INTERESTS TO SUPPLEMENT TRIS-BASE EXTENDER WITH CHOLESTEROL / α-TOCOPHEROL PRELOADED IN CYCLODEXTRINS AND VITAMIN-C TO CHILL RABBIT SEMEN AT 4°C

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ABSTRACT

Semen refrigeration deteriorates the motile quality of rabbit semen and limits its usefulness over 48h of storage. The oxidant stress associated with the diminution of temperature leads to the high production of Reactive Oxygen species (ROS) and harmful alterations of the cell membrane. In this context, The objective of the present study was to supplement the extender with Vitamine C (VitC) and Cholesterol (CLC) or α-tocopherol (TLC) both Loaded in Cyclodextrins, to explore the quality of rabbit semen preserved at 4°C for 48h. Rabbit semen was collected, pooled and then treated by CLC (2.5mg), TLC (0.625) and VitC (0.125mg) for 80-100 million spz/ml; and by different duals (CLC+TLC), (CLC+VitC), (TLC+VitC) and (CLC+TLC+VitC). We analyzed motility kinematic parameters by a Computer-assisted semen analysis (CASA SPA®); and the oxidative status by measuring the amount of lipid peroxidation (TBARS). The results showed a diminution of motility during the time of preservation. However when compared to the control, CLC and TLC treatment improved significantly ($p<0.05$) the kinematic parameters after 24h of 4°C conservation. Velocity Curvilinear (VCL) and Velocity of Average Path (VAP) in TLC treatment (56.0±23.9 and 32.2±15.3 µm/s, respectively) and CLC-TLC (VCL 57.9±22.5, VAP 36.4±15.3 µm/s) were significantly higher compared to the control (VCL: 46.9±24.0, VAP: 24.8±14.7 µm/s). After 48h, VAP (34.9±17.0 µm/s) was significantly ($P<0.05$) higher in TLC-TLC-VitC treatment than in control (22.9±11 µm/s). With concern to the oxidative status of refrigerated semen, we noticed a similar level of TBARS at 0h in all treatments. However, we did not notice a significant elevation of TBARS levels in all treatment after 24h or 48h compared to the control. The benefit of CLC and TLC was highly attributed to the higher solubility of cholesterol and α-tocopherol through cyclodextrins.

Key words: CLC, α-Tocophérol, Vitamine C, Rabbit Semen, 4°C.

INTRODUCTION

Refrigerated semen in rabbit artificial insemination (AI) has good fertility/prolificacy results; however, it is limited to a short time, which generally does not exceed 36h of storage (Roca *et al*., 2000). Refrigerated spermatozoa of mammals show reduced motility and low viability (Rodriguez-Gil *et al*., 2006). Boar and stallion are the most studied species for sperm chilling; Gączarzewicz *et al.* (2015) showed that low motility of boar semen was consistent with a decrease of mitochondrial transmembrane potential and oxido-reductive capability; Gibb & Aitiken 2016 reviewed that stallion sperm stored at low temperature, are subjected to high production of free radicals and membrane peroxidation.

Cholesterol-loaded-cyclodextrin (CLC) was widely experimented to enhance semen cryo survival among different species (Mocé *et al*., 2010) but weakly studied for rabbits. Tris-Glucose-Based extenders are adequate to chill rabbit semen at 5° (Johinke *et al.*, 2014). Although, few studies have

reported the extender enrichment with antioxidant substances on rabbit chilled semen (Rosato *et al.*, 2012).

The present study aimed to explore the effects of cholesterol-loaded-Cyclodextrin, α-Tocophérol Loaded-Cyclodextrins and Vitamine C on the quality of 4°C refrigerated Rabbit semen for 48h.

MATERIALS AND METHODS

Animals and experimental design

Semen was collected from 10 mature rabbit bucks by using artificial vagina (IMV, France ®)-. Each ejaculate was priorly examined for Mass and motility percentage (Mm ; %Mot respectively) (Brun *et al*., 2002). Retained ejaculates were pooled (n= 6), examined for concentration and further diluted to obtain pools with 160-200 million spermatozoa/ml. Pools were aliquoted into 8 tubes and submitted by additional dilution (v:v) to eight treatments: 1). Control (Cntr); 2). CLC (5mg/ml); 3). Vitamin C (VitC) (0.25mg/ml); 4). α-Tocophérol-Loaded-Cyclodextrins TLC (1.25mg/ml) ;5). TLC+VitC; 6). CLC+VitC; 7). CLC+TLC and 8). CLC+TLC+VitC. The treatments were then directly placed into a refrigerator at 4°C for 48h. The kinematic parameters were determined at 0h, 24h and 48h using a computer-assisted semen analyzer (CASA) (SCA, 4.0, Microptic ®, Spain) Castellini & Lattailiolli, (1999). Lipid peroxidation was also analyzed at 0h, 24h and 48h using Thio-BarbituricAcid-Reactive-Substances (TBARS) method (Buegue, 1978).

