Small Ruminant Research 141 (2016) 29–35

Contents lists available at ScienceDirect

Small Ruminant Research

journal homepage: www.elsevier.com/locate/smallrumres

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

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ARTICLE INFO

Article history:
Received 10 December 2015
Received in revised form 19 June 2016
Accepted 20 June 2016
Available online 21 June 2016

Keywords:
Cold shock
HOST
Lipid peroxidation
Motility
Oxidative stress

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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http://dx.doi.org/10.1016/j.smallrumres.2016.06.009
0921-4488/

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

E, respectively). All samples were incubated at 22 °C for 15 min and then 0.5 ml of Fraction B was added to each aliquot to give 1 ml as a final volume for each aliquot. The final concentrations of glycerol and egg-yolk were 5% and 15%, respectively. All aliquots were then cooled to 4 °C for 2 h, packaged into 0.25 straws and frozen horizontally suspending straws 4 cm above the liquid nitrogen for 12 min, and then completely immersed into nitrogen liquid. Two straws of each aliquot were thawed in water bath (37 °C) for 30 s. Motility (CASA assessment), hypo-osmotic swelling test (HOST) and membrane lipid peroxidation were evaluated immediately after thawing.

2.6. CASA assessment

Motility was assessed using a computer-assisted sperm analyzer (CASA) (Sperm class analyzer, SCA Microptic, S.L., Version 3.2.0, Barcelona, Spain). To facilitate the image capture, the samples were diluted (10–20 × 10^6 Spermatozoa/ml) by fraction A. 10 μl of each sample was placed in Makler® chamber (10 μm depth; Sefi Medical Instruments, Haifa, Israel) previously heated (37 °C). The chamber was placed under phase contrast microscope (Nikon E200®-LED microscope) with a warmed stage (37 °C) and images were captured using a video camera (Caméra Digital Basler A312 fc Germany) at magnification x10. Four sequences were scanned and at least 200 spermatozoa were analyzed. The standard settings were set at 25 frames/s, 20–90 μm^2 for head area and VCL > 10 μm/s to classify a spermatozoa as motile (Tamayo-Canul et al., 2011). Measured kinetic parameters were: total motility (TM%) progressive motility (PM%), linearity (LIN%); straightness (STR%); wobble (WOB%); curvilinear velocity (VCL μm/s); straight linear velocity (VSL μm/s); average path velocity (VAP μm/s); amplitude of lateral movement of the head (ALH μm); beat cross frequency (BCF Hertz). Total motility (TM) was defined as the percentage of spermatozoa with VCL > 10 μm/s, and progressive motility (PM) was defined as the percentage of spermatozoa with VCL > 25 μm/s and STR > 80% (Mortimer, 1997).

2.7. Analysis of sperm membrane functionality

Functional integrity of sperm membrane was evaluated using the hypo-osmotic swelling test (HOST). Briefly, 20 μl of sperm was incubated with 200 μl of 100 mOsM hypo-osmotic solution for 60 min. Hypo-osmotic solution was prepared by mixing 9 g of fructose and 4.9 g of sodium citrate diluted in 1 l of distilled water at 37 °C. The spermatozoa possessing a swollen and coiled tail were assumed to have functional membrane. At least 150 spermatozoa were taken into account to calculate the percentage of reacted spermatozoa (Bucak et al., 2009).

2.8. Lipid peroxidation test (LPO)

The content of two straws (500 μl containing 10^8 Spz) was centrifuged immediately after thawing at 1500*g* for 10 min and the supernantant was eliminated. The sperm pellets were washed three times by resuspending in 1 ml of PBS and recentrifuging. Sperm was then suspended in 500 μl of PBS and sonicated with a probe using Sonics Vibra Cell VCX-750, at 20 kHz for 15 s on ice. The procedure was repeated six times at intervals of 30 s. The sonicated sperm were used to measure LPO.

Lipid peroxidation was measured based on the malondialdehyde (MDA) concentration. The MDA, an end product of lipid peroxidation, was quantified using the thiobarbituric acid (TBA) assay according to the method described by Buege and Aust (1978). One milliliter of TTH solution (trichloracetic acid 15%, w/v, thiobarbituric acid 0.375%, w/v in hydrochloric acid 0.25N) was added to the sonicated sperm. The mixture was boiled for 60 min and then cooled in an ice bath. After cooling, the suspension was centrifuged at 18,000*g* for 15 min. The supernatant was then separated, kept in an ice bath, and the absorbance measured at 532 nm at 25 °C. The molar extinction coefficient for MDA is 1.56 × 10^5 M^{-1} cm^{-1}. The results were expressed as nmoles MDA/10^8 SPZ.

