Interest of vitamin E and cholesterol preloaded in cyclodextrins on motility of cryopreserved rabbit semen

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SUMMARY

The present study aimed to investigate the effect of cholesterol and α-tocopherol, loaded in cyclodextin, on motility of cryopreserved rabbit semen. Rabbit sperm was collected from seven mature bucks. The pooled sperm was initially evaluated and then submitted -prior freezing- to seven dilution treatments. The control aliquot was diluted with Tris-based extender; the six others were treated with Tris-based extenders containing: β-cyclodextrins, cholesterol, α-tocopherol, cholesterol-loaded β-cyclodextrins, α-tocopherol-loaded β-cyclodextrins or a treatment of both complexes. Kinematic motility parameters were assessed by a computer assisted semen analyzer system before and after conventional freezing-thawing process. The results showed that both cholesterol and α-tocopherol complexed to β-cyclodextrins improved significantly ($p<0.05$) the post-thaw kinematic parameters. However, no significant effect was recorded when complexes were simultaneously combined. In conclusion, the present results revealed that the most significant impact on rabbit cryopreserved sperm was observed when cholesterol and α-tocopherol were loaded in cyclodextrins.

Keywords : Cyclodextrins, Cholesterol, α-Tocopherol, Cryopreservation, Semen, Rabbit.

RESUME

Intérêt de la vitamine E et du cholestérol préchargé dans des cyclodextrines sur la motilité du sperme congelé du lapin

La présente étude a comme objectif d'étudier l›effet du cholestérol et de l'α-tocophérol, chargé dans des cyclodextines sur la motilité du sperme congelé du lapin. Le sperme a été collecté à partir de sept mâles matures. Les éjaculats ont été regroupés sous forme de mélanges puis dilués dans sept traitements différents. Le témoin est un dilueur à base de Tris et les six autres ont été traités avec des diluants à base de Tris contenant : β-cyclodextrines, cholestérol, α-tocophérol, β-cyclodextrines-cholestérol, β-cyclodextrines-α-tocopherol, ou une association (v/v) des deux derniers complexes. Les paramètres de motilité ont été évalués par un système informatique d'analyse de semence avant et après le processus de congélation-décongélation. Les résultats ont montré que le cholestérol et l'α-tocophérol complexés améliorent de manière significative ($p <0,05$) les paramètres de motilité après décongélation. Aucun effet significatif n'a été enregistré lorsque les deux complexes ont été simultanément utilisés. En conclusion, les présents résultats ont révélé que l'impact le plus significatif a été observé lorsque le cholestérol et l'α-tocophérol sont chargés dans les cyclodextrines, probablement par l'augmentation de leur solubilité.

Mots-clés : Cyclodextrines, Cholestérol, α-Tocophérol, Cryopreservation, Sperme, Lapin.

Introduction

Rabbit artificial insemination (AI) is usually performed with fresh or cooled semen stored for a short time period [20, 28], while rabbit cryopreserved sperm has been limited essentially to experimental purposes due to lower fertility/prolificacy outputs [23]. During cryopreservation, alterations in sperm cell integrity are observed and attributed to intracellular ice formation, toxicity of cryoprotectants, osmotic stress and to oxidative damages [36].

Poly-unsaturated fatty acids and cholesterol/phospholipids ratio are determinant factors in sperm cell membrane fluidity and resistance to cold-shock [2, 15]. Semen in cold shock-sensitive species (boar, bull, ram or stallion) expresses low cholesterol/phospholipids ratio level, while semen in cold shock-resistant species (rabbit, human) exhibits higher level ratio with more polyunsaturated fatty acids [10, 24]. During cryopreservation, lipids are subjected to intense peroxidation process through reactive oxygen species (ROS) causing consequently sperm motility alterations and low fertilizing outputs [14]. Since spermatozoa lose cytoplasmic defenses through spermatogenesis, the antioxidant protection is barely handled by membrane antioxidant system. Vitamin E is the major natural lipid-soluble antioxidant acting as a scavenge system of peroxyl radicals and breaks the propagation of peroxidation chain reaction [12].

