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THE PRESENCE OF VITAMIN C ENHANCES THE PROTECTION OF CHOLESTEROL-CYCLODEXTRIN AND VITAMIN E-CYCLODEXTRIN IN CRYOPRESERVATION OF BULL SEMEN

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Abstract

BACKGROUND: Cryopreservation of sperm cells is a well-established technique. However, cryopreserved sperm cells can have significant cellular damage, primarily due to oxidative stress. **OBJECTIVE:** To investigate the synergistic effects of vitamin C with vitamin E-loaded cyclodextrins on the quality of cryopreserved bull sperm cells in combination with cholesterol-loaded cyclodextrins. **MATERIALS AND METHODS:** The ejaculates from nine mature bulls were divided into six equal aliquots for different treatments, including vitamin C (VitC), vitamin E loaded cyclodextrin (CD-VitE), CD-VitE+VitC, cholesterol loaded cyclodextrin (CD-CHL), CD-CHL + CD-VitE + VitC and the control. The sperm motility (CASA), membrane integrity (HOST) and lipid peroxidation (TBARS) were assessed after freezing and thawing. **RESULTS:** The combination of CD-CHL + CD-VitE + VitC retained the highest sperm motility and membrane integrity, as well as resulted in the least lipid peroxidation. **CONCLUSION:** The study shows the synergistic effects of vitamin C, vitamin E and cholesterol in improving the cryopreservation outcomes of bull sperm.

Keywords: antioxidant; bull sperm; cholesterol; cryopreservation; vitamin C; vitamin E.

INTRODUCTION

Cryopreservation has many advantages for storing sperm resources. However, many stresses in the freeze/thaw process may alter spermatozoa functionalities, particularly irreversible damages in plasma membrane structure, loss of motility, reduced viability, and DNA fragmentation (1, 2), which has negative impact on the success of artificial insemination resulting in economic loss. Many freeze-caused damages are associated with cold shock (3, 4) and oxidative stresses (5, 6). Since the spermatozoa's resistance to freezing damages is related to the composition of plasma membrane, the low cholesterol:phospholipid ratio is thought to be responsible for the susceptibility of bull sperm (7). For this reason, cholesterol has been added to protect bull sperm against freezing damages (8). The protective effect was even more pronounced when cholesterol was encapsulated in cyclodextrin (CD-CHL) due to the enhanced solubility and permeability into the sperm

membrane (9, 10). On the other hand, bull sperm has a large amount of polyunsaturated fatty acid (PUFA) that is suceptible to lipid peroxidation under free radical attack (11). Vitamin E (VitE) and vitamin C (VitC) are two major protectors due to their antioxidant activity (12, 13). There is a synergic interaction between VitE and VitC because VitE could be regenerated by VitC after its oxidation (14). VitC acts as a reducing agent for VitE regeneration (15). It has been reported that the combination of VitE and VitC improves sperm motility, viability and membrane integrity of bull sperm after cryopreservation (16). Due to the insolubility of VitE, vitamin E was loaded into cyclodextrin to form complexes (CD-VitE) that exhibit more protection during cryopreservation compared to vitamin E alone (17, 18).

In this study, we investigated the effect of VitC supplementation to CD-CHL and CD-VitE on bull sperm cryopreservation via the evaluation of sperm motility, membrane integrity, and lipid peroxidation after freezing and thawing.

MATERIALS AND METHODS

Cholesterol and vitamin E complexes

Permethyl-β-cyclodextrin-cholesterol (CD-CHL) and permethyl-β-cyclodextrin-vitamin E (CD-VitE) complexes were prepared by the co-evaporation method in 1:1 and 2:1 w/w ratios, respectively. For each complex, cyclodextrin and the guest substance (cholesterol or vitamin E) were dissolved in 75 mL ethanol and continuously stirred for 24 h in the dark at room temperature. The solvent was then evaporated under vacuum at 40°C in a rotary evaporator and the residue was kept in a desiccator until use.