2.9. Statistical analyses

Calculation of means, SEM, and statistical analysis were performed using Statview 4.02 software (Abacus Concepts Inc., Berkeley, CA, USA). Values of each parameter were expressed as the mean± SEM. Variables used for comparison purposes were the used treatments (Control, CD, CHL, Vit E, CD-CHL, CD-Vit E, CD-CHL-Vit E). Differences between treatments were assessed using a one-way ANOVA, followed by posthoc Fisher's test. Values were considered significant when P < 0.05.

3. Results

3.1. Percentages of total motility, progressive motility and membrane functionality

The total motility (TM), progressive motility (PM), and membrane functionality (MF) of all analyzed samples, after the freezing-thawing process, are presented in Fig. 1.

Concerning TM, no significant difference (P > 0.05) was observed between the three tested complexes (CD-CHL-Vit E = 71.48 ± 2.58%, CD-CHL = 67.49 ± 4.79 % and CD-Vit E = 64.04 ± 0.32%). Also, no significant difference (P > 0.05) was observed between the individual molecules (CD = 61.76 ± 3.03%, Vit E = 53.78 ± 2.25%, CHL = 52.51 ± 2.58%). However, the values were significantly (P < 0.05) higher in complexes treated samples compared to the

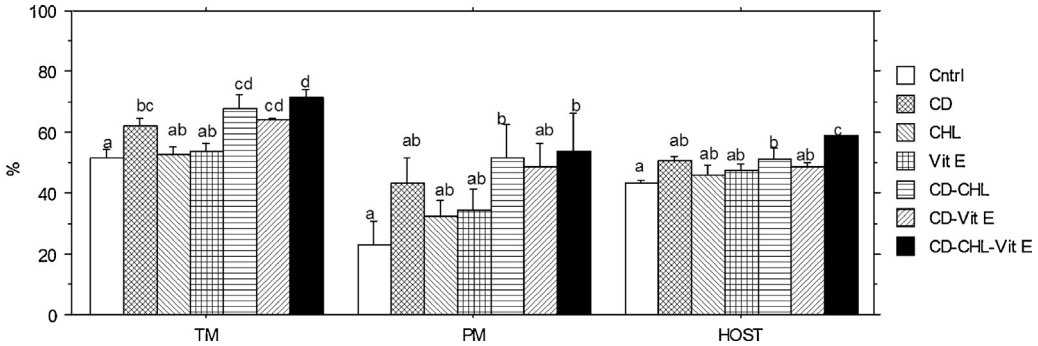

Fig. 1. Percentages (Mean ± S.E.M.) of total motility (TM), progressively motility (PM) and hypo-osmotic swelling test (HOST) after cryopreservation of epididymal ram semen in the control (Cntrl) group and groups pretreated with cyclodextrins (CD), cholesterol (CHL), vitamin E (Vit E), cholesterol-loaded cyclodextrins (CD-CHL), vitamin E-loaded cyclodextrins (CD-Vit E) and cholesterol-loaded cyclodextrins with vitamin E-loaded cyclodextrins (CD-CHL-Vit E). Different letters indicate significant differences (P < 0.05).

individual molecules. The control group showed the lowest total motility values. Concerning the progressive motility (PM), as a semen quality parameter, the differences between treatments were not statistically significant at all, and the only significance was when comparing the complexes to control (CD-CHL-Vit E = 53.56 ± 2.51%, CD-CHL = 51.72 ± 0.73%, CD-Vit E = 48.23 ± 7.82% and Cntrl = 22.72 ± 7.86 %)

Membrane functionality test (Fig. 1), revealed that CD-CHL-Vit E complex (58.85 ± 0.02%) maintained membrane functionality significantly higher (P < 0.05) than all used treatments.

