of the TCG extender at different dose levels resulted in a significant decrease in the percentage of premature acrosome-reacted sperm cells during 72 h of storage ($P < 0.001$). The maximum inhibitory effect on the occurrence of the premature acrosome reaction was obtained in the group pretreated with 3 mg CLC (Fig. 2). There was an interaction between the CLC dose level and period of storage on the occurrence of premature acrosome reactions in sperm cells ($P < 0.001$).

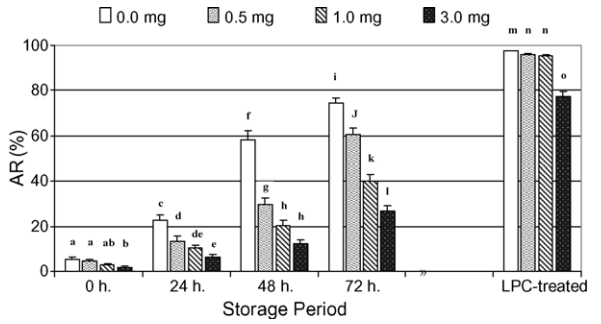

Fig. 2. Effect of different dose levels of CLC-pretreatment (0, 0.5, 1.0 or 3.0 mg per 120×10^6 spermatozoa) on the occurrence of premature acrosome reactions in sperm cells during storage and the ability of sperm cells to undergo acrosome reactions induced by LPC following 72 h of storage (a, b: $P < 0.05$; c, e: $P < 0.001$; f, h: $P < 0.001$; i, l: $P < 0.001$; m, o: $P < 0.0001$).

3.3. Experiment 3

Following 72 h of storage, a considerable proportion of CLC-pretreated sperm cells (between 68.7% and 91.8%) underwent the acrosome reaction after LPC treatment (Fig. 2). However, the ability of the sperm cells to undergo acrosome reactions varied with regards to the dose levels of CLC pretreatment ($P < 0.001$).

4. Discussion

The seminal plasma is regarded as the main source of various factors that are involved in the decapacitation or acrosome stabilisation in sperm cells (Dukelow et al., 1967). Dilution of those factors beyond a certain limit is expected to alter the structure and function of sperm membranes. In practice, excessive dilution of semen causes loss of motility, metabolic activity and fertilising ability of sperm cells (Maxwell and Johnson, 1999). In the present study, the effect of the dilution rate (even high rates up to 1:50) on the percentage of sperm cells prematurely undergoing acrosome reactions was not as prominent as the effect of storage temperature ($P < 0.05$ vs. $P < 0.0001$). This might be a result, at least in part, of the relatively high cholesterol concentration and considerably high cholesterol to phospholipid (0.88) molar ratio (Watson, 1981) of rabbit sperm membranes. Moreover, the presence of a large number of tocopherol-rich (De Lamirande et al., 1997) and cholesterol-rich (Castellini et al., 2006) seminal granules are reported as a peculiar property in rabbit semen. Castellini (2008) speculated that those seminal granules may act to prevent premature capacitation and to reduce the ability of sperm cells to undergo acrosome reactions in rabbits.

Elevating the storage temperature from 4 °C to 35 °C in the first experiment resulted in a drastic increase in the percentage of sperm cells prematurely undergoing acrosome reactions. Although it has not been documented in rabbit spermatozoa, the stimulatory effect of elevated incubation temperatures (between 37 and 44 °C) on sperm activation and acrosome reaction was reported previously in hamster (Mahi and Yanagimachi, 1973), guinea pig (Fleming and Kuehl, 1985) and human (Kraemer et al., 1998) spermatozoa. Theoretically, Bedford and Yanagimachi (1991) described capacitation as an escape from the stable state imposed as a function of sperm storage in the cauda epididymis. They have postulated that elevated sperm storage temperatures (such as 37 °C) may lead to a suppression of the stabilising function of the particular macromolecules that normally bind the sperm surface during the epididymal passage.

Cholesterol plays an important role in stabilising sperm membranes. The sperm membranes lose cholesterol during capacitation. Loss of sterols begins just after the removal of sperm cells from seminal plasma and is obligatory for the capacitation of human sperm (Cross, 1996). Incubation of sperm cells in media containing high level of sterols, to maintain high sterol concentrations in spermatozoa, inhibits progesterone and calcium ionophore-induced acrosome reactions in human (Cross, 1996). The sperm cholesterol content gradually decreases when sperm cells

are incubated in a capacitating medium *in vitro* (Go and Wolf, 1985). The loss of cholesterol is initially linear, but acrosomal responsiveness begins after several hours, suggesting that cholesterol loss precedes the development of responsiveness (Zarintash and Cross, 1996).

Likewise, in the second experiment of the present study, supplementation of the TCG extender with CLC reversibly inhibited the acrosome reaction in a dose-dependent manner in sperm cells during storage. This data may have some practical implications for sperm storage technology because both cryopreservation (Cormier et al., 1997) and liquid storage (Zou and Yang, 2000) induce capacitation-like changes in sperm cells. In some wild ruminants those membrane changes may be so influential that the percentage of prematurely capacitated sperm cells may increase up to 80% in post-thaw samples and, thus, may reduce pregnancy rates (Mejia et al., 2009). Moreover, elevation of the environmental temperature (Murase et al., 2007) or exposing the sperm cells to relatively high temperatures, such as body temperature of 37 °C (Bedford and Yanagimachi, 1991), was reported to increase the sensitivity of acrosomal membranes, leading to premature capacitation in sperm cells. Therefore, it is possible that increasing the cholesterol concentration of sperm membranes with CLC pretreatment may minimise fertility problems resulting from the hot season ejaculates and problems with inseminations performed using frozen-thawed semen, including the high proportion of sperm cells that prematurely underwent acrosome reactions. A similar inhibitory effect of quercetin, an antioxidant flavonoid, and the low bush blueberry extracts on capacitation of stallion (McNiven and Richardson, 2006) and boar (Desroches et al., 2005) spermatozoa has been demonstrated in previous studies. However, CLC technology may have additional advantages over them because it is easily stored at room temperature and includes a constant level of active substance in all batches or preparations.

In experiment 3 of this study, the ability of sperm cells to undergo acrosome reactions induced by LPC after storage varied significantly with regards to the dose level of CLC pretreatment. Although the inhibitory effect of CLC treatment on premature acrosome reactions increased in correlation with the dose levels (from 0.5 to 3 mg) the reduction in the ability of sperm cells to respond to LPC treatment was not equally correlated with CLC levels. In this study, 1 mg CLC provided a relatively high number of sperm cells (compared with 3 mg) capable of undergoing acrosome reactions after 72 h of storage (data is not presented). Thus, calculation of the optimum CLC dose level that prevents sperm capacitation for a transient period during storage without impairing the final capacitation and acrosome reaction processes that take place following deposition of semen seems to be critical in CLC technology. At higher dose levels, the inhibitory effect of CLC on the ability of sperm cells to undergo capacitation or acrosome reactions was reported in bovine (Purdy and Graham, 2004a), horse (Spizziri et al., 2010), human (Khorasani et al., 2000) and rabbit (Aksoy et al., 2010) spermatozoa.

In conclusion, CLC supplementation prevents premature acrosome reactions in liquid-stored rabbit spermatozoa.

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