Cryobiology 89 (2019) 104–108

Contents lists available at ScienceDirect

Cryobiology

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

Effect of different concentrations of L-carnitine in extender for semen cryopreservation in sheep

Clara Vieira de Souza[a], Felipe Zandonadi Brandão[a], Juliana Dantas Rodrigues Santos[a], Vivian Angélico Pereira Alfradique[a,1], Vanessa Moreira Barbosa dos Santos[a], Maria Clara da Cruz Morais[a], Paulo Sergio Cerqueira Rangel[b], Andreza Amaral da Silva[c], Joanna Maria Gonçalves Souza-Fabjan[a,*]

[a] Faculdade de Medicina Veterinária, Universidade Federal Fluminense, Rua Vital Brasil Filho, 64, Niterói, RJ, 24320-340, Brazil
[b] Faculdade de Medicina Veterinária, Universidade do Grande Rio, Rua Professor José de Souza Herdy, 1160, Duque de Caxias, RJ, 25071-202, Brazil
[c] Faculdade de Medicina Veterinária, Universidade Federal Rural do Rio de Janeiro, Rodovia BR 465, Km 07, Seropédica, RJ, 23890-000, Brazil

ARTICLE INFO

Keywords:
Andrology
Antioxidant
Ovis aries
Semen preservation
Lipid peroxidation
CASA
Hypoosmotic
Oxidative stress

ABSTRACT

This study assessed the effect of L-carnitine (LC) in sheep semen extenders containing or not egg yolk for cryopreservation in sheep. Two extenders (TRIS-egg yolk or the commercial optiXcell™ IMV medium) were used, totaling six groups: IMV – (0, 5 and 10 mM LC) and TRIS – (0, 5 and 10 mM LC). After the freezing-thawing process and throughout incubation at 38 °C for up to 3 h, several parameters were evaluated: sperm kinetics, hypoosmotic, plasma membrane integrity, capacitation status and lipid peroxidation level. The supplementation of either 5 or 10 mM LC randomly affected some parameters and, overall, TRIS was superior ($P < 0.05$) than IMV extender. In the LC-groups, IMV had greater ($P < 0.05$) oxidative stress than TRIS. In conclusion, although LC affected isolated parameters, its supplementation in semen extender had no consistently beneficial effect on freezing-thawing ram sperm.

The advance in biotechnologies of sperm preparation has focused on preserving the fertilization capacity of frozen spermatozoa. The process of freezing and thawing leads to injuries in the sperm cell [12] and the overall success of the technique depends on the freezing medium composition. Different strategies have been established to optimize cryopreservation efficiency; several extenders have been developed and various cryoprotective substances have been tested [3,10]. Out of those, the most commonly used are egg yolk and glycerol [8]. Egg yolk has a membrane protective role, and vitamin E and ascorbic acid, known for their antioxidant properties.

The cryopreservation process causes oxidative stress to the sperm cell and the addition of antioxidants to the semen freezing media helps to protect the sperm against these damages [3,4]. Carnitine is an endogenous compound limiting the β-oxidation pathway, acting in the transport of fatty acids to mitochondria, maintaining cellular homeostasis and contributing to the reactive oxygen species production in physiological levels [11]. The use of L-carnitine (LC) in the extender for cryopreservation enhanced semen quality and had a greater post-thawing sperm motility in goat, rabbit and buffalo species [3,7,9] but did not have effect on boar semen [6]. Hence, the role of LC may be contradictory depending on the species; and the literature is still incipient regarding its use for the cryopreservation of sheep semen. We hypothesized that LC supplementation in the semen extender could improve the post-thawing ram sperm viability and its longevity, especially in the commercial extender medium without the presence of egg yolk (which naturally contains high quantity of antioxidants). Hence, to check the role of LC, this study aimed to assess the effect of different LC concentrations in both extenders, containing or not egg yolk, in sheep semen extenders.