3.2. Kinematic parameters (VCL, VAP, VSL, LIN, ALH, BCF)

The impact of investigated treatments on VCL, VSL, VAP and LIN are presented in Fig. 2. The lowest value of VCL was observed in the control group (68.97 ± 2.51 μm/s). Among all treatments, CD-CHL-Vit E showed the highest value (110.47 ± 1.91 μm/s) followed by CD-CHL (104.35 ± 2.17 μm/s) and CD-Vit E (88.82 ± 2.21 μm/s).

Regarding VAP, results were similar to tendencies expressed by VCL, except that CD-CHL-Vit E complex, showed values (82.21 ± 1.75 μm/s) significantly higher to all other treatments. In addition, VAP in CD-Vit E and CD-CHL complexes was significantly higher (P < 0.05) than those observed in Vit E and CHL alone. Concerning VSL, expressing not only the aptitude of the gametes to present high speeds, but also the aptitude to progress in a straight line manner, the results demonstrated that CD-CHL-Vit E complex expressed the highest values statistically different from all treatments.

LIN reduction in addition to ALH and BCF increase are considered as a group of parameters indicating sperm capacitation (Chamberland et al., 2001; Mortimer and Maxwell, 1999). In the present results, after thawing, the linearity (LIN) of sperm treated with CD-CHL-Vit E (58,99 ± 1,22%), CD-CHL (49,94 ± 1,43%) and CD-Vit E (46,03 ± 1,49%) was significantly augmented (P < 0.05) compared to all treatments including the control (39,83 ± 1,69%). LIN was particularly higher in CD-CHL-VitE complex compared to all treatments. The main results showed also that ALH and BCF values (Fig. 2) were significantly lower in CD-CHL-VitE indicating the absence of premature capacitation. Finally, the marked result drawn from Figs. 1 and 2 was that CD-CHL-Vit E increased all mobility parameters and membrane functionality of thawed sperm compared to all investigated treatments.

3.3. Lipid peroxidation

From the data shown in Fig. 3, the MDA concentration was significantly lower (P < 0.05) in sperm pretreated with the CD-CHL-Vit E complex (0.682 ± 0/092 nmole/10^8spz) compared to

