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Cholesterol-loaded cyclodextrin plus trehalose improves quality of frozen-thawed ram sperm

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Abstract: The objective of this study was to determine effects of supplementing Tris-based semen extenders with either cholesterol-loaded cyclodextrin (CLC) or 7-dehydrocholesterol loaded cyclodextrin (7-DCLC) plus trehalose (T) for cryopreservation of ram semen. Semen was collected with an artificial vagina from five Merino rams (2–3 years of age) during the non-breeding season. Ejaculates were pooled, divided into eight equal portions, diluted with a standard Tris-based extender containing: no additive (control); T (50 mM); or T (50 mM) + 1.5, 2.5 or 3.5 mg of either 7-DCLC or CLC. Semen was chilled from 37°C to 4°C, placed in 0.25 ml French straws, held 5 cm above liquid nitrogen for 12 minutes, then plunged into liquid nitrogen. After thawing, a computer-aided semen analyzer system (CASA) was used to assess motility, whereas plasma membrane and acrosome integrity (PMAI) and high mitochondrial membrane potential (HMMP) were assessed with flow cytometry. Sperm supplemented with 2.5 mg and 3.5 mg CLC + T had the highest ($P < 0.05$) total and progressive motility (65.2 ± 4.7 and 19.0 ± 1.0% respectively, mean ± SEM), albeit with no significant differences from sperm with 1.5 or 3.5 mg CLC + T. Sperm with 2.5 mg CLC + T had the highest ($P < 0.05$) PMAI (59.3%; not different from 3.5 mg CLC + T) and highest ($P < 0.05$) HMMP (64.6%; not different from 1.5 or 3.5 mg CLC + T). The lowest ALH value, 2.8 ± 0.3 μm was in the 2.5 mg 7-DCLC + T group; otherwise, there were no significant differences among groups for any other CASA end point. In conclusion, adding CLC + T to a tris-based extender optimized quality of frozen-thawed ram semen. Therefore, extenders including CLC + T have potential to improve quality of frozen-thawed ram sperm.

Keywords: 7-dehydrocholesterol; semen cryopreservation; computer-assisted semen analyzer; flow cytometry

Inefficiencies in collection, cryopreservation and insemination of ram semen compared to bull semen (Cognie et al. 2003) have limited use of artificial insemination in the sheep industry, prompting research to develop novel diluents and freezing methods (Blackburn 2004). Semen cryopreservation alters the cholesterol-phospholipid ratio in sperm plasma membranes, which can decrease sperm fertility (Khan et al. 2017). Ram sperm have a low phospholipid-cholesterol ratio, increasing sensitivity to cryopreservation-induced damage (Parks and Lynch 1992; White 1993). Furthermore, lipid/

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cholesterol ratios of sperm membranes also affect ability to capacitate and undergo an acrosome reaction (Moore et al. 2005).

Various compounds have been added to extenders to enhance freezability of ram semen (Moce et al. 2010). For example, cholesterol decreases phase changes and membrane permeability, providing a more physiological environment for membrane proteins (Aksoy et al. 2010). Addition of cholesterol loaded cyclodextrin (CLC) to semen increased morphological integrity (Mansour 2009), reduced acrosomal and DNA damage (Katanbafzadeh et al. 2014) and semen lipid peroxidation (Lopez-Revuelta et al. 2006) and improved sperm viability, membrane integrity, motility and mitochondrial activity (Moce et al. 2010). Furthermore, 7-dehydrocholesterol (7-DCLC) also improved quality of frozen-thawed ram and chilled bull semen (Inanc et al. 2017; Inanc et al. 2018).

Various monosaccharides and disaccharides have improved quality of frozen-thawed semen (Quan et al. 2012; Panyaboriban et al. 2015). The disaccharide trehalose (T) protects sperm membranes from lipid peroxidation and oxidative damage (Iqbal et al. 2016), perhaps by increasing activity of antioxidant enzymes such as superoxide dismutase, glutathione and catalase (Iqbal et al. 2016).