This project was approved by the Ethics Committee for the Use of Animals (#1009) and followed the guidelines of Animal Research: Reporting of *In Vivo* Experiments (ARRIVE). All reagents used were from Sigma Chemical Co. (St. Louis, MO, USA); except for the optiXcell ™ extender (IMV Technologies, L'Aigle, France).

The study was conducted at UniPECO (22°S, 42°W), in Cachoeiras de Macacu, Brazil, in the breeding season. After clinical and andrological examination, four Santa Ines rams with proven fertility were used. The animals were under natural light, having access to the pasture and

* Corresponding author.
E-mail address: joannavet@gmail.com (J.M.G. Souza-Fabjan).
[1] Present address: Faculdade de Medicina Veterinária, Av. Peter Henry Rolfs, s/n, Viçosa, MG, 36570–900, Brazil.

https://doi.org/10.1016/j.cryobiol.2019.05.009
Received 12 January 2019; Received in revised form 9 May 2019; Accepted 20 May 2019
Available online 21 May 2019

Table 1
Ram sperm kinetics and plasma membrane integrity values before and immediately after (0 h) frozen-thawed sperm subjected to different extenders (IMV or TRIS) and concentrations of L-carnitine (LC) for cryopreservation (Mean +SEM).

		Before freezing			0 h		
		0 mM LC*	5 mM LC*	10 mM LC*	0 mM LC*	5 mM LC*	10 mM LC*
Total motility (%)	IMV**	79.9 ± 10.8 ^{Aa}	79.7 ± 13.2 ^{Aa}	77.8 ± 6.1 ^{Aa}	27.5 ± 5.2 ^{Aa}	30.4 ± 2.0 ^{Aa}	33.7 ± 12.6 ^{Aa}
	TRIS**	76.6 ± 15.9 ^{Aa}	88.2 ± 3.9 ^{Aa}	80.2 ± 10.5 ^{Aa}	41.7 ± 8.1 ^{Ba}	32.8 ± 12.5 ^{Aa}	42.3 ± 9.0 ^{Aa}
Fast sperm (%)	IMV	45.4 ± 11.5 ^{Aa}	50.0 ± 18.4 ^{Aa}	37.9 ± 10.9 ^{Aa}	1.9 ± 1.1 ^{Ba}	8.3 ± 6.7 ^{Aa}	10.9 ± 6.1 ^{Aa}
	TRIS	51.1 ± 11.2 ^{Aa}	52.8 ± 15.2 ^{Aa}	51.7 ± 1.4 ^{Ba}	13.4 ± 7.9 ^{Aa}	8.8 ± 5.8 ^{Aa}	8.3 ± 6.2 ^{Aa}
Medium sperm (%)	IMV	7.7 ± 1.7 ^{Aa}	8.2 ± 2.3 ^{Aa}	5.5 ± 1.9 ^{Aa}	1.2 ± 0.9 ^{Aa}	2.5 ± 0.6 ^{Aa}	3.9 ± 2.3 ^{Aa}
	TRIS	7.6 ± 2.1 ^{Aa}	8.2 ± 3.0 ^{Aa}	8.8 ± 4.3 ^{Aa}	4.4 ± 2.4 ^{Aa}	2.4 ± 0.5 ^{Aa}	7.8 ± 7.5 ^{Aa}
Slow sperm (%)	IMV	26.7 ± 4.4 ^{Aa}	21.5 ± 5.9 ^{Aa}	27.2 ± 6.5 ^{Aa}	24.3 ± 4.3 ^{Aa}	18.2 ± 3.6 ^{Aa}	18.9 ± 5.1 ^{Aa}
