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Effect of cyclodextrins, cholesterol and vitamin E and their complexation on cryopreserved epididymal ram semen

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ABSTRACT

The aim of the present study was to investigate the potential benefit of vitamin E and cholesterol when preloaded in cyclodextrins, alone or in association to protect ram epididymal sperm during the freezing-thawing process. Epididymal sperm were collected from Twenty four testes; sperm from the two testicles of each ram was pooled and divided in 7 aliquots. The control aliquot was diluted with Fraction A (Tris-citric acid-fructose) without further supplementation. The Six (6) other aliquots were diluted with fractions A containing cyclodextrins (CD), cholesterol (CHL), vitamin E (Vit E), cholesterol-loaded cyclodextrins (CD-CHL), vitamin E-loaded cyclodextrins (CD-Vit E) and CD-CHL and CD-Vit E (CD-CHL-Vit E), respectively. After incubation at 22 °C for 15 min and addition of Fraction B (Fraction A-egg yolk-glycerol), all aliquots were equilibrated at 4 °C for 2 h and then frozen in liquid nitrogen. A Computer Aided Semen Analysis (CASA) was used to investigate the impact on different motility parameters and the hypo-osmotic swelling test (HOST) to quantify membrane functionality. The Oxidative stress impact on sperm membrane was investigated through lipid peroxidation (LPO) measurement. After thawing, CD-Vit E and CD-CHL treatments improved significantly ($P < 0.05$) the total motility, VAP and linearity (LIN), compared to the control, Vit E and CHL samples. However, the association of CD-CHL and CD-Vit E (CD-CHL-Vit E) exhibited a significant effect on total motility, progressive motility, membrane functionality, sperm velocities (VCL, VSL and VAP) and LIN ($P < 0.05$). Membrane lipid peroxidation was significantly lower in semen pretreated with CD-Vit E than in control and Vit E alone. Among all treatments, the association of CD-CHL and CD-Vit E (CD-CHL-Vit E) showed the highest protection against LPO ($P < 0.05$). The present results revealed that the significant impact was observed when vitamin E, cholesterol and cyclodextrins are all used in the same treatment, thus demonstrating the complementary effect of solubilized vitamin E and cholesterol in protecting concomitantly spermatozoa against cold shock and oxidative stress.

1. Introduction

Cryopreservation of epididymal sperm remains a useful method to create germplasm bank, particularly concerning dead elite animals and endangered wild species (Ehling et al., 2006; Fickel et al., 2007). Collecting epididymal sperm from slaughtered animal is also an interesting alternative in the experimentation context with an ease access to semen compared to collection from lived animals (Nichi et al., 2007). In the present study, ram epididymal sperm was used to investigate a new approach to optimize semen freezing by testing concomitantly three molecules: cholesterol, vitamin E and cyclodextrins.

The cryopreserved sperm from most species yield unsatisfactory fertility after artificial insemination compared to fresh sperm (Watson, 2000). Cell cryodamage caused by intracellular ice formation (Mazur, 1977), osmotic shock (Holt and North, 1994), cold shock (Darin-Bennett and White, 1977) and oxidative stress (Agarwal et al., 2014; Aitken et al., 1991) alter normal sperm structure and decrease motility, viability and fertilizing potential (Hammerstedt et al., 1990).

Different strategies have been explored to reduce spermatozoa injuries during the freezing thawing process; in this respect,

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cholesterol was particularly reported as a key factor to combat cold shock. Nevertheless, related to cholesterol lipophilicity, only little positive impacts have been observed in cholesterol supplemented extenders (Graham and Foote, 1987), more significant effects are reported when the solubility of this molecule is increased. In fact, cholesterol-loaded cyclodextrins increases significantly cryosurvival of epididymal stallion semen (Pamornsakda et al., 2011) and ejaculated sperm of different animal species, including boar, goat, bull and stallion (Blanch et al., 2012; Konyali et al., 2013; Moore et al., 2005; Purdy and Graham, 2004). In Ram particularly, cyclodextrins-cholesterol complex has been reported to improve motility, viability and membrane integrity (Ahmad et al., 2013; Awad, 2011; Mocé et al., 2010b; Motamedi-Mojdehi et al., 2014). Cyclodextrins are oligosaccharides with an internal hydrophobic cavity which forms inclusion complexes with various hydrophobic guest molecules, including cholesterol and lipophilic vitamins, and an external hydrophilic face increasing their solubility in semen extenders (Dodziuk, 2006; López-Nicolás et al., 2012).

Oxidative stress occurring during the freezing thawing process, is also one of the major factors affecting gametes integrity and functionality (Agarwal et al., 2014; Aitken et al., 1991). Oxidative stress is established as an excess production of reactive oxygen species (ROS) with a failing of sperm antioxidant molecules (Sies, 1986). The main target of ROS is cell membrane causing lipid peroxidation which then alters membrane fluidity and permeability (Jones and Mann, 1977; Aitken, 1999). Vitamin E, a lipophilic molecule present in cell membrane, is considered as both a membrane-stabilizer and a potent antioxidant molecule protecting cell membrane against lipid peroxidation and ROS attacks (Urano et al., 1987, 1988; Niki and Noguchi, 2004). During cryopreservation, vitamin E is not synthesized by spermatozoa once consumed (Zhang et al., 2001). Consequently, in ram and other animal species, semen extenders supplemented with vitamin E reduce significantly lipid peroxidation and improve post-thawed semen quality (Beconi et al., 1993; Hu et al., 2011; Silva et al., 2013). Nevertheless, we hypothesized that the positive impacts could be significantly improved by increasing vitamin E solubility through cyclodextrins complexation. This has been successfully used in different domains including food and cosmetic industries (Koontz et al., 2009; Regiert, 2005). In our knowledge, the impact on cryopreserved sperm had never been reported, whatever the animal species.