Regarding cryopreservation of ram semen, there are limited studies of CLC plus T and apparently no studies of varying concentrations of 7-DCLC plus T. The objective was to determine effects of T alone, or in combination with various concentrations of CLC or 7-DCLC, on viability, acrosome integrity, mitochondrial membrane potential, motility and kinetic end-points of frozen-thawed ram sperm.

MATERIAL AND METHODS

Chemicals. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, USA).

Cyclodextrin preparation. Methyl-beta-cyclodextrins (4555) were loaded with cholesterol (C8667) or 7-dehydrocholesterol (SC 214398), as described (Purdy and Graham 2004). Briefly, 1 g of methyl-beta-cyclodextrins was dissolved in 2 ml of methanol in two test tubes. Cholesterol and 7-dehydrocholesterol (200 mg) were each dissolved in 1 ml chloroform. Then, 450 ml cholesterol or 7-dehydrocholesterol were mixed with methyl-beta-cyclodextrins; solvents were evaporated with nitrogen vapor and finally CLC and 7-DCLC powders were obtained. Working solutions were prepared with 50 mg of CLC or 7-DCLC; these were dissolved in 1 ml of stock Tris solution in test tubes at 37°C and vortexed.

Rams. During the non-breeding season, semen was collected with an artificial vagina from five Merino rams (2–3 years old) that belonged to the Bahri Dagdas International Agricultural Research Institute (Konya, Turkey). Five ejaculates were collected from each ram. This study was replicated six times. Animal use was reviewed and approved by the institutional animal care committee (2016/58).

Semen processing. A Tris-based extender (3.63 g Tris (T1503), 1.82 g citric acid (C0759), 0.5 g glucose (G7528)/100 ml distilled water) with 20% egg yolk (v/v) and 6% glycerol was used. Ejaculates were selected according to their initial quality (mass activity ≥ 4; volume ≥ 0.5 ml; sperm concentration ≥ 2×10^9/ml; and motility ≥ 80%) and pooled. Based on a preliminary study and previous experiments, appropriate concentrations of T (T0167), CLC and 7-DCLC were determined. Two groups, one with no additive (control group) and another with only T (50 mM) were diluted in a single stage. However, the remaining six groups were first diluted with a CLC or 7-DCLC working solution of 1.5, 2.5, or 3.5 mg/120 × 10^6 sperm, incubated for 15 minutes at 22°C, and then diluted with Tris-extender (T 50 mM) at 500 × 10^6 sperm/ml. For all groups, extended semen was cooled to 4°C over 2 hours, then loaded into 0.25 ml French straws that were held 5 cm above liquid nitrogen for 12 minutes before being plunged into liquid nitrogen (–196°C). After storage for at least 6 months, straws were thawed in a water bath at 37°C for 30 seconds.

Computer-aided sperm analysis. A computer-aided semen analyzer (CASA; Microptic®, Barcelona, Spain) was used for evaluation of motility and other kinetic end points at 37°C (10 × objective). Motility was evaluated as fast (> 120 μm/s), medium (> 90 μm/s), slow (> 60 μm/s), or static. A 5μl aliquot of semen was placed onto a slide and cover slipped for motility analysis. Progressive motility (%), total motility (%), non-progressive motility (%), beat cross frequency (Hz), amplitude of lateral head displacement (ALH, μm), velocity curve linear (VCL, μm/s), velocity straight line (VSL, μm/s), velocity average path (VAP, μm/s), straightness (STR, VSL/VAP × 100), linearity (LIN,

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VSL/VCL × 100), and wobble (WOB, %) were assessed. For each sample, 250 to 320 sperm were evaluated in six fields (Tirpan and Tekin 2015).

Flow cytometry analysis. Flow cytometry analyses were performed with a Cytoflex Flow Cytometer (Beckman Coulter, Fullerton, USA). Assessments of semen samples were made with a laser beam at 488 nm (50 mW laser output), with 525 ± 40, 585 ± 42 and 610 ± 20 nm emission filters. Data were collected from 10 000 events.