	TRIS	26.3 ± 2.6 ^{Aa}	20.8 ± 4.9 ^{Aa}	17.0 ± 5.3 ^{Aa}	28.3 ± 9.4 ^{Aa}	27.2 ± 11.7 ^{Aa}	24.7 ± 10.5 ^{Aa}
VCL (μm/s)***	IMV	94.7 ± 7.2 ^{Aa}	100.9 ± 23.3 ^{Aa}	91.1 ± 22.0 ^{Aa}	31.2 ± 7.9 ^{Aa}	56.5 ± 26.8 ^{Aa}	57.8 ± 12.4 ^{Aa}
	TRIS	102.9 ± 15.5 ^{Aa}	109.6 ± 11.7 ^{Aa}	119.1 ± 8.6 ^{Aa}	46.6 ± 5.4 ^{Ba}	48.0 ± 9.3 ^{Aa}	47.5 ± 19.2 ^{Aa}
VAP (μm/s)***	IMV	60.8 ± 7.7 ^{Aa}	67.6 ± 21.3 ^{Aa}	50.9 ± 15.1 ^{Aa}	72.4 ± 75.5 ^{Aa}	27.7 ± 9.9 ^{Aa}	27.4 ± 5.3 ^{Aa}
	TRIS	64.5 ± 16.1 ^{Aa}	75.6 ± 8.8 ^{Aab}	89.8 ± 11.1 ^{Bb}	27.5 ± 2.5 ^{Aa}	30.5 ± 8.5 ^{Aa}	23.7 ± 5.0 ^{Aa}
VSL (μm/s)***	IMV	39.0 ± 9.0 ^{Aa}	44.6 ± 15.1 ^{Aa}	30.1 ± 9.2 ^{Aa}	10.6 ± 2.4 ^{Aa}	14.8 ± 1.6 ^{Aab}	17.9 ± 2.9 ^{Ab}
	TRIS	44.0 ± 13.2 ^{Aa}	52.3 ± 7.8 ^{Aa}	43.6 ± 12.5 ^{Aa}	20.9 ± 4.8 ^{Ba}	23.6 ± 9.3 ^{Aa}	17.2 ± 3.1 ^{Aa}
ALH (μm/s)***	IMV	4.3 ± 0.3 ^{Aa}	4.4 ± 0.4 ^{Aa}	3.8 ± 0.2 ^{Aa}	3.2 ± 0.5 ^{Aa}	2.9 ± 0.8 ^{Aa}	2.6 ± 1.6 ^{Aa}
	TRIS	3.7 ± 1.0 ^{Aa}	4.0 ± 0.7 ^{Aa}	4.1 ± 0.2 ^{Aa}	3.2 ± 0.8 ^{Aa}	3.3 ± 0.8 ^{Aa}	2.6 ± 0.7 ^{Aa}
STR (%)***	IMV	59.4 ± 7.7 ^{Aa}	69.6 ± 4.3 ^{Ab}	59.5 ± 3.2 ^{Aa}	64.4 ± 11.5 ^{Aa}	62.7 ± 9.7 ^{Aa}	59.0 ± 13.2 ^{Aa}
	TRIS	68.0 ± 8.0 ^{Aa}	69.3 ± 7.0 ^{Aa}	62.3 ± 13.4 ^{Aa}	67.9 ± 11.2 ^{Aa}	80.5 ± 4.1 ^{Ba}	76.5 ± 9.8 ^{Aa}
BCF (Hz)***	IMV	8.2 ± 2.0 ^{Aa}	9.0 ± 2.6 ^{Aa}	8.4 ± 2.1 ^{Aa}	5.9 ± 0.3 ^{Aa}	6.3 ± 3.5 ^{Aa}	5.3 ± 3.9 ^{Aa}
	TRIS	9.4 ± 1.3 ^{Aa}	9.0 ± 2.7 ^{Aa}	8.1 ± 0.8 ^{Aa}	8.0 ± 2.3 ^{Aa}	9.4 ± 2.8 ^{Aa}	7.7 ± 1.9 ^{Aa}
LIN (%)***	IMV	40.8 ± 6.8 ^{Aa}	47.5 ± 1.0 ^{Aa}	38.3 ± 8.2 ^{Aa}	36.8 ± 15.2 ^{Aa}	34.1 ± 12.8 ^{Aa}	28.6 ± 8.7 ^{Aa}
	TRIS	47.7 ± 11.8 ^{Aa}	48.6 ± 11.9 ^{Aa}	45.1 ± 14.6 ^{Aa}	38.4 ± 11.9 ^{Aa}	55.1 ± 7.8 ^{Ba}	51.3 ± 20.3 ^{Aa}
WOB (%)***	IMV	64.1 ± 4.6 ^{Aa}	66.2 ± 6.9 ^{Aa}	64.2 ± 11.6 ^{Aa}	55.4 ± 14.2 ^{Aa}	52.9 ± 12.1 ^{Aa}	47.8 ± 4.0 ^{Aa}
	TRIS	69.3 ± 9.3 ^{Aa}	69.8 ± 12.5 ^{Aa}	75.3 ± 5.1 ^{Aa}	55.5 ± 8.2 ^{Aa}	63.6 ± 12.0 ^{Aa}	65.2 ± 17.5 ^{Aa}
PM integrity (%)***	IMV	33.6 ± 9.1 ^{Ab}	16.5 ± 10.4 ^{Aa}	17.0 ± 8.8 ^{Aa}	4.4 ± 2.2 ^{Aa}	2.0 ± 2.4 ^{Aa}	3.2 ± 2.5 ^{Aa}
	TRIS	30.2 ± 4.4 ^{Aa}	31.9 ± 15.6 ^{Aa}	28.8 ± 10.4 ^{Aa}	6.7 ± 1.5 ^{Aa}	4.2 ± 2.6 ^{Aa}	7.4 ± 10.7 ^{Aa}