Several strategies have been proposed to minimize cryo-injuries either by adjusting sperm cell environment (diluents, protocols) or by modifying sperm cell membrane composition essentially through dietary supplementation. In this respect, previous reports demonstrated that supplementation of vitamin E to rabbit diet (> 200 mg/kg) increases significantly α-tocopherol level in fresh semen [8-9]; Being so, the susceptibility of semen to oxidation, expressed by the amount of thio-barbituric acid reactive substances (TBARS), showed a high negative correlation with α-tocopherol intake (-0,8) and its seminal level (-0,9).

Cyclodextrins are cyclical oligosaccharides with a hydrophobic interior core expressing high affinity to guest lipophilic molecules such as cholesterol and α-tocopherol and a hydrophilic enclosure, increasing its solubility in semen extenders. Using cholesterol-loaded cyclodextrin (CLC) treatment for cold-sensitive sperm to a high threshold could improve gametes cryosurvival rates [24]. Semen pre-treatment with CLC has been used successfully to insert cholesterol into cell membranes [25-27, 37] in a view to improve sperm resistance to cold shock. Similarly, it has been shown that semen pre-treatment with α-tocopherol has demonstrated positive effects on post-thaw motility, viability and oxidation protection in boar [4, 30-31] ; ram [13, 32] ; fish [6] ; dog [21] and human cryopreserved semen [33]. In rabbit, in our knowledge, there is no previous report concerning the interest of extender supplementation with antioxidants, especially α-tocopherol. We hypothesized that preloading α-tocopherol in cyclodextrins will increase its solubility and will consequently enhance rabbit sperm motility through fighting against oxidative stress.

On the basis of the presented background, the current study aimed to investigate the interest of a double protection on post thawed rabbit sperm motility using simultaneously cholesterol and α-tocopherol, both preloaded in cyclodextrins to increase their solubility in semen extenders.

Materials and methods

REAGENTS AND PREPARATION OF TREATMENTS COMPLEXES

All reagents used were from Sigma–Aldrich Company (Prochima Sigma – Tlemcen, Algeria).

Cholesterol loaded methyl-β-cyclodextrins complex (CLC) was prepared as described previously by Purdy and Graham [27]. In a glass test tube, 1g of methyl-β-cyclodextrins was dissolved in 2ml of methanol. In a second glass test tube, 200mg of cholesterol was dissolved in 1ml of chloroform. A 0.45ml portion of the cholesterol solution was added to the cyclodextrins solution and mixed. The obtained mixture was maintained under stirring for 24h 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.

α-tocopherol loaded Methyl-β-cyclodextrinscomplex (TLC) was prepared in 1:1 molar ratios (α-tocopherol:methyl-β-cyclodextrins) by co-evaporation method. The methyl-β-cyclodextrins (309.11 mg) and α-tocopherol (100mg) were dissolved in 50ml of ethanol. The obtained mixture was maintained under stirring for 24h 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 [17].

SEMEN TREATMENT AND PROCESSING

Preparation of treatment and freezing extenders

A Tris based extender was used to prepare treatment and freezing solutions. Tris extender contained: Tris (hydroxymethylaminomethane) (3.025g), citric acid (1.7g), glucose (1.25g), Penicillin G (0.1g), dihydrostreptomycin (0.1g), and distilled water (QS 100ml). The freezing extender was obtained by adding dimethylsulfooxide DMSO (20%) to Tris based extender, representing the control treatment. The tested treatments were: 1). Control (Cntr); the six treatments prepared using Tris extender and their concentrations were determined as follows: 2). β-cyclodextrins (Cdx) (0.71mg/ml); 3). cholesterol (Chl) (4mg/ml); 4). α-tocopherol (Toc) (0.12mg/ml); 5). cholesterol-loaded cyclodextrins (CLC) (10.86 mg/ml); 6). α-tocopherol-loaded cyclodextrins (TLC) (0.92mg/ ml) and 7). CLC+TLC with equal proportion (v/v). All solutions were filtered by syringe filter (0.45µm) and stored at 4°C until used.