A working solution of 100 µg/ml fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA [L7381], 2.99 mM propidium iodide (PI, [L7011], molecular probes, Invitrogen, Carlsbad, USA) and 0.153 mM 5,5',6,6'-tetrachloro1,1'3,3'-tetramethylbenzimidazolyl-carbocyanine iodide (JC-1, T3198, molecular probes, Invitrogen) was prepared with dimethyl sulfoxide (DMSO), filtered with a 0.22 µm Millipore Millex CV filter, divided equally into 30-µl portions and stored at –20°C.

An FITC-PNA/PI double staining method was used to determine sperm acrosome and plasma membrane integrity. For this, 5 µl of FITC-PNA (100 µg/ml) and 3 µl PI (2.99 mM) were added to 496 µl of buffer solution, prepared with 10 µl of semen previously diluted in phosphate-buffered saline solution, to obtain a final concentration of 5×10^6 sperm/ml.

Mitochondrial status was assessed with JC-1/PI dual staining. Mitochondrial probe (JC-1) fluoresces orange or green when mitochondrial membrane potential is high or low, respectively (Ly et al. 2003). For this assessment, 10 µl JC-1 (0.153 mM) and 3 µl PI (2.99 mM) were added to 487 µl of buffer solution prepared with 10 µl of semen previously diluted in phosphate-buffered saline, to obtain a final concentration of 5×10^6 sperm/ml.

All semen samples were incubated at 37°C for 30 minutes. Debris (non-sperm events) were gated out, enabling analysis of sperm plasma membrane and acrosome integrity (Figure 1) and viable sperm with high mitochondrial membrane potential (Figure 2) with CytExpert 2.2 software (Beckman Coulter, Indianapolis, USA).

Statistical analysis. Prior to data analysis, normality and homogeneity of variances to meet assumptions of parametric tests were verified with a Shapiro-Wilk test. As nonprogressive motility, wobble, plasma membrane and acrosome integrity and high mitochondrial membrane potential violated parametric test assumptions (not normally

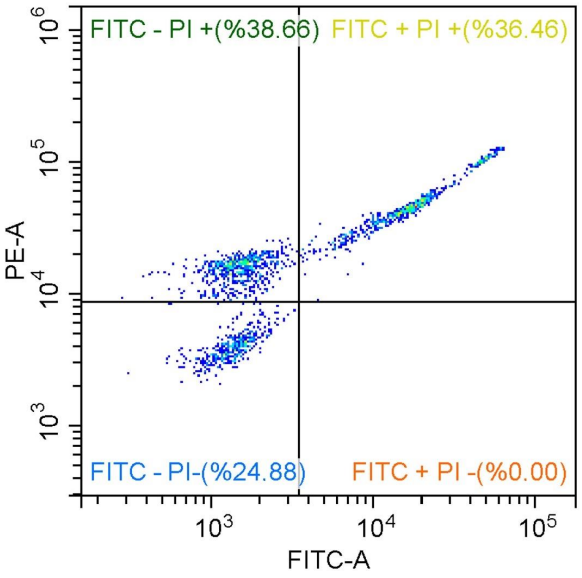

Figure 1. Flow cytometric examination of frozen-thawed ram semen stained with FITC-PNA/PI

FITC-A = fluorescein isothiocyanate-conjugated axis; FITC-PNA/PI = fluorescein isothiocyanate-conjugated peanut agglutinin/propidium iodide; FITC - PI - = plasma membrane and acrosome intact; FITC - PI + = plasma membrane damaged sperm without acrosome staining; FITC + PI - = plasma membrane intact with acrosome staining; FITC + PI + = plasma membrane damaged sperm with acrosome staining; PE-A = propidium iodide axis