Cyclodextrins complexes preparation

All reagents were from SIGMA-ALDRICH ® (PROCHIMA- SIGMA – Tlemcen, Algérie). CLC complex was prepared according to Purdy & Graham, (2004). Briefly, a mix of methyl-β-cyclodextrin solution (0.5g/ml in methanol) and cholesterol solution (200mg/ml in chloroform) was maintained under agitation for 24h at 25°C and shielded from light. The solvent was then rotary evaporated under vacuum and the residue crystalline powder was kept in a desiccator. The complex TLC was prepared using the method of co-evaporation described by Koontz *et al*. (2009). Methyl-β-cyclodextrin (309.11mg) and α-Tocopherol (100mg) were dissolved in 50ml éthanol. The mix was maintained under agitation for 24h at 25°C and shielded from light. The solvent was then rotary evaporated under vacuum and the residue crystalline powder was kept in a desiccator.

Statistical Analysis

The statistical analysis was performed using StatView ® statistical program (version 5.0). Values of motility percentages, kinematic parameters and TBARS of each treatment were expressed as means ± standard deviation of the mean (SD). Data for chilling treatments were analyzed by one-way analysis of variance (ANOVA) for comparison, followed by Post-hoc Fisher's test. A P- value <0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

The concentration and the percentage of motility of the pooled semen (Concentration: 683,42 million spz/ml; MOT:67.62%; PMOT:24.2%) were approachable to those reported by Brun *et al.* (2002) (716 million spz/ml and 77.4% of MOT). The kinematic parameters of pooled semen (VCL: 59.3µm/s; VAP: 41.4µm/s and VSL:27.1µm/s) were similar to the industrial findings of Lavara *et al*. (2005) (VCL, VAP, and VSL: 54.8µm/s, 36µm/s and 26.1µm/s respectively). Our findings showed the good quality of the pooled semen.

Figure 1 showed the percentages of total motility (MOT) and progressive motility (PMOT) for different treatments. Our findings showed a decrease of motility during refrigeration for all groups; the decrease was significant at 48h of preservation compared to 0h. However when Comparing treatments to the control, the results showed that CLC and CLC-VitC treatments presented –respectively- higher percentages ($p<0.05$) of PMOT at 24h and MOT at 48h.

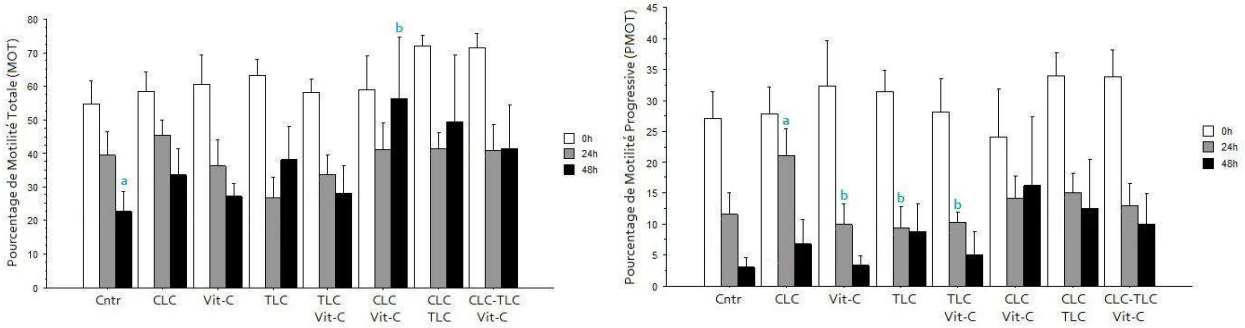

Figure 1: Effect of CLC, TLC and Vitamin E and their combinations on Total and progressive motility percentages. (Means ± SE), different letters indicate P-value <0.05 in comparison with control.

The effect of CLC, TLC and VitC on kinematic parameters is set out in Table 1. Compared to control, curvilinear velocity (VCL), average path velocity (VAP) and velocity of the straight line (VSL) of 4°C preserved sperm were significantly improved (p>0.05) by all treatments after 24h; in fact, the most effective at 24h was the combination CLC-TLC and CLC-TLC-VitC. At 48h of 4°C preservation, only the dualistic treatments CLC-TLC, CLC-VitC and CLC-TLC-VitC enhanced significantly (p<0.05) the VAP and VSL values. For the peroxidation levels (table2), our results did not show any significant effect of different treatment in comparison to control.

Table 1: Effect of CLC, TLC, VitC and their combinations on Kinematic parameters and TBARS level.