Within a column, values with different superscripts differ significantly ($P < 0.05$).
a,b differ among groups treated with different concentrations of LC (0, 5, 10 mM) at the same moment (before freezing or 0 h).
A,B differ between extenders (IMV *vs.* TRIS) at the same moment (before freezing or 0 h) and at the same concentration.
* Concentrations of L-carnitine (Sigma C0158) used (either 0, 5 or 10 mM) in the semen extender for cryopreservation.
** Extenders: IMV: OptiXcell™; TRIS egg yolk.
*** VCL: curvilinear velocity; VAP: average path velocity; VSL: straight-line velocity; ALH: amplitude of lateral head displacement; STR: straightness; BCF: beat/cross frequency; LIN: linearity; WOB: wobble; PM integrity: plasma membrane integrity.

receiving commercial concentrate according to their nutritional requirements, with mineralized water and salt available *ad libitum*.

After semen collection, macroscopic (volume, odor, color, appearance) and microscopic (sperm kinetics, sperm membrane integrity, sperm capacitation, hypoosmotic test) assessments were performed. Then, a pool of semen of all males was formed, and then diluted according to the treatment. Two extenders were used: the commercial optiXcell™ medium (IMV Technologies, L'Aigle, France) or TRIS egg yolk (3.63 g TRIS, 0.50 g fructose, 1.99 g citric acid, 14 mL egg yolk, 100.000 IU penicillin, 100 mg streptomycin, 5% glycerol, glass-distilled water to 100 Ml; pH: 7.4 and mOsm: 886). For each extender, different concentrations of LC [0, 5 and 10 mM; (Sigma C0158)] were supplemented, totaling six groups. A final concentration of 100 x 10^6 sperm/straw was done. After dilution, the same microscopic evaluations and sperm concentration were performed. Later, semen was packed in 0.25 mL straws and cryopreserved in liquid nitrogen. Immediately after thawing (0 h), semen samples from each treatment were evaluated (the same microscopic analyses) and lipoperoxidation of sperm cell was quantified. Then, semen was incubated in Fert-TALP medium (0.33 g NaCl, 0.011 g KCl, 100 μL NaH_2PO_4, 93 μL Na lactate, 0.105 g $NaHCO_3$, 100 μL Phenol Red, 0.0135 g caffeine, 0.0147 g $CaCl_2$ $2H_2O$, 50 μL $MgCl_2$, 0.119 g Hepes) at 38 °C in 5% CO_2, and the same parameters were assessed at 1 h, 2 h and 3 h of incubation.