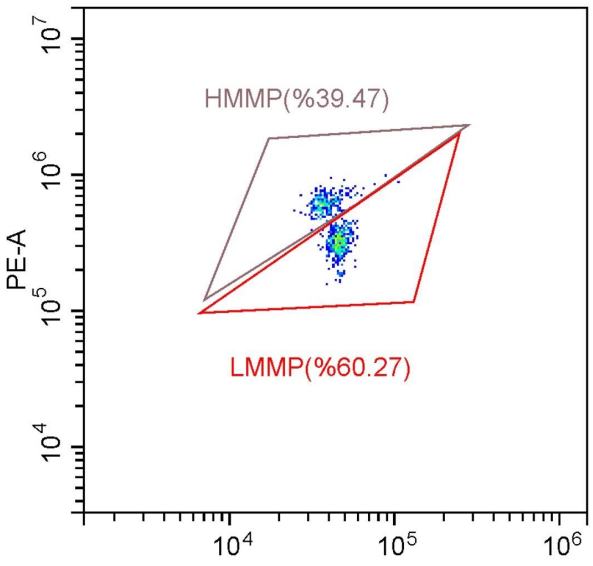

Figure 2. Flow cytometric examination of frozen-thawed ram sperm stained with JC-1

HMMP = high mitochondrial membrane potential; JC-1 = 5,5',6,6'-tetrachloro1,1'3,3'-tetramethylbenzimidazolyl-carbocyanine iodide; LMMP = low mitochondrial membrane potential; PE-A = propidium iodide axis

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Table 1. Mean (± SEM or range) of sperm motility (computer-aided semen analyzer system; CASA), plasma membrane and acrosome integrity (PMAI) and high mitochondrial membrane potential (HMMP) of frozen-thawed ram sperm cryopreserved with various extenders

End point (%)	Control (%)	T (%)	CLC + T 1.5 mg (%)	CLC + T 2.5 mg (%)	CLC + T 3.5 mg (%)	7-DCLC + T 1.5 mg (%)	7-DCLC + T 2.5 mg (%)	7-DCLC + T 3.5 mg (%)
Total motility	25.0 ± 4.2^{a}	21.9 ± 5.0^{a}	50.3 ± 7.7^{bcd}	65.2 ± 4.7^{d}	53.1 ± 4.4^{cd}	27.4 ± 0.5^{ab}	30.0 ± 5.4^{abc}	34.1 ± 4.8^{abc}
Progressive motility	11.6 ± 0.7^{ab}	8.6 ± 0.9^{ab}	15.5 ± 2.3^{bc}	17.0 ± 1.5^{bc}	19.0 ± 1.0^{c}	7.6 ± 0.2^{a}	9.3 ± 1.3^{a}	11.2 ± 1.4^{ab}
Nonprogressive motility	17.5 (11.9–23.7)^{a}	19.1 (12.2–27.8)^{a}	40.5 (33.6–48.7)^{b}	48.2 (40.5–56.4)^{b}	38.0 (33.7–43.0)^{b}	22.8 (18.6–20.4)^{a}	18.3 (9.6–29.6)^{a}	27.4 (14.2–33.7)^{a}
PMAI	10.3 (10.0–10.7)^{b}	9.3 (6.1–13.6)^{a}	45.4 (43.6–46.7)^{c}	59.3 (57.8–62.0)^{d}	56.1 (49.3–66.7)^{d}	18.0 (14.5–22.8)^{ab}	23.4 (16.1–28.6)^{b}	26.7 (21.4–34.7)^{b}
HMMP	10.4 (7.8–15.5)^{a}	14.2 (13.5–14.6)^{a}	51.5 (41.8–64.1)^{b}	64.6 (61.1–66.6)^{b}	56.2 (40.8–76.6)^{b}	11.0 (8.4–13.0)^{a}	23.0 (4.4–46.9)^{a}	24.5 (16.7–33.9)^{a}

7-DCLC = 7-dehydrocholesterol loaded cyclodextrin; CLC = cholesterol-loaded cyclodextrin; T = trehalose

$^{a–d}$Within a row, means without a common superscript differed ($P < 0.05$)

distributed), they were analysed with a Kruskal Wallis test and data reported as mean and range. All other end points met parametric test assumptions and were analyzed with one-way analysis of variance (ANOVA), with a Tukey test used to locate differences between groups and data reported as arithmetic mean ($\bar{x}$) ± standard error (SEM). For all analyses, SPSS® 22.0 (IBM, New York, USA) for Windows was used and $P < 0.05$ was considered significant.

