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Short communication

Effect of different sugars supplemented to the extender in combination with cholesterol-loaded cyclodextrin (CLC) on post-thaw quality of ram spermatozoa

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ABSTRACT

The present study was designed to determine the effect of different sugars supplemented to the extender on post-thaw quality of CLC treated ram sperm. The ejaculates ($n = 10$) were divided into two aliquots: first aliquot was treated with CLC (3 mg/120 × 10^6 sperm), whereas, second aliquot remained untreated and served as control. The samples were then incubated at 35 °C for 15 min. After incubation, each aliquot was again divided into 3 equal subgroups. Each of the subgroups was then diluted with one of the extenders containing fructose, sucrose or trehalose (28 mM) to make a final sperm concentration 200 × 10^6/ml. After dilution, the semen samples were loaded into 0.25 ml straws and cryopreserved using standard procedure. The data showed that type of the sugar did not affect post-thaw sperm parameters ($P > 0.05$). However, the CLC pre-treatment improved ($P < 0.05$) motile, live, live-intact, total intact and the number of total active sperm per straw. A non-significant interaction ($P > 0.05$) was determined between type of sugar and CLC. In conclusion, CLC pre-treatment significantly improved post-thaw sperm parameters in ram and has no interaction with any of the used sugars.

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1. Introduction

Cryopreservation is a common biotechnology which allows preserving living cells, organisms and tissues for extended period of time. Different types of cells including sperm have been preserved successfully by using various freezing protocols. In order to improve post-thaw sperm quality and to overcome the detrimental effects of cryopreservation, many efforts have been made by optimizing sperm freezing technology in variety of species such as bull (Taşdemir et al., 2013), ram (Ahmad et al., 2015), goat (Küçük et al., 2014), stallion (Fayrer-Hosken et al., 2008) and dog (Yildiz et al., 2000).

Cryopreservation exerts detrimental effects to sperm in terms of thermal, mechanical, chemical and osmotic stressors (Ollero et al., 1998). The cryodamage is generally associated with the phase change of water in extra and intracellular environment during freezing and thawing (Mazur, 1984). This in- or outflow of water adversely affects the sperm plasma membrane which is the primary vulnerable site for cryodamages (Bailey et al., 2003). However, the susceptibility of sperm membrane to cryodamage is varying among different species. This variation might be owing to the unique fatty acid composition of sperm plasma membrane in each species (Mandal et al., 2014). The ram sperm are highly sensitive to the cryo-induced damages as its membrane is composed of higher unsaturated phospholipids and has lower in cholesterol/phospholipid molar ratio (Curry and Watson 1994).

Until now, a variety of extenders have been used aiming to enhance cryosurvival rate with reduced membrane damages of ram spermatozoa (Jafaroghli et al., 2011). Additionally, different monosaccharaides (glucose, fructose) or/and disaccharides (sucrose, trehalose) have also been incorporated into the extenders (Bucak et al., 2007; Quan et al., 2012; Cirit et al., 2013; Panyaboriban et al., 2015) to achieve better post-thaw results. The impact of monosaccharaides and disaccharides sugars supplementation is variable during the cryopreservation. The disaccharides are advantageous over the monosaccharaides due to better osmotic dehydration induction and circumvention of intracellular ice crystallization capacity (Molinia et al., 1994; Aisen et al., 2002).

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Amongst disaccharides, the trehalose provides a better protection during freezing and thawing by interacting with the phospholipid polar head groups of plasma membrane by reducing the van der Waals interactions among the hydrocarbon chain (reviewed by Ahmad and Aksoy, 2012). In addition, trehalose increases membrane fluidity (Aboagla and Terada, 2003), osmotic tolerance and decreases premature acrosome reaction (Ahmad et al., 2015) of sperm during freezing and thawing. Likewise, the CLC has been reported as modulator of sperm membrane which enhances the sperm cryosurvival rate (Purdy and Graham, 2004a) by increasing membrane fluidity (Purdy et al., 2005), widening osmotic tolerance limit (Ahmad et al., 2013), reducing premature acrosome reactions (Amidi et al., 2010; Serin et al., 2011) and protecting against oxidative stress (Naseer et al., 2015). In this background, it was hypothesized that the sugars might have positive interaction with CLC in order to enhance the cryosurvival rate of ram sperm. Therefore, the present study was designed to investigate the effects of supplementation of different sugars (fructose, sucrose and trehalose) to the extender on post-thaw quality of CLC treated ram sperm.

2. Materials and methods

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

2.1. CLC preparation

The CLC was prepared as described previously (Purdy and Graham, 2004b). The CLC powder was diluted in TCG buffer (300 mM Tris, 95 mM citric acid, 28 mM glucose). The solution was vortexed, sonicated and finally BSA (3 mg/ml) was added to obtain a working CLC solution (Aksoy et al., 2010).