		Cntr	**CLC[1]**	**TLC[2]**	**VitC[3]**	**CLC TLC**	**CLC VitC**	**TLC VitC**	**CLC-TLC VitC**
VCL	0h	59.6±20.5	56.0*±24.4	55.4*±18.5	59.0±21.6	62.1±21.9	60±20.6	56.3*±19.4	57.3±19.5
(µm/s)	24h	46.9±24.0	57.7*±22.5	56.0*±23.9	58.1*±30.5	57.9*±22.5	51.1±20.3	51.1*±20.4	57.7*±22.8
	48h	45.3±17.4	51.7±21.9	49.8±24.7	48.9±21.6	54.7±24.6	61.4*±28.4	54±24.3	58.1*±24.5
VAP	0h	46.1±15.6	42.4*±15.8	44.7±15.2	47.8±18.5	48.2*±17	45.4±16.4	45.2±15.8	47.2±17.1
(µm/s)	24h	24.8±14.7	33.2*±13.9	32.2*±15.3	30.7*±16.1	36.4*±15.3	31.6*±13	31.2*±15.9	34.0*±14.4
	48h	22±11.	26.7±13.4	27±13.6	23.5±11.4	33.0*±16.8	32.3*±14.4	27.0±12.1	34.9*±17
VSL	0h	33±18.5	30*±18.1	32.7±17.5	34.7±20.6	31.9±19.1	29.5*±17.8	34.2±19.6	32.7±18.7
(µm/s)	24h	13.4±10.5	21.6*±12.5	19.4*±13.9	17.5*±11.3	21.8*±13.6	22.6*±12.2	20.0*±15.7	22.6*±13.5
	48h	13.6±10.3	14.8±9.8	16.2±10.3	13.8±9	19.0*±13.1	18.6*±10	16.4±9.5	21.1*±15.5
ALH	0h	1.80±0.9	1.81±1.0	1.61*±0.8	1.6*±0.9	1.91±1.0	1.89±0.9	1.59*±0.9	1.56*±0.8
(Hz)	24h	2.0±0.9	2.40*±0.9	2.32*±1.0	2.53*±1.3	2.35*±1.0	2.05±0.9	2.32*±0.8	2.37*±0.9
	48h	1.96±0.7	2.2±0.8	2.1±1	2.14±0.9	2.27±1.0	2.63*±1.2	2.39*±1.1	2.46*±1.1
TBARS[4]	0h	0.67±0.09	0.62±0.3	1.0±1.0	0.78±0.4	0.41±0.2	0.91±0.7	1.43±1.5	0.9±0.9
(nmol/ml)	24h	0.83±0.05	1.43±1.0	0.63±0.1	0.7±0.1	0.83±0.3	0.51±0.1	0.93±0.1	1.0±0.6
	48h	0.69±0.2	0.62±0.3	1.16±0.9	0.94±0.5	0.76±0.5	0.49±0.2	0.79±0.3	1.69±0.2

Means with (*) on the same row differ significantly to control (P-value<0.05). [1]CLC(Cholesterol-Loaded-Cyclodextrin). [2]TLC (α-tocopherol-laoded-Cyclodextrin). [3]VitC (Vitamin C). [4]TBARS/MDA (ThioBarbituric Acide Reactive Sustances/Malondealdehyde).

Cholesterol and α-tocopherol are important components of the spermatozoa plasma membrane. Cholesterol affected the fluidity of phospholipid bilayer (Mocé *et al*., 2009). Vitamine E remains the natural membrane's inserted antioxidant system (Mourvaki *et al.*, 2008). Few studies investigated the effect of cholesterol on rabbit sperm preserved at 4°C. Whereas, the use of cyclodextrins to treat rabbit semen at 4°C with α-tocopherol has never been investigated before. Our findings showed that CLC, TLC or their combination enhanced significantly the motility and kinematic parameters after 24h at 4°C. Aksoy *et al.* (2010) found that CLC enhanced osmotic tolerance in fresh rabbit semen and inhibited premature acrosome reaction in liquid storage at 4°C. Crispilho *et al.* (2013) and Nasseer *et al*. (2015) demonstrated also a positive effect on stallion and ram. Besides, our treatments did not show a significant impact of Cyclodextrin/Vitamin E complex on lipid peroxidation, in discordance to Castellini *et al.* (2000) where they found that motility parameters were not improved but only a

reduced lipid oxidation. The use of soluble Vitamin E (TROLOX) reported a positive impact especially in boar (Mendes *et al*. (2013). The combination of TLC-VitC showed no synergetic effect, in contrast to Castellini *et al.* (2000), Youcef *et al.* (2003). However, combine CLC-VitC enhanced the motility compared to CLC.

CONCLUSIONS

In conclusion, cholesterol and α-tocopherol complexed to cyclodextrins showed to be effective to enhance kinematic parameters after 24h at 4°C preservation. This technology has interesting perspectives in combination with other molecules such as LDL or liposomes.