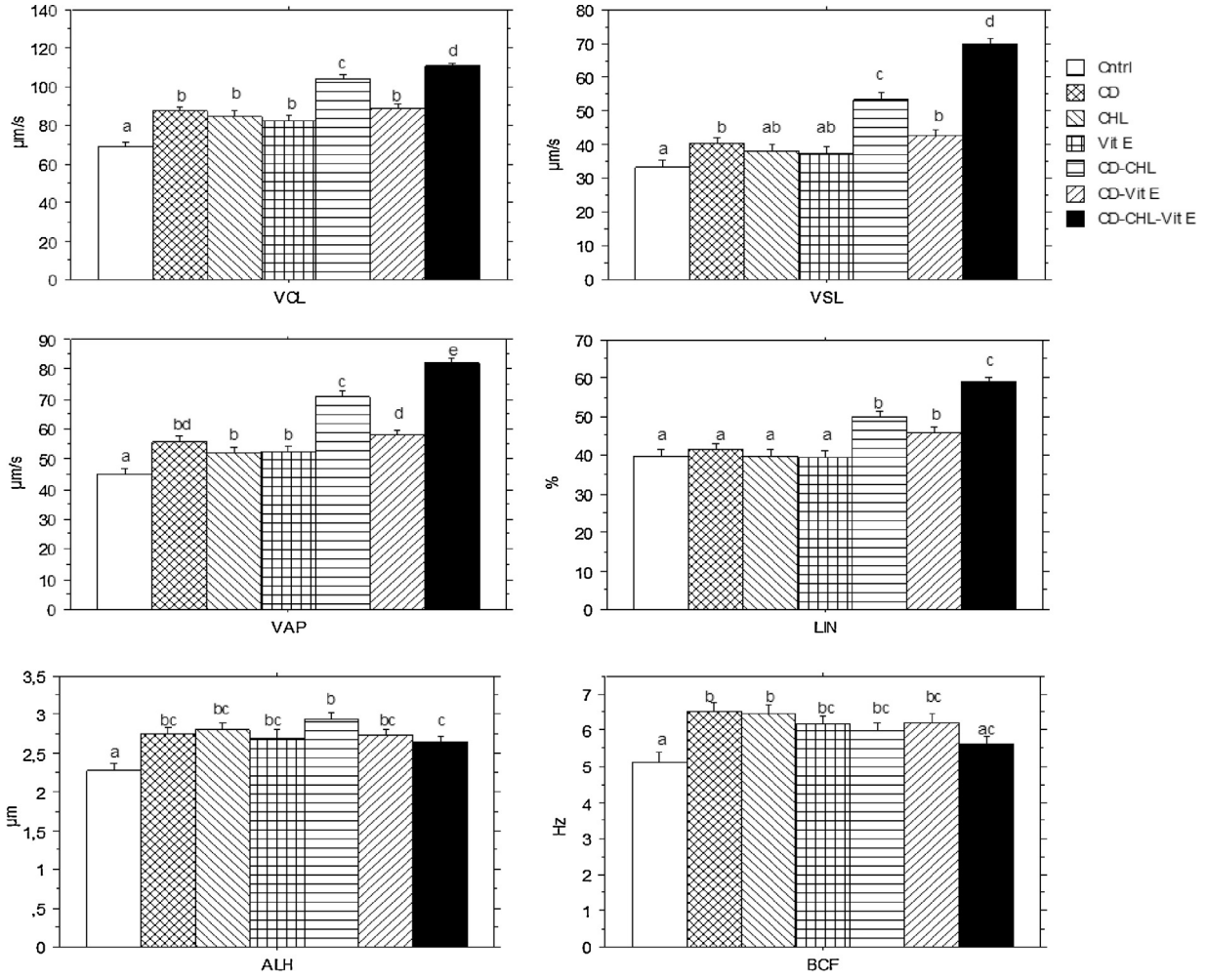

Fig. 2. Mean (±S.E.M) of curvilinear velocity (VCL), straight linear velocity (VSL), average path velocity (VAP), linearity percentage (LIN%), amplitude of lateral movement of the head (ALH) and beat cross frequency (BCF) after cryopreservation of epididymal ram semen in the control (Cntrl) group and groups pretreated with cyclodextrins (CD), cholesterol (CHL), vitamin E (Vit E), cholesterol-loaded cyclodextrins (CD-CHL), vitamin E-loaded cyclodextrins (CD-Vit E) and cholesterol-loaded cyclodextrins with vitamin E-loaded cyclodextrins (CD-CHL-Vit E). Different letters indicate significant differences (P < 0.05).

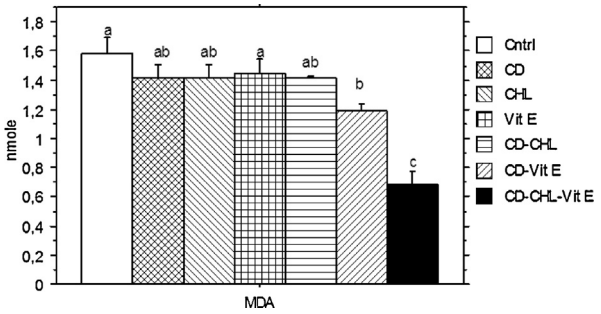

Fig. 3. Mean (±S.E.M) of lipid peroxidation (MDA) after cryopreservation of epididymal ram semen in the control group (Cntrl) and groups pretreated with cyclodextrins (CD), cholesterol (CHL), vitamin E (Vit E), cholesterol-loaded cyclodextrins (CD-CHL), vitamin E-loaded cyclodextrins (CD-Vit E) and cholesterol-loaded cyclodextrins with vitamin E-loaded cyclodextrins (CD-CHL-Vit E). Different letters indicate significant differences ($P < 0.05$).

all tested treatment indicating cell membrane protection against lipid peroxidation damages. At lesser degree, CD-Vit E showed the same protective effect with values significantly lower than those observed in the other treatments, especially in Vit E alone ($P < 0.05$). CD, CHL and CD-CHL, when compared to the control, showed a protection effect but not at the same extent as CD-Vit E and CD-CHL-Vit E.

4. Discussion

The objective of the present study was to explore a new alternative in the preservation of epididymal ram semen. The experimental approach consisted on a simultaneous protection against cold shock and oxidative stress using cholesterol and vitamin E. As the two molecules present a limited solubility in semen extenders, their solubility was increased through preloading in cyclodextrins.