Sperm collection

Nine mature bulls (2 years old) were housed in Centre National d'Insémination Artificielle et amélioration Génétique at Algiers, Algeria. Ejaculates were collected via an artificial vagina. Immediately after collection, the sperm quality of each ejaculate was evaluated. Only ejaculates that exhibited a volume ≥ 4 mL, motility ≥ 70%, massal motility ≥ 3 and sperm concentration ≥ 1×10^9 / mL were used in experiments.

Extenders and cryopreservation

Each ejaculate was divided into six equal aliquots of 0.1 mL, which were then diluted with 0.4 mL Extender A (1.0 g fructose + 2.42 g Tris (hydroxymethylaminomethane) + 1.38 g citric acid + 100 mg penicillin in 100 mL water). The dilution contained 5 mg CD-CHL, 0.5 mg CD-VitE, 0.25 mg Vit C, 0.5 mg CD-VitE + 0.25 mg VitC, or 5 mg CD-CHL + 0.5 mg CD-VitE + 0.25 mg VitC. The control aliquot was diluted without any supplementation. After 15 min incubation at 22°C, aliquots were further diluted in 0.5 mL Extender B (Fraction A + 10% glycerol + 30% egg-yolk) to obtain finally 100×10^6 cells/mL. Sperm was finally equilibrated at 4°C for 2 h, packaged in 0.25 mL and frozen in liquid nitrogen.

Motility analysis

For sperm motility assessment, one straw per treatment group was thawed and analyzed using a Computer Assisted Sperm Analyzer (CASA) (SCA Microptic, S.L., Barcelona, Spain). To optimize image capture and minimize sperm cell overlap, samples were diluted to a concentration below 40×10^6 cells/mL. The aliquot of 5 µL sperm samples were placed in a pre-warmed Makler® chamber and examined with a phase-contrast microscope. Images were captured by a video camera at a 10× magnification (negative phase contrast). At least 200 motile spermatozoa were evaluated for parameters including total motility (TM), progressive motility (PM), 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), beat cross frequency (BCF) and linearity % [LIN = (VSL / VCL) × 100] (19).

Membrane integrity

Plasma membrane integrity was assessed by the hypo-osmotic swelling test (HOST). A 150 mOsmL/L hypo-osmotic solution was prepared by dissolving 9 g of fructose and 4.9 g of sodium citrate in 1 L distilled water. The aliquots of 20 µL thawed semen were incubated in 200 µL hypo-osmotic solution at 37°C for 60 min. Spermatozoa were examined under a microscope at 400× magnification. At least 200 sperm cells were counted and those with swollen tails were considered HOS positive (20).

Thiobarbituric acid reaction assay

For malondialdehyde (MDA) content assay in thawed samples, 100×10^6 cells/mL was washed three times by centrifugation at 1500×g for 15 min each, and re-suspended in 500 µL distilled water. The final pellet was sonicated for 15 s,