Sperm motility parameters were analyzed by objective computer-assisted semen analysis (CASA) using the SCA® system (Sperm Class Analyzer Microptic, Nikon Eclipse Ci – Tokyo, Japan). The software configuration was adjusted for ram sperm. The standard configuration of the parameters was as follows: the spermatic head dimensions detectable by the system between 18 and 60 μm^2. Spermatozoa were identified as immobile with curvilinear velocity (VCL) below 10 μm/s; between 10 and 45 μm/s were classified as slow, between 45 and 75 μm/s were classified as medium; and above 75 μm/s were classified as fast. Spermatozoa presenting straightness (STR) above 80% were considered progressive [1]. Nine selected fields were automatically evaluated for the kinetics parameters: Total Motility (TM, %); Average path velocity (VAP, μm/s); Curvilinear Velocity (VCL, μm/s); Straight-line velocity (VSL, μm/s); Amplitude of lateral head displacement (ALH, μm/s); Beat/Cross Frequency (BCF, Hz); Straightness (STR, %); Linearity (LIN, %); WOB is defined as the mean value of the ratio between VAP and VCL (WOB, %). Ejaculates with motility ≥70% were selected for freezing. Sperm concentration was determined by Neubauer chamber.

The straws were placed in an automated freezing system (TK 3000®; TK Tecnologia em Congelação Ltda, Uberaba, Brazil), preheated at 32 °C. A cooling rate of 0.25 °C/min until 5 °C was applied, and this temperature was maintained for 4 h. The freezing rate used was 20 °C/min from 5 to −120 °C, when the straws were immersed in liquid nitrogen (−196 °C) [8].

The capacitation status was assessed by chlortetracycline test (CTC) [1]. A 0.75 mM CTC solution was prepared daily in a buffer containing 20 mM Tris, 130 mM NaCl and 5 mM cysteine, pH 7.8. For staining, 0.01 mL of sperm sample was mixed with 0.01 mL of CTC solution in one slide. Then, a drop of antifouling solution (0.22 M 1,4-diazabicyclo [2.2.2] octane, DABCO) was mixed to retard loss of CTC fluorescence. The samples were observed in a microscope under epifluorescence (1000x) and were classified as: noncapacitated, capacitated or acrosome reacted cells. Cells were observed using 2A blue-violet filter (BV-2A) with emission in the 400–440 nm range and 470 nm fluorescence

Table 2
Ram sperm kinematic and plasma membrane integrity values after incubation (1–3 h) of frozen-thawed sperm subjected to different extenders (IMV or TRIS) and concentrations of L-carnitine (LC) for cryopreservation (Mean +SEM).