RESULTS

Sperm supplemented with 2.5 mg CLC + T and with 3.5 mg CLC + T had the highest ($P < 0.05$) total and progressive motility (65.2 ± 4.7 and 19.0 ± 1.0% respectively, mean ± SEM); however, for these two end points, there were no significant differences among sperm with 1.5 to 3.5 mg CLC + T (Table 1). Sperm with 2.5 mg CLC + T had the highest ($P < 0.05$) plasma membrane and acrosome integrity (59.3%; not different from 3.5 mg CLC + T) and the highest ($P < 0.05$) high mitochondrial membrane potential (64.6%; not different from 1.5 or 3.5 mg CLC + T; Table 1). With the exception of ALH, there were no significant differences among groups for any other CASA end point (Figure 3). ALH values of CLC 1.5 mg + T and CLC 2.5 mg + T were higher ($P < 0.05$) than 7-DCLC 2.5 mg + T (Figure 4).

DISCUSSION

In the present study, novel approaches were investigated to improve quality of frozen-thawed ram sperm collected and cryopreserved during the non-breeding season. Semen supplemented with CLC + T had highest motility, plasma membrane and acrosome integrity, high mitochondrial membrane potential and ALH. However, supplementation with various concentrations of 7-DCLC + T or T alone resulted in poorer membrane integrity and motility end-points.

Supplementing extender with CLC + T significantly increased motility. In previous studies, supplementing extender with other cholesterol conjugates and 7-DCLC increased motility and membrane integrity of cryopreserved ram sperm (Moraes et al. 2010; Inanc et al. 2018). Additionally,

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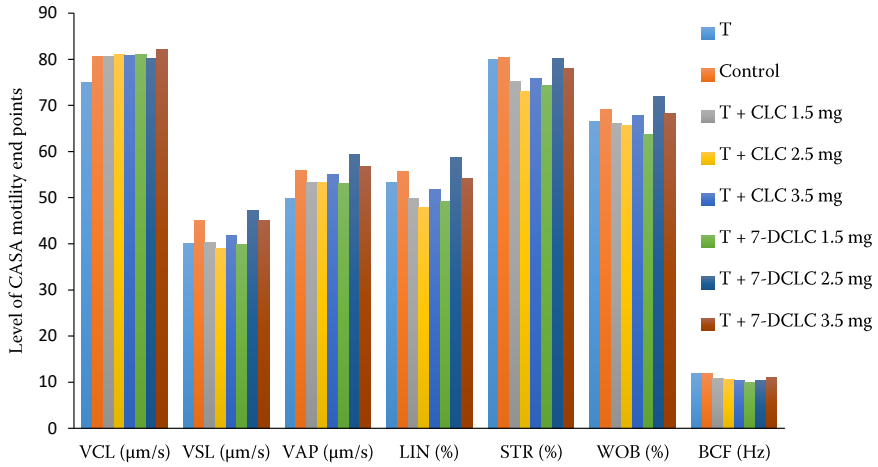

Figure 3. Mean CASA motility end points of frozen-thawed ram sperm cryopreserved with various extenders

7-DCLC = 7-dehydrochwarnolesterol loaded cyclodextrin; BCF = beat cross frequency; CASA = computer-aided semen analyzer system; CLC = cholesterol-loaded cyclodextrin; LIN = linearity; STR = straightness; T = trehalose; VAP = velocity average path; VCL = velocity curve linear; VSL = velocity straight line; WOB = wobble

either trehalose or CLC improved various aspects of ram sperm (Purdy and Graham 2004; Bucak et al. 2007; Moce et al. 2010; Konyali et al. 2013; Goularte et al. 2014; Pelufo et al. 2015; Gungor et al. 2017). In another study (Ucan et al. 2016), motility of cryopreserved ram sperm was improved by 3 mg CLC in combination with any one of three sugars (fructose, trehalose or sucrose). In humans, VSL, VCL, LIN and ALH were correlated with fertility (Krause 1995). In addition, ALH values were a more reliable method to predict success of fertilization in humans than other conventional semen quality criteria (Hirano et al. 2001). Although fertility was not evaluated in the present study, we inferred that a combination of CLC and trehalose have potential to increase fertility of frozen-thawed ram sperm.