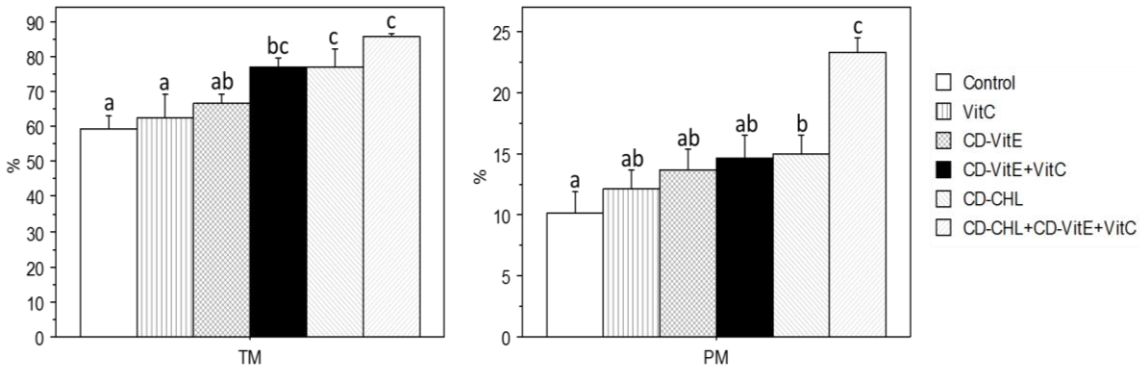

Figure 1. The percentages (mean ± SE) of total motility (TM) and progressive motility (PM) of frozen-thawed bull semen in extenders containing vitamin C (VitC), vitamin E loaded into cyclodextrins (CD-VitE), vitamin C with vitamin E loaded into cyclodextrins (CD-VitE + VitC), cholesterol loaded into cyclodextrins (CD-CHL), and the combination of CD-VitE, CD-CHL and vitamin C (CD-CHL+CD-VitE+VitC). Different letters indicate significant differences ($P < 0.05$).

repeated six times at 30 s intervals (21). The thiobarbituric acid reaction (TBAR) assay was used to measure MDA in sonicated sperm samples. Briefly, 0.5 mL sonicated sample was mixed with 1 mL TBA-TCA-HCl solution (15% trichloracetic acid + 0.375% thiobarbituric acid + 0.25 N HCl) and heated at 95°C for 60 min. After cooling and centrifugation (18000 ×g for 15 min), the absorbance of the supernatant was determined at 535 nm. The MDA level was expressed as nmoles MDA per 1×10^8 cells using a molar extinction coefficient of 1.56×10^5/M·cm (22).

Statistical analyses

Statistical analysis was performed using StatView 4.02 software (Abacus Concepts Inc., Berkeley, CA, USA). One-way ANOVA with the post-hoc Fisher's test was used for comparison in among treatment groups. Significance was set at $P < 0.05$. Results are expressed as mean ± SE.

RESULTS

Total motility and progressive motility

Sperm samples treated with VitC, CD-VitE, CD-CHL or their combination showed higher total motility (TM) and progressive motility (PM) after thawing than that of the control sperm sample (Figure 1). No significant differences ($P > 0.05$) were observed in TM among sperm samples treated with VitC, CD-VitE and the control sample. However, the combinations of VitC and CD-VitE with CD-CHL increased significantly the motility values (85.6 ± 1.1%) as compared to the control group (59.1 ± 4.0%). For PM, CD-CHL + CD-VitE + VitC treatment had the highest values (23.3 ± 1.2%, $P < 0.05$) among all treatments. In addition, a slight improved PM was obtained in sperm samples treated with VitC or CD-VitE and their combination (CD-VitE + VitC), but not in a significant manner compared to the control (10.1 ± 1.9%), whereas the sperm sample treated with CD-CHL increased PM significantly.

Membrane integrity

Sperm membrane integrity was not affected by VitC and CD-CHL supplementation (Figure 2). Nevertheless, sperm samples treated with CD-VitE and CD-VitE+VitC (29.1 ± 6.4% and 31.9 ± 4.8%, respectively), had greater membrane integrity than the control group (24.2 ± 2.7%).

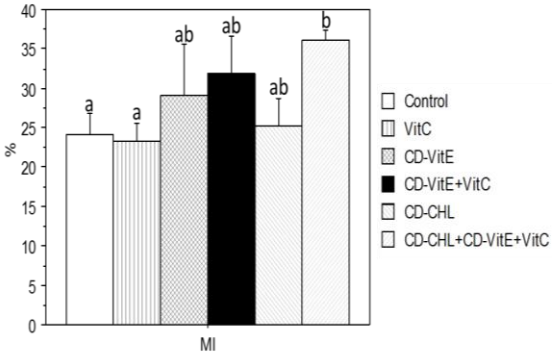

Figure 2. The membrane integrity of frozen-thawed sperm samples in extenders containing vitamin C (VitC), vitamin E loaded into cyclodextrins (CD-VitE), vitamin C with vitamin E loaded into cyclodextrins (CD-VitE + VitC), cholesterol loaded into cyclodextrins (CD-CHL), and the combination of CD-VitE, CD-CHL and vitamin C (CD-CHL+CD-VitE+VitC). Different letters indicate significant differences ($P < 0.05$).