Sperm collection, evaluation and cryopreservation

Seven mature local population rabbit bucks were used as sperm donors during a period of two months. The bucks were housed in individual cages; water and food were provided ad-libitum. Semen samples were collected by artificial vagina and gel plugs were removed immediately after collection.

Semen ejaculates from each buck were examined for volume and masse motility (0-9) [3, 5]. Individual sperm motility was assessed subjectively at 400x magnification to arbitrary score progressive motility (0-4) and estimate motility percentage [28]. Concentration was estimated using a Thomas-Zeiss cell chamber (final dilution 1:200). Only sperm collection with a mass motility score over six (≥6), progressive motility score over three (≥3) and subjective motility percentage over sixty percent (>60%) were pooled and allocated to cryopreservation treatments.

Cryopreservation protocol was addressed according to Rosato and Laffaldano [29]. Semen pools were initially cooled at 5°C for 90 minutes to minimize cold-chock damage, then diluted to a ratio 1:5 in Tris buffer containing 20% dimethylsulfooxide (DMSO). The pools were placed at 5°C for 45 mn for equilibration. Pools were then split and diluted to a ratio 1:1 (v/v) with the seven treatments extenders ; including the control (Tris buffer), the treated semen was packaged in 250µl plastic straws and frozen by exposure to liquid nitrogen vapor (5 cm above the liquid nitrogen surface) for 10 minutes. The straws were then plunged into liquid nitrogen at -196°C for one hour. Sperm samples were thawed by immersing the straws in a water bath at 50°C for 10 seconds.

Sperm motion characteristics were determined before freezing and after thawing using sperm class analyser SCA

4.0 (Microptic® spain). Briefly 10µl drop of sperm was placed onto a pre-warmed Makler chamber. A minimum of 200 cells, using at least three microscopic fields were measured using the previously established parameters: Frame to frame rate: (16-25/s); cell size (min/max): (10,5/24 µm²); Low VAP (20µm/s); Medium VAP (40µm/s); [7, 18]. The sperm parameters recorded were: curvilinear velocity (VCL, µm/s); straight line velocity (VSL, µm/s); average path velocity (VAP, µm/s); amplitude of lateral movement of the head (ALH, µm) and beat cross frequency (BCF, Hertz).The overall percentage of motile spermatozoa (TM), and the percentage of spermatozoa with a progressive motility (PM) i.e., cells with an average path velocity > 50 µm/s and straightness > 75%.

STATISTIC ANALYSIS

The statistical analysis was performed using StatView ® statistical program (version 5.0). Values are presented as means ± standard error of the mean (SEM). Comparisons between percentages of motility and sperm kinematic parameters were analyzed by one-way analysis of variance (ANOVA), followed by Post-hoc Fisher's test. A P- value <0.05 was considered to be statistically significant.

Results

FRESH SPERM CHARACTERISTICS

Table 1 represents the initial characteristics of fresh sperm used in the experiment. Mean values for volume and concentration were lower than values observed by brun et al., [5] within the same range of masse motility (Mm≥6) ; (respectively: volume (0.67), concentration (716 · 10^6 spz)) [5]. The motility of the used sperm (Mm and Mi) showed a medium score (6.7 and 2.9) with an acceptable motility percentages.