2.2. Animals and semen collection

Five mature Kıvırcık rams were used in this study. The animals were maintained at the Experimental Animal Unit, Department of Reproduction and Artificial insemination, Faculty of Veterinary Medicine, Adnan Menderes University under optimum managemental conditions. A total of ten ejaculates (2 ejaculates per each ram) were collected using electroejaculator. An approval was taken from the local ethical committee of Adnan Menderes University (2012/025) to use animals for the experiments.

2.3. Cryopreservation of semen

The ejaculates ($n = 10$) with a progressive motility ≥70%, concentration $\geq 1000 \times 10^6$ sperm/ml and abnormal sperm rate ≤20% were used for cryopreservation. Each ejaculate was divided into two aliquots. First aliquot was treated with CLC (3 mg/120 $\times 10^6$ spermatozoa), whereas, in the second aliquot, an equal volume of Tris-citric acid-glucose (TCG) was added to equalize the total volume of both groups. The samples were then incubated at 35 °C for 15 min. After incubation, each aliquot was further divided into 3 equal subgroups. Each of the subgroups was then diluted with one of the extenders containing fructose, sucrose or trehalose (28 mM) to a concentration of 200×10^6 sperm/ml. All extenders were composed of Tris (300 mM), citric acid (95 mM), egg yolk (14% v/v) and glycerol (5% v/v) (Molinia et al., 1994). After dilution, the semen samples (50×10^6 sperm/straw) were loaded into 0.25 ml straws and were equilibrated at 4 °C for 2 h. The straws were placed horizontally over liquid nitrogen vapour (5 cm above the liquid nitrogen level) for 10 min and then plunged into the liquid nitrogen for storage.

2.4. Determination of post-thaw sperm quality

For post-thaw sperm analysis, two straws per each replicate for each subgroup were thawed for 30 s in a water bath at 37 °C. The sperm quality parameters were evaluated immediately after thawing.

Motility was assessed subjectively by using a phase contrast microscope equipped with a warm stage adjusted 37 °C. Percentage of live sperm was determined using eosin-nigrosin staining. A total of 200 sperm counted and classified as live (unstained heads) or dead (stained heads) from each slide at ×400. Morphologically abnormal sperm rate was determined by fixing 10 μl portion of sperm sample in 1 ml of Hancock's solution. A 10 μl of this suspension was observed under a phase-contrast microscope at ×1000 and 200 sperm were counted for abnormalities. Sperm plasma membrane integrity was assessed by a modified hypoosmotic swelling test in combination with eosin (HE-test) staining (Aksoy et al., 2008). A total of 200 spermatozoa were examined from each slide to determine percentage of total intact (curled tails with stained or unstained heads of sperm) and live-intact (unstained heads of sperm only with curled tails) sperm under a bright field microscope. The proportion of acrosome reacted sperm was determined by the method described by Coy et al. (2002) with minor modifications. At least 200 sperm from each sample were counted for the presence or absence of fluorescence emitted by FITC-PNA. The sperm with a green fluorescence were classified as acrosome-reacted, whereas sperm with intact acrosome in the same field did not appear under epifluorescent optic and hence counted under differential interference contrast optic.

2.5. Determination of total active sperm per straw (sperm $\times 10^6$/straw)

After the initial statistical analysis, when no interaction was found between the groups, thus the total active sperm number were calculated. The applied formula is as follows: [Total active sperm number per straw = number of sperm per straw (50×10^6) × motile sperm (%) × live sperm (%) × morphologically normal sperm (%) × live-intact sperm (%) × non reacted sperm (%)].

2.6. Statistical analysis

All the data were analyzed using a statistical program (SPSS version 17; SPSS Inc., Chicago, IL). Initially, the two-way ANOVA was applied to determine the possible interactions between types of sugar × CLC treatment and ram × all treatment. The data were then averaged and all the post-thaw sperm parameters (motility, viability, membrane integrity, reacted acrosome, abnormal sperm rate and the number of total active sperm per straw) were compared between groups by using one-way ANOVA. Duncan's multiple range test (DMRT) was used to determine significance by fixing probability level at $P < 0.05$. In case, no interaction was determined between the variables i.e. types of sugar × CLC, the independent-samples *t*-test was used to compare the post-thaw sperm parameters between the experimental groups. Significance was determined by fixing probability level at $P < 0.05$. All the results are presented as mean ± SEM.

3. Results

Type of the sugar did not affect post-thaw sperm parameters ($P > 0.05$). However, CLC pre-treatment of the sperm significantly ($P < 0.05$) improved the studied post-thaw parameters compared to control irrespective of sugar supplementation (Table 1). A non-significant interaction ($P > 0.05$) was determined between sugar type and CLC. Therefore, the main effect of CLC was calculated and

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