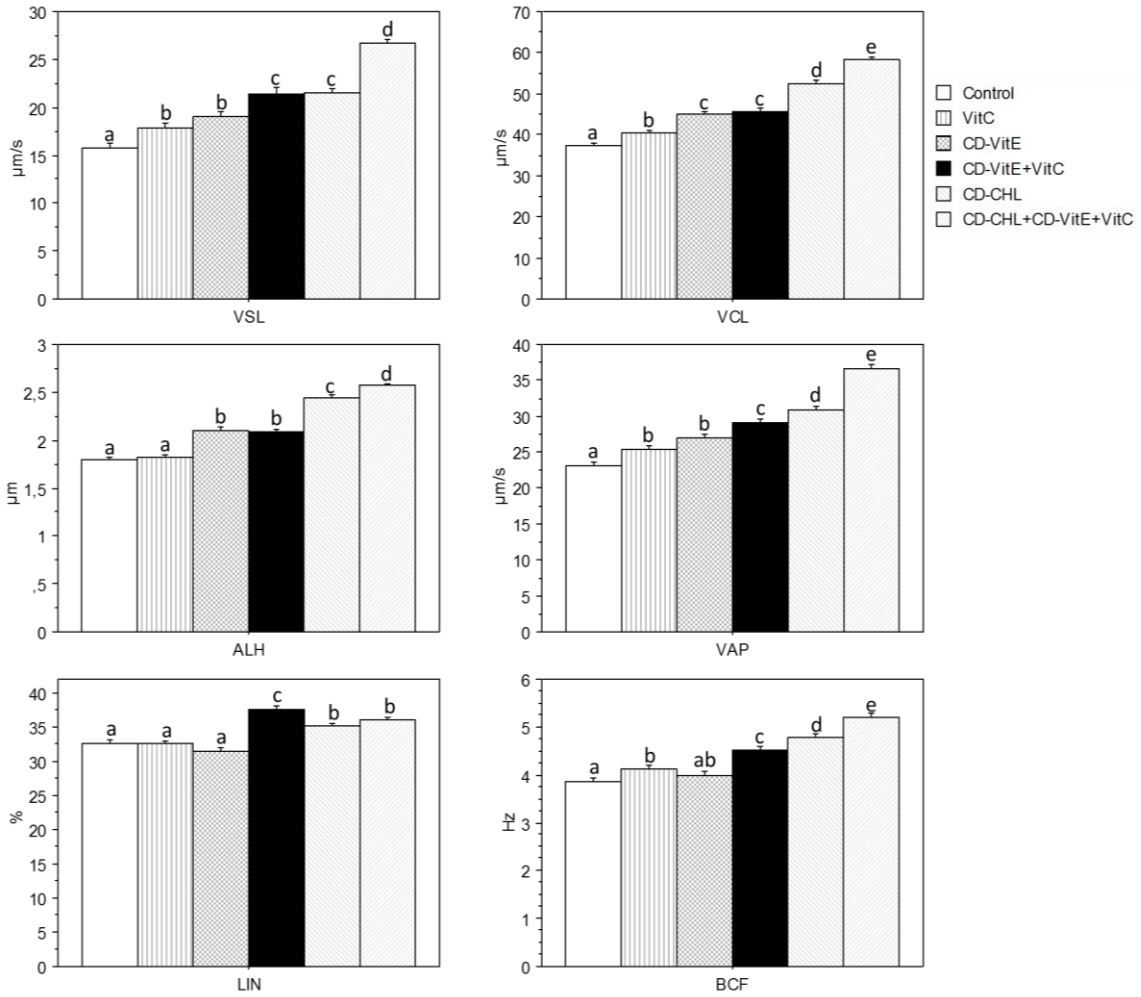

Figure 3. Sperm motility parameters of frozen-thawed bull semen samples in extenders containing vitamin C (VitC), vitamin E loaded into cyclodextrins (CD-VitE), vitamin C with vitamin E loaded into cyclodextrins (CD-VitE + VitC), cholesterol loaded into cyclodextrins (CD-CHL), and the combination of CD-VitE, CD-CHL and vitamin C (CD-CHL+CD-VitE+VitC). Different letters indicate significant differences ($P < 0.05$).