It is well established that cholesterol plays a determinant role in maintaining cell membrane structure, fluidity and function over the range of physiological temperatures (Amann and Pickett, 1987; Darin-Bennett and White, 1977). During cryopreservation, sperm membrane is exposed to cold shock damages with significant cholesterol depletion affecting cell membrane integrity, gametes motility and fertility outcomes. The susceptibility of mammalian sperm to cold shock depends on the proportion of cholesterol/phospholipids ratio in cell membrane. In ram, this ratio is lower compared to bull and human sperm, explaining the highest susceptibility of ram sperm membrane to cold shock (Muiño-Blanco et al., 2008).

In the current study, supplementation of freezing extender with cholesterol alone (CHL), improved only VCL, VAP, ALH and BCF values when compared to the control. This effect against cold shock injury has been previously reported describing the same little effect against cold shock injuries (Graham and Foote, 1987). This is in relation to the hydrophobic characteristic of cholesterol limiting its solubility in semen extenders. When cholesterol was preloaded in cyclodextrins (CD-CHL), all velocities and linearity were significantly higher ($p < 0.05$) compared to all used treatments except CD-CHL-Vit E (Fig. 2). The total and progressive motility as well as viability (HOST) were also significantly improved, particularly, when compared to the control (Fig. 1). The present results are in accordance with the previous studies reporting the same positive effects of cholesterol-loaded cyclodextrins on gametes motility and/or membrane integrity in ram (Morrier et al., 2004), bull (Mocé and Graham, 2006; Purdy and Graham, 2004) and stallion (Moore et al., 2005; Pamornsakda et al., 2011).

Cyclodextrins act by increasing the transfer of cholesterol into spermatozoa membrane (Moore et al., 2005; Pamornsakda et al., 2011) and consequently enhance the cell permeability to cryoprotectants such as glycerol (Glazar et al., 2009; Mocé et al., 2010a). In this respect, it is shown recently that treating ram sperm with cholesterol-loaded cyclodextrins reduced the level of glycerol required for cryopreservation from 5% to 3% (Awad, 2011; Motamedi-Mojdehi et al., 2014).

In the present study, the total motility and the sperm velocities in samples treated with cyclodextrins alone, prior to cryopreservation, were significantly higher than those of the control. Zeng and Terada (2000, 2001) and Madison et al. (2013) reported the same beneficial effects as those of the current results, suggesting that further depletion of cholesterol by cyclodextrins, enhances post-thawed sperm motility and velocities. The mechanism by which cyclodextrins protect sperm against cryoinjury is unclear. However, it is shown that depleting membrane cholesterol enhances membrane fluidity and permeability (Cooper, 1986; Grunze and Deutick, 1974) and then possibly decreases intracellular lethal ice formation. Conversely, our results are in contrast to the report by Mocé et al. (2010b) in ram, they showed that after thawing, the cyclodextrins decrease spermatozoa motility by making the sperm more sensitive to cold shock damage. The effect of CD on membrane cholesterol depletion is dependent to the incubation time with cyclodextrins and the concentration of CD (Iborra et al., 2000; Nagao et al., 2010). In our study, the short time of incubation of sperm with CD (15 min) and the possibility of CD to bind with cholesterol of egg yolk may limit the sensitivity of sperm to cold shock by restricting membrane cholesterol depletion or by facilitating the interaction of egg yolk cholesterol with sperm membrane. Nevertheless, further studies are required to elucidate the specific biological mechanisms through which prefreezing treatment with CD enhances sperm motility after thawing.

Sperm membrane in ram is the first target of damages caused by oxidative stress due to its high proportion on unsaturated fatty acids. Thus, lipid peroxidation is the major injury caused by ROS in sperm membrane but also in other cell components such as acrosome and cell genome (Peris et al., 2007). Vitamin E in cell membrane is the first antioxidant molecule combating the oxidative stress (Peris et al., 2007; Zhang et al., 2001) by interrupting the chain reaction of LPO and scavenging the ROS (Niki and Noguchi, 2004). Moreover, Vitamin E has been proposed to act as a structural component which stabilizes cell membranes containing polyunsaturated lipids (Diplock and Lucy, 1973; Fukuzawa et al., 1977). This is due to its localization in the lipid core of membrane and its binding with phospholipids (Urano et al., 1987, 1988). Nevertheless, once vitamin E is exhausted, the cell becomes vulnerable to the attacks of ROS, especially during the freezing-thawing process (Peris et al., 2007; Zhang et al., 2001).