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REFERENCES

Aksoy M., Akman O., Lehimcioğlu N.C., Erdem H. 2010: Cholesterol-loaded cyclodextrin enhances osmotic tolerance and inhibits the acrosome reaction in rabbit spermatozoa. *Anim. Reprod. Sci., 120, 166-172.*

Brun J.M., Theau-Clément M., Bolet G. 2002: The relationship between rabbit semen characteristics and reproductive performance after artificial insemination. *Anim. Reprod. Sci., 70, 139-149.*

Buege J.A., Aust, S.D. 1978: Microsomal lipid peroxidation. *Methods in Enzymology. 52: 302–310.*

Castellini C., Lattaioli P. 1999: Effect of number of motile sperms inseminated on reproductive performance of rabbit does. *Anim. Reprod. Sci., 57, 111-120.*

Crespilho A.M., Sprizziri B.E., Meyers M., Graham J.K. 2013: The effect of cholesterol addition, buffer, and pH on equine sperm stored at 5°C. *Equine veterinary Sci., 33, 663-666.*

Dad S., Bisby R.H., Clark I.P., Parker A.W. 2006: Formation of singlet oxygen from solutions of vitamin E. *Free. Radic. Res., 40, 333-338.*

Gączarzewicz D., Udała J., Piasecka M., Błaszczyk B., Stankiewicz T. 2015: Storage temperature of boar semen and its relationship to changes in sperm plasma membrane integrity, mitochondrial membrane potential, and oxidoreductive capability. *Turk J Biol.,39, 582-594.*

Gibb Z., Aitken R.J. 2016: The Impact of Sperm Metabolism during In Vitro Storage: The Stallion as a Model. *BioMed Research International., ID 9380609, 8 pages.*

Johinke D., de Graff S.P., Bathgate R. 2014: Investigation of *in vitro* parameters and *in vivo* fertility of rabbit spermatozoa after chilled storage. *Anim. Reprod. Sci., 147, 3-4, 135-143.*

Koontz J.L., Marcy J.E., O'Keefe S.F., Duncan S.E. 2009: Cyclodextrin Inclusion Complex Formation and Solid-State Characterization of the Natural Antioxidants α- Tocopherol and Quercetin. *J. Agr. Food. Chem., 57, 1162–1171.*

Lavara R., Moce E., Lavara F., Viudes de Castro M.P., Vicente S.J. 2005: Do parameters of seminal quality correlate with the results of on-farm inseminations in rabbits?. *Theriogenology., 64, 1130–1141.*

Mendez M. F. B., Zangeronimo M. G. Rocha L. G. P., Faria B. G., Pereira, B. A. C. D. Fernandes, Chaves B. R., Murgas L. D. S.. Sousa R. V. 2013: Effect of the addition of IGF-I and vitamin E to stored boar semen. *Animal, 7:5, 793–798.*

Mocé E., Blanch E,. Tomas C., Graham J.K. 2010: Use of Cholesterol in Sperm Cryopreservation: Present Moment and Perspectives to Future. *Reprod. Domes. Anim., 45, 57-66.*

Mourvaki E., Collodel G., Moretti E., Cosci E., Castellini C. 2008: Distribution of alpha-, gamma (+beta)- and delta-tocopherol in the seminal plasma, spermatozoa and seminal vesicles of rabbit. *Andrologia., 40, 282-285.*

Naseer Z., Ahmed E., Aksoy M., Kuçuk N., Serin I., Ceylan A., Boyacioglu M., Kum C. 2015: Protective effect of cholesterol-loaded cyclodextrin pretreatment against hydrogen peroxide-induced oxidative damage in ram sperm. *Cryobiology., 71, 18-23.*

Purdy P.H., Graham J. 2004: Effect of cholesterol-loaded cyclodextrin on the cryosurvival of bull sperm. *Cryobiology, 48, 36-45.*

Roca J., Martinez S., Vazquez J.M., Lucas X., Parrilla I., Martinez E.A. 2000: Viability and fertility of rabbit spermatozoa diluted in tris-buffer extenders and stored at 15°C. *Anim. Reprod. Sci., 64, 103-112.*

Rosato M.P., Di lorio M., Manchisi A., Gambacorta M., Petrosino G., Centoducati G., Santacroce M.P., Laffaldano N., 2012: In vitro survival and lipid peroxidation status of rabbit spermatozoa after both chilled and frozen storage in lycopene enriched extenders. *Livest. Sci., 146, 199-202.*

Rodríguez-Gil J. E. 2006: Mammalian sperm energy resources management and survival during conservation in refrigeration. *Reprod. Domest. Anim., 41(2), 11-20.*

Yousef M.I., Abdallah G.A., Kamel K.I. 2003: Effect of ascorbic acid and Vitamin E supplementation on semen quality and biochemical parameters of male rabbits. *Anim. Reprod. Sci., 76, 99–111.*