The greatest membrane integrity was obtained in the sperm samples treated with CD-CHL + CD-VitE + VitC (36.1 ± 1.2%) among all treatments ($P < 0.05$).

Sperm motility - CASA

Figure 3 shows CASA motility parameters after cryopreservation. VSL, VCL and VAP increased significantly after VitC, CD-VitE or their combination treatments as compared to the control ($P < 0.05$). CD-CHL + CD-VitE + VitC, followed by CD-CHL, had the highest significant effect ($P < 0.05$) on all motility motions, except LIN (%). VitC or VitE supplementation improved the motility parameters as compared to control. However, their combination exhibited more efficient protection.

Lipid peroxidation

The MDA contents as tested by the TBARS method are shown in Figure 4. Sperm samples treated with CD-CHL + CD-VitE + VitC had the lower MDA content (0.05±0.08 nmol). A slight, but not statistically significant, decrease in MDA level was obtained in sperm samples treated with VitC (0.12 ± 0.17 nmol) and CD-VitE (0.10 ± 0.26 nmol) as compared to the control (0.15 ± 0.04 nmol). However, the combination of CD-VitE and VitC had a significantly larger effect (0.06 ± 0.11 nmol).

DISCUSSION

The resistance of sperm cells to cryo-damage depends mainly on its membrane structure and antioxidative activity. Our earlier study reported

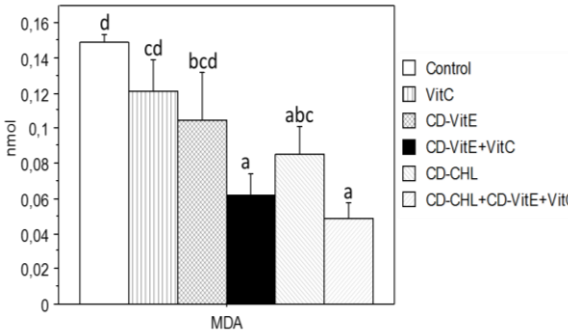

Figure 4. The malondialdehyde content of frozen-thawed sperm samples in extenders containing vitamin C (VitC), vitamin E loaded into cyclodextrins (CD-VitE), vitamin C with vitamin E loaded into cyclodextrins (CD-VitE + VitC), cholesterol loaded into cyclodextrins (CD-CHL), and the combination of CD-VitE, CD-CHL and vitamin C (CD-CHL+CD-VitE+VitC). Different letters indicate significant differences ($P < 0.05$).

that cholesterol and vitamin E, preloaded in cyclodextrins, had complementary protective effect on bull sperm, resulting in increased sperm quality and reduced lipid peroxidation (21). The present study demonstrated that the addition of vitamin C in extenders further improves the protective effects of CD-CHL and CD-VitE. Indeed, all sperm parameters were improved in treatments containing Vitamin C compared to the control group. The best sperm parameters were observed in extender containing simultaneously CD-CHL, CD-VitE and VitC.

Cold shock is a critical factor that disturbs sperm membrane during cryopreservation. It can alter cell morphology and promote lipid peroxidation (1, 23). Spermatic plasma membrane having low cholesterol:phospholipid ratio such as ram, stallion, and bull sperm, are more sensitive to cold shock than sperm which have high cholesterol: phospholipid ratio (human and rabbit sperm) (24). Several studies indicate that sperm treated with CD-CHL has improved motility and membrane integrity after freezing (25, 26, 27). Our results are in accordance with these previous studies. We report significant positive effects of CD-CHL on progressive (15.1 ±1.5%), total motility (76.9 ± 4.9%) and various velocity parameters. Salmon et al. (9) suggested that cholesterol enrichment of sperm via cyclodextrins enhances sperm tolerance to cold shock and osmotic stresses without attenuating capacitation and acrosomal exocytosis.