EFFECT OF CHOLESTEROL AND α-TOCOPHEROL ON POST THAW SPERM MOTILITY PARAMETERS

Total motility (TM) and progressive motility (PM)

The effect of cholesterol and α-tocopherol on post-thaw total motility (TM) and progressive motility (PM) are set out in table 2 and represented in figure 1. Compared to the control, the results showed that cholesterol and α-tocopherol complexation with β-cyclodextrin (CLC and TLC) presented higher but not significant difference on post-thaw total (TM) and progressive motility (PM). The same tendency was observed when comparing CLC or TLC to cholesterol (Chl) or α-tocopherol (Toc) alone. In contrast, cholesterol (Chl) and α-tocopherol (Toc) presented no observable effect compared to the control. When CLC and TLC where concomitantly associated (CLC+TLC), no significant improvement on post thaw TM and PM was observed compared to the control or to CLC and TLC alone.

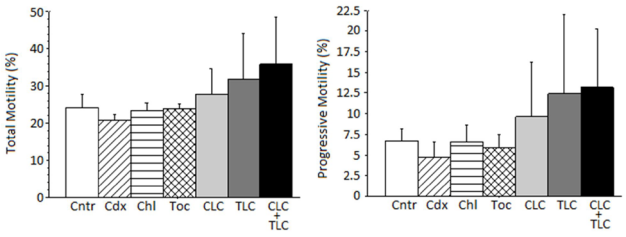

Figure 1: Effect of cholesterol and α-tocophérol on total motility (TM) and progressive motility (PM) of rabbit semen after thawing in: Control (Cntr), β-cyclodextrins (Cdx), cholesterol (Chl), α-tocopherol (Toc), cholesterol-loaded cyclodextrins (CLC), α-tocopherol-loaded cyclodextrins (TLC) and CLC and TLC association (CLC+TLC), (Mean ± SEM).

(n=5)	Volume	Mm	Mi	% Mot	Cn(X10⁶)
	0.42 ± 0.49	6.7 ± 0.2	2.95 ± 0.16	78.3 ± 2.78	468.98 ± 27.77

Mm : masse motility ; Mi : individual motility ; %Mot : percentage of motility ; Cn: concentration

Table I: The mean ± SEM values of fresh rabbit sperm parameters.

Parameter	Cntr	Cdx	Chl	Toc	CLC	TLC	CLC+TLC
TM (%)	24.2 ± 3.3	20.6 ± 1.5	23.4 ± 1.9	23.7 ± 1.2	27.5 ± 7.1	31.7 ± 12.5	35.8 ± 12.7
PM (%)	6.6 ± 1.5	4.7 ± 1.8	6.5 ± 2.1	5.8 ± 1.6	9.6 ± 6.5	12.4 ± 9.5	13.1 ± 7
VCL (µm/s)	23.2 ± 0.8 ^{a}	23.2 ± 1.0 ^{a}	24.1 ± 0.9 ^{a}	24 ± 1.0 ^{a}	30.8 ± 1.2 ^{b}	31.1 ± 0.9 ^{b}	31.8 ± 0.9 ^{b}
VAP (µm/s)	10.1 ± 0.5 ^{a}	10.2±0.7 ^{a}	10.3 ± 0.6 ^{a}	10.3 ± 0.6 ^{a}	13 ± 0.6^{b}	13.5 ± 0.5^{bc}	14.6 ± 0.5^{c}
VSL (µm/s)	5.3 ± 0.4 ^{a}	5.7±0.6 ^{ac}	5.5 ± 0.5 ^{a}	5.6 ± 0.5 ^{a}	7.3 ± 0..4^{b}	7.1 ± 0.3^{bc}	7.8 ± 0.4^{b}
ALH (µm)	1.3 ± 0.04 ^{a}	1.3±0.05 ^{a}	1.4 ± 0.04 ^{a}	1.4 ± 0.04 ^{a}	1.7 ± 0.05^{b}	1.8 ± 0.04^{bc}	1.8 ± 0.04^{c}
BCF (Hz)	1.6 ± 0.10^{a}	1.3±0.09^{a}	1.8 ± 0.12^{a}	1.5 ± 0.11^{b}	2.4 ± 0.13 ^{b}	2.5 ± 0.10 ^{b}	2.7 ± 0.10 ^{b}

abcValues in rows with different letters differ significantly (P<0.05). TM: total motility; PM: progressive motility; VCL, curvilinear velocity; VAP, average path velocity; VSL, straight-line velocity; ALH, amplitude of lateral head displacement; BCF, beat-cross frequency. Control (Cntr), β-cyclodextrins (Cdx), cholesterol (Chl), α-tocopherol (Toc), cholesterol-loaded cyclodextrins (CLC), α-tocopherol-loaded cyclodextrins (TLC) and CLC and TLC association (CLC+TLC).