In the present work, the supplementation of sperm extender by vitamin E alone (Vit E) had increased significantly VCL, VAP, ALH and BCF when compared to the control. This is in accordance with the previous reports where vitamin E was found to increase sperm motility and membrane integrity (Breininger et al., 2005; Hsu et al., 1998; Silva et al., 2013; Vasconcelos Franco et al., 2014, 2013). However, such positive effects are not systematically observed in ram sperm (Da Silva Maia et al., 2009; Sarlós et al., 2002; Upreti et al., 1997). These divergent results could be related to the solubility and to the concentrations of vitamin E used in these experimentations.

Over the last years, several works have been carried out to improve the antioxidant efficiency of animal semen extenders. These studies were based, in part, on the substitution of the vitamin E by herbal antioxidants (Motlagh et al., 2014), antioxidant enzymes such as glutathione (Salmani et al., 2013) and on combination between vitamin E and other antioxidant molecules (Hsu et al., 1998; Krishnamoorthy et al., 2007; Da Silva Maia et al., 2009). However, in our knowledge, no study has investigated the interest of vitamin E-loaded cyclodextrins in semen extenders supplemen-

tation. As vitamin E shares the same hydrophobic characteristic with cholesterol, it had been hypothesized that preloading vitamin E in cyclodextrins could increase its solubility with a positive impact on cell membrane. In fact, the obtained results showed that CD-Vit E decreased LPO of sperm membrane and enhanced motility parameters (total motility, CASA parameters) when compared to control and Vitamin E alone. These results suggest that cyclodextrins enhanced the beneficial effect of Vitamin E on sperm probably by inserting vitamin E as it does for cholesterol in sperm membrane. However, further studies are required to determine if preloading vitamin E in cylodextrins enhances vitamin E level in cell membrane.

The most relevant results had been obtained when using simultaneously vitamin E and cholesterol, both preloaded in cyclodextrins (CD-CHL-Vit E). This treatment had improved significantly the membrane functionality (HOST) and all CASA motility parameters compared to all other tested treatments. When Compared to the control, CD-CHL-Vit E complex enhanced significantly the total (71% *vs* 51%) the progressive motility (53% *vs* 22%), membrane functionality (58% *vs* 43%) and all CASA motility parameters. In addition, CD-CHL-Vit E was the most treatment that decreased significantly membrane lipid peroxidation. This is probably due to the effects of both Vitamin E, as antioxidant and membrane stabilizer (Urano et al., 1987, 1988) and cholesterol as a hindrance to ROS propagation (López-Revuelta et al., 2007) and protector against peroxide radical (Naseer et al., 2015).

During cryopreservation, membrane damage, particularly due to the depletion of cholesterol and oxidative stress, causes premature capacitation and acrosome reaction which may compromise fertility (GaArtiga et al., 1993; Pérez et al., 1996). The capacitation status is characterized by high VCL, ALH and BCF with low VSL and LIN (Chamberland et al., 2001; Mortimer and Maxwell, 1999; Verstegen et al., 2002). In the current results, such observations had not been found suggesting that the tested complexes, particularly CD-CHL-Vit E, protected the gametes against premature capacitation. In our study, lipid peroxidation was negatively correlated ($P > 0.05$) with VCL, VSL, LIN and membrane functionality. This result showed that protection against LPO could explain in part the beneficial effects of complexes on sperm motility, membrane functionality and preventing premature capacitation.

In conclusion, the present results demonstrated that increasing vitamin E solubility with cyclodextrins improved significantly both post-thawed sperm parameters and antioxidant status. The most relevant results were observed when the solubility of both vitamin E and cholesterol was improved and when a complementary protection was provided, cholesterol to reinforce the cell membrane and vitamin E to combat ROS. This new approach opens interesting perspectives in the semen cryopreservation, particularly in species with low cholesterol: phospholipids ratio.

Conflict of interests

The authors declare that there are no conflicts of interest.

Acknowledgment

The authors thank Salah Adrar, responsible of slaughterhouse in Bejaia (Algeria), for providing the testes of slaughter rams.

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