Sperm membrane with high levels of polyunsaturated fatty acid also tends to be more susceptible to lipid peroxidation during the freeze-thaw process (28). Our results indicated that sperm treated with cholesterol reduce MDA level, a product of lipid peroxidation. The protective effect against lipid peroxidation can be explained by the antioxidant proprieties of cholesterol, which was first proposed in 1991 in the study of Smith LL (29). Later it was established that cholesterol attenuated the oxidative damage through preserving the antioxidant enzyme system and limiting ROS propagation (30, 31).

ROS is needed for normal sperm function such as capacitation and the acrosome reaction (32). However, the unrestrained ROS generation reduces the antioxidant capacity of sperm cells and increases oxidative stress (33). Previous studies demonstrated that ROS is considerably increased during sperm cryopreservation, with high lipid peroxidation damage, DNA injury, and cell death (34, 35). Thereby, to reduce oxidative stress and limit radical attacks, many antioxidant molecules have been used in sperm cryopreservation extenders. Vitamin E is one of the best protective molecules (36, 37). Sperm samples pretreated with vitamin E have a capability against ROS, and the quality of frozen-thawed sperm are higher (38, 39, 40). Conversely, in previous studies vitamin E did not improve the frozen-thawed motility in stallion (41) or ram sperm (42). These divergent observations could correspond to the low solubility of vitamin E in sperm extenders.

The low solubility of vitamin E in extender reduces its bioavailability (43). Encapsulation with cyclodextrin for vitamin E improves sperm motility. In line with our results, it was reported that the addition of CD-VitE to cryopreserved sperm resulted a significant increase in motility parameters, membrane functionality and acrosome integrity (17). The beneficial effect could be correlated with the improved solubility without affecting its antioxidant activity (43, 44). Nevertheless, once vitamin E is depleted, cells become vulnerable to ROS attacks again. Vitamin C has been studied as a vitamin E regenerator to ensure a continued effect of its antioxidant power (45, 46). Vitamin C constitutes a strong line of defense by retarding free radicals which induce cellular damage. In addition, it was reported that vitamin C prevents sperm chromatin abnormalities and apoptosis during cryopreservation (47).

The mechanism of the synergistic activity between vitamin E and vitamin C have been

reported in bull sperm cryopreservation (16). However, in our knowledge, the effect of the combination between CD-VitE and VitC on sperm cryopreservation has not been studied yet. The present study showed that supplementation with CD-VitE or VitC, did not affect sperm parameters, whereas when used simultaneously they had a significant improvement in sperm characteristics, particularly in the oxidative state. As can be seen in Figure 4, the combined use of vitamin C and vitamin E (CD-VitE + VitC) decreases the MDA level. The synergy between two vitamins ensures lipophilic and hydrophilic radical scavenging, whereas VitE is involved as the primary scavenger of hydroperoxyl radicals in the lipid bilayer (36, 48). VitC on the other hand gives an electron to the oxidized form of α-tocopheroxyl radical (49, 50). This role contributes to the level of VitE and its antioxidant activity. However, VitC can be oxidized to an ascorbyl radical, a relatively stable free radical. Two molecules of ascorbyl radical rapidly disproportionate into completely oxidized and reduced components of VitC (dehydroascorbic acid and ascorbate, respectively) (51, 52). To minimize VitC loss, the dehydroascorbic acid is enzymatically reduced intracellularly to ascorbate by glutathione (53). Glutathione reductase (GR) is an enzyme reducing oxidized glutathione (GSSG) by using nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) as an electron donor (54).

When the sperm sample was treated with CD-VitE, CD-CHL and VitC at the same time, total motility (85.6 ± 1.1%) and progressive motility (23.3 ± 1.2%) was the greatest, along with higher sperm velocity (VCL, VSL and VAP) and lower lipid peroxidation damage. In conclusion, the optimal protection against cryo-damages were achieved when vitamin E and cholesterol were both preloaded in cyclodextrins and vitamin C were also added to sperm extender.

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