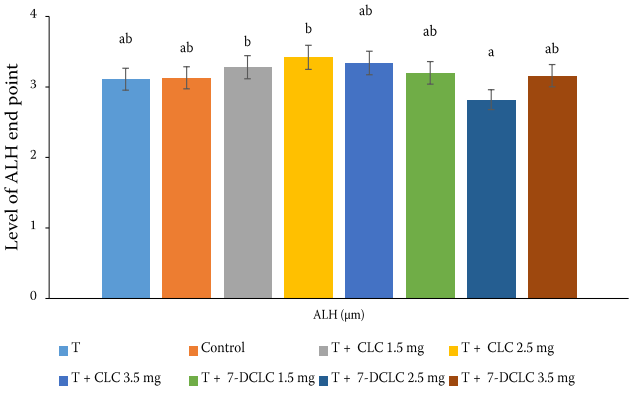

Figure 4. Mean ± SEM values of amplitude of lateral head displacement (ALH; µm), measured by computer-aided semen analyzer system, of frozen-thawed ram sperm cryopreserved with various extenders

7-DCLC = 7-dehydrocholesterol loaded cyclodextrin; CLC = cholesterol-loaded cyclodextrin; T = trehalose

[a,b]Columns without a common superscript differ ($P < 0.05$)

In the present study, addition of CLC + T to semen extender also resulted in the highest membrane integrity, consistent with positive effects of cholesterol on membrane fluidity (Ahmad et al. 2015). In previous studies, addition of trehalose and CLC decreased occurrence of premature acrosome reactions while increasing osmotic tolerance and membrane fluidity during cryopreservation (Aboagla and Terada 2003; Ahmad et al. 2015). Furthermore, addition of CLC to the semen extender reduced cryo-damage of membrane integrity, attributed to enrichment of phospholipid composition in sperm membranes (Purdy and Graham 2004). Various research groups reported positive effects of CLC on ram (Moce et al. 2010; Purdy et al. 2010; Motamedi-Mojdehi et al. 2014), rabbit (Aksoy et al. 2010), swine (Blanch et al. 2012) and bull (Moce and Graham 2006) sperm. Also, plasma membrane and acrosome integrity is essential for fertilization, as acrosomal enzymes are sequestered in the acrosome region.

Values for high mitochondrial membrane potential were significantly higher in all CLC + T groups compared to control, T, and 7-DCLC groups. In contrast, 1.5 mg CLC did not improve mitochondrial activity of frozen-thawed stallion or ram sperm (Spizziri et al. 2010; Inanc et al. 2018). Nevertheless, there were positive correlations between high mitochondrial membrane potential and plasma membrane integrity (Zuge et al. 2008; Korkmaz et al. 2017) as well as motility (Kasai et al. 2002; Bucak et al. 2015). Sperm have dense fibrils in the axoneme of their mitochondria that produce intracellular ATP and are involved in sperm mo-

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tility (Garner and Hafez 1993). Cryopreservation can cause axonemal damage, followed by decreases in mitochondrial membrane potential, ATP and sperm motility (Cummins et al. 1994; De Lamirande and Gognon 1999). We inferred that the combination of CLC + T mitigated this damage, thereby resulting in greatest motility, high mitochondrial membrane potential and plasma membrane and acrosome integrity.

In conclusion, addition of CLC + T to a Tris-based extender protected frozen-thawed ram sperm, manifested as significant improvements in plasma membrane integrity, motility and higher mitochondrial membrane potential. Addition of 2.5 mg CLC + T resulted in highest total motility, whereas 3.5 mg CLC + T yielded highest progressive motility. Conversely, 1.5 to 3.5 mg of 7-DCLC + T, or T alone, resulted in poorest membrane integrity and motility. We concluded that adding CLC + T to extenders has potential to enhance cryopreservation of ram semen, improve fertility of frozen-thawed sperm and thereby expand the use of artificial insemination in sheep.

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