Based on the presented background, the aim of this study was to investigate the impact of vitamin E-loaded cyclodextrins on ram epididymal semen after freezing–thawing process. A new approach was also investigated by combining a complementary protection against cold shock and oxidative stress using simultaneously vitamin E-loaded cyclodextrins and cholesterol-loaded cyclodextrins in semen extender.

2. Materiel and methods

2.1. Chemicals

All chemicals were obtained from Sigma–Aldrich Company groups (St. Louis, MO): Benzylpenicillin (Cat.P3032), Chloroform (Cat.C2432), Cholesterol (Cat.C8503), Citric acid (Cat.C2404), Ethanol (Cat.24103), Fructose (Cat.F3510), Glycerol (Cat.G15523), Hydrochloride acid 37% (Cat.258148), Methanol (Cat.24229), Methyl-β-cyclodextrin (Cat.C4555), Phosphate buffer saline (PBS; Cat.79378), Sodium citrate (Cat.24216), Streptomycin sulfate (Cat.S6501), Thiobarbituric acid (Cat.T5500), Trichloracetic acid (Cat. 27242), Tris-(hydroxymethylaminomethane) (Cat. 93352), Vitamin E (α-tocoppherol; Cat.T3251).

2.2. Preparation of methyl-β-cyclodextrin-vitamin E complex

Methyl-β-cyclodextrin-vitamin E complex (CD-Vit E) was prepared in 1:1molar ratios (α-tocopherol: methyl-β-cyclodextrin) by co-evaporation method. The methyl-β-cyclodextrin (309.11 mg) and α-tocopherol (100 mg) were dissolved in 50 ml of ethanol. The obtained mixture was maintained under stirring for 24 h at room temperature and shielded from light. The solvent was then evaporated under vacuum by rotary evaporation and the residue was kept in desiccator (Koontz et al., 2009).

2.3. Preparation of methyl-β-cyclodextrin-cholesterol complex

Methyl-β-cyclodextrin-cholesterol complex (CD-CHL) was prepared as described previously by Purdy and Graham (2004). In a glass test tube, 1 g of methyl-β-cyclodextrin was dissolved in 2 ml of methanol. In a second glass test tube, 200 mg of cholesterol was dissolved in 1 ml of chloroform. A 0.45 ml portion of the cholesterol solution was added to the cyclodextrin solution and mixed. The obtained mixture was maintained under stirring for 24 h at room temperature and shielded from light. The solvent was then evaporated under vacuum by rotary evaporation and the residue was kept in a desiccator.

2.4. Post mortem sperm recovery

Twenty four testes were collected from 12 adult rams (Berber breed). Immediately after slaughtering, testes were transported at room temperature (22 °C) to the laboratory. The sperm was collected by retrograde flushing method as reported by Martinez-Pastor et al. (2006) within 1:30 h from testes recovery. Briefly, the epididymis and vas deferens were dissected and separated from the testis and both cauda epididymis and vas deferens were isolated from the whole epididymis. Superficial blood vessels were cut and their contents removed by rinsing and wiping. The sperm was recovered in glass tube by making a cut near the junction of the corpus and the proximal cauda. Then, the vas deferens was catheterized with a blunted 22 G needle and flushed with 1 ml of warmed extender **(Fraction A)** (37 °C) followed by air injection to recover a maximum amount of sperm.

2.5. Semen dilution, freezing and thawing

The freezing extender was composed of two fractions, **Fraction A**: Tris (hydroxymethylaminomethane) 3.028 g + fructose 1.25 g + citric acid 1.70 g + penicillin G sodium 800 i.u./ml + streptomycin sulphate 1 mg/ml in 100 ml of distilled water; **Fraction B**: fraction A + glycerol 10% (v/v) + egg-yolk 30% (v/v). Each one of the 6 treatment solutions (CD, CHL, Vit E, CD-CHL, CD-Vit E and CD-CHL-Vit E) was prepared by adding corresponding complexes to 10 ml of fraction A. The final concentrations in 1 ml of fraction A were for CD: 9.17 mg, CHL: 0.83 mg, VitE: 1 mg, CD-CHL: 9.17-0.83 mg, CD-Vit E: 3.02–1 mg and CD-CHL-Vit E (CD-CHL (9.17-0.83 mg) + CD-Vit E (3.02–1 mg)), respectively. The control solution consisted of 10 ml of fraction A without any supplementation.

The epididymal sperm concentration was determined by a haemocytometer and a CASA system. Collected samples presenting the following characteristics: volume ≥ 0.8 ml, massal motility ≥ 3, individual motility ≥ 70% and sperm concentration ≥ 2×10^9 (Silva et al., 2013) were included in the experimentation. Semen samples collected from each ram (2 testes) were pooled and divided into 7 equal aliquots (0.1 ml/aliquot containing ≈200×10^6spz). The control was diluted with 0.4 ml of Fraction A (control solution). The remaining aliquots were diluted with 0.4 ml of corresponding treatment solutions (CD, CHL, Vit E, CD-CHL, CD-Vit E and CD-CHL-Vit