Table II: The mean ± SEM effect of cholesterol and α-tocopherol on post-thaw sperm motility parameters.

Kinematic parameters (VCL, VAP, VSL, ALH, BCF)

The effect of cholesterol and α-tocopherol on post-thaw kinematic parameters are set out in table 2 and represented in figure 2. Compared to the control, curvilinear velocity (VCL), average path velocity (VAP) and straight line velocity (VSL) of post thawed sperm were significantly improved ($p < 0.05$) by CLC. Also, α-Tocopherol-loaded cyclodextrins (TLC) enhanced significantly ($p<0.05$) post-thaw kinematic parameters including VCL, VAP and VSL. The amplitude of lateral head displacement (ALH) and beat-cross frequency (BCF), where significantly ($p<0.05$) higher in CLC and TLC compared to the control. No significant effect was recorded when cholesterol (Chl) and α-Tocopherol (Toc) are used without complexation.

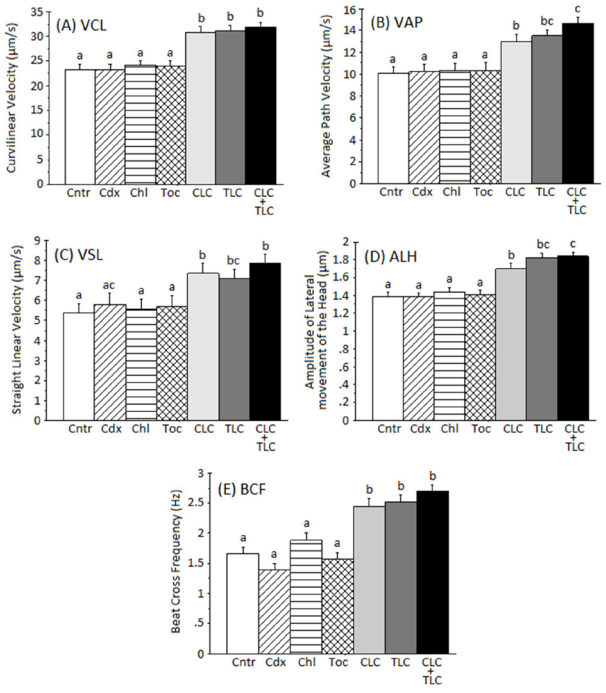

Figure 2: Effect of cholesterol and α-tocopherol on curvilinear velocity (VCL) ; straight line velocity (VSL) ; average path velocity (VAP); amplitude of lateral movement of the head (ALH) and beat cross frequency (BCF) of rabbit semen after thawing in: Control (Cntr), β-cyclodextrins (Cdx), cholesterol (Chl), α-tocopherol (Toc), cholesterol-loaded cyclodextrins (CLC), α-tocopherol-loaded cyclodextrins (TLC) and CLC and TLC association (CLC+TLC). (abc: $P<0.05$), (Mean ± SEM).

When CLC and TLC are simultaneously added to semen (CLC+TLC), an enhancement effect on kinematics parameters was observed with significant difference ($P<0.05$) compared to control concerning VCL, VAP, VSL, ALH and BCF. However, no improvement was observed when compared to CLC or TLC alone.

Discussion

The aim of the current study was to investigate the interest of cholesterol and α-tocopherol (vitamin E) as complementary molecules to improve post thawed rabbit sperm motility. Cyclodextrins was proposed to solubilize and enhance bioavailability of hydrophobic components into aqueous solutions. Through the present study, it is presumed that cholesterol and α-tocopherol could work in a complementary manner; cholesterol by reinforcing the sperm cell membrane and facing consequently cold shock injuries, while α-tocopherol ensures an antioxidant protection over the phase transition stress.

Cholesterol is an important structural component of spermatozoal plasma membrane. Cholesterol:phospholipids ratio may establish differences among mammalian species toward sperm cold-chock resistance [24]. Rabbit spermatozoa presents high Cholesterol:phospholipids ratio (0.88) [35] and consequently only few studies investigated the interest of cholesterol on post-thaw motility. Mocé et al., [22] reported no CLC effect on rabbit semen cryopreservation, however, Aksoy et al. [1] found that CLC enhanced osmotic tolerance in fresh rabbit semen and inhibited premature acrosome reaction in liquid storage at 4°C. Despite that Aksoy et al. [1] did not cryopreseved rabbit sperm treated by CLC, the improvement of post-thaw kinematic parameters of our study would be associated to Aksoy et al. [1] findings ; in fact, when osmotic tolerance of cells are increased this improves their cryotolerance.

In the present experimental, treatment with cyclodextrins alone was ineffective on most of post-thawed sperm motility parameters. Cyclodextrins cholesterol-efflux force appeared to be canceled in rabbit sperm, these findings are in concordance with those reported by Mocé et al., [22] demonstrating that rabbit sperm treated with cyclodextrins alone do not show depressed cryosurvival rates, but exhibit similar cryosurvival rates to untreated sperm. These observations could be related to the already cholesterol-rich membrane and probably also to the presence of rich-sterol prostate granules known as cholesterol efflux modulator [10, 11].

As spermatozoa discard and lose the majority of the cytoplasmic antioxidants during the final stages of spermatogenesis, vitamin E remains the most liposoluble and naturally membrane's integrated system of defense against peroxidative damage. Alternatively, supplementing α-tocopherol to sperm extenders would constitute an effective alternative against cryo-produced ROS [4, 6, 20]. In rabbit sperm, effect of α-tocopherol (Vitamin E) on sperm was explored but exclusively through nutritional supplementations [8, 9]; Castellini et al. [8], particularly, increased 1.9 fold the amount of vitamin E in fresh semen and 2.19 fold in 5°C stored semen by 4 fold α-tocopherol dietary supplementation. However, this enrichment did not improve spermatozoa characteristics but only reduced membrane lipid oxidation during storage. In the current work, direct incorporation of α-tocopherol presented also no improvement effect on motility parameters. In contrast, previous studies on other animal species, using the same tocopherol derivate, reported a positive impact

on cryopreserved sperm motility and lipid peroxidation, especially in boar [31] and sea bass [20].

Cyclodextrins was majorly proposed to solubilize α-tocopherol in a view to enhance the suppressive effect of α-tocopherol on lipid oxidation [34], in our knowledge; the use of this complexed form of α-tocopherol has never been investigated in rabbit semen diluents. In our study, α-tocopherol preloaded in β-cyclodextrins enhances significantly post thaw motility in the same manner as when using CLC treatment. cyclodextrins α-tocopherol inclusion would ensure a gradual release of α-tocopherol and constitute a short cut of nutritional supplementation with more efficiency on gametes motility. The protective effect of α-tocopherol is presumably due to preserving bio-membranes from lipid peroxidation [16].

When sperm was concomitantly treated with cholesterol and α-tocopherol, both preloaded in cyclodextrins, an additive effect was observed compared to CLC and TLC indicating probably a dual protection against membrane damages and oxidative injuries during cryopreservation.

In conclusion, cyclodextrins appeared as effective in improving cholesterol and α-tocopherol solubility in semen extenders and represent an interesting alternative to enhance post-thaw kinematic parameters.

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