		1 h			2 h			3 h		
		0 mM LC*	5 mM LC*	10 mM LC*	0 mM LC*	5 mM LC*	10 mM LC*	0 mM LC*	5 mM LC*	10 mM LC*
Total motility (%)	IMV**	35.5 ± 18.8 ^{Aa}	30.4 ± 1.0 ^{Aa}	23.1 ± 3.6 ^{Aa}	22.2 ± 7.8 ^{Aa}	25.2 ± 3.1 ^{Aa}	40.4 ± 20.8 ^{Aa}	27.7 ± 13.6 ^{Aa}	30.8 ± 19.4 ^{Aa}	32.2 ± 19.3 ^{Aa}
	TRIS**	37.9 ± 10.9 ^{Aa}	28.3 ± 4.1 ^{Aa}	23.1 ± 4.1 ^{Aa}	38.3 ± 16.0 ^{Aa}	37.1 ± 12.1 ^{Aa}	28.4 ± 6.3 ^{Aa}	18.6 ± 8.7 ^{Aa}	42.2 ± 21.0 ^{Aa}	28.2 ± 4.8 ^{Aa}
Fast sperm (%)	IMV	1.9 ± 1.1 ^{Aa}	5.6 ± 5.9 ^{Aa}	2.5 ± 0.9 ^{Aa}	2.1 ± 1.9 ^{Aa}	4.0 ± 2.3 ^{Aa}	6.6 ± 5.0 ^{Aa}	4.5 ± 5.6 ^{Aa}	8.2 ± 9.9 ^{Aa}	3.6 ± 3.8 ^{Aa}
	TRIS	4.0 ± 2.4 ^{Aa}	3.3 ± 0.7 ^{Aa}	1.9 ± 1.6 ^{Aa}	5.0 ± 4.9 ^{Aa}	6.8 ± 4.4 ^{Aa}	2.0 ± 0.8 ^{Aa}	1.1 ± 0.7 ^{Aa}	2.4 ± 2.5 ^{Aa}	1.9 ± 0.6 ^{Aa}
Medium sperm (%)	IMV	3.0 ± 2.4 ^{Aa}	3.5 ± 2.3 ^{Aa}	1.4 ± 0.8 ^{Aa}	1.0 ± 0.6 ^{Aa}	1.7 ± 0.9 ^{Aa}	3.2 ± 1.5 ^{Aa}	1.8 ± 0.9 ^{Aa}	2.3 ± 2.1 ^{Aa}	2.2 ± 1.5 ^{Aa}
	TRIS	1.7 ± 1.2 ^{Aa}	2.5 ± 1.8 ^{Aa}	1.6 ± 0.4 ^{Aa}	1.6 ± 1.0 ^{Aa}	2.4 ± 1.3 ^{Aa}	3.1 ± 0.8 ^{Aa}	0.9 ± 0.7 ^{Aa}	0.8 ± 0.4 ^{Aa}	1.2 ± 0.7 ^{Aa}
Slow sperm (%)	IMV	18.6 ± 2.4 ^{Aa}	24.7 ± 8.6 ^{Aa}	18.4 ± 2.6 ^{Aa}	19.1 ± 6.1 ^{Aa}	19.6 ± 1.8 ^{Aa}	30.5 ± 16.4 ^{Aa}	21.4 ± 8.3 ^{Aa}	20.4 ± 7.9 ^{Aa}	26.3 ± 14.4 ^{Aa}
	TRIS	36.2 ± 7.5 ^{Ba}	21.3 ± 9.8 ^{Aa}	20.0 ± 8.1 ^{Aa}	31.6 ± 17.9 ^{Aa}	27.9 ± 10.5 ^{Aa}	22.0 ± 6.0 ^{Aa}	21.7 ± 10.8 ^{Aa}	36.3 ± 16.3 ^{Aa}	24.0 ± 3.0 ^{Aa}
VCL (μm/s)***	IMV	30.1 ± 5.2 ^{Aa}	48.1 ± 22.5 ^{Aa}	29.5 ± 3.0 ^{Aa}	27.9 ± 6.9 ^{Aa}	43.4 ± 12.8 ^{Aa}	38.3 ± 10.4 ^{Aa}	34.9 ± 18.2 ^{Aa}	42.0 ± 22.0 ^{Aa}	29.4 ± 7.1 ^{Aa}
	TRIS	30.0 ± 12.2 ^{Aa}	35.7 ± 10.2 ^{Aa}	29.0 ± 7.7 ^{Aa}	36.1 ± 15.9 ^{Aa}	44.1 ± 4.5 ^{Aa}	31.7 ± 3.9 ^{Aa}	37.7 ± 19.5 ^{Aa}	28.3 ± 9.0 ^{Aa}	23.6 ± 2.5 ^{Aa}
VAP (μm/s)***	IMV	113.3 ± 106.0 ^{Aa}	38.2 ± 23.5 ^{Aa}	19.8 ± 2.6 ^{Aa}	18.5 ± 7.3 ^{Aa}	34.6 ± 13.6 ^{Aa}	28.7 ± 10.0 ^{Aa}	27.9 ± 18.9 ^{Aa}	32.3 ± 24.3 ^{Aa}	20.2 ± 10.4 ^{Aa}
	TRIS	31.2 ± 5.7 ^{Aa}	26.8 ± 9.6 ^{Aa}	21.1 ± 8.1 ^{Aa}	30.8 ± 13.9 ^{Aa}	38.1 ± 4.9 ^{Aa}	25.7 ± 4.6 ^{Aa}	32.0 ± 18.7 ^{Aa}	23.1 ± 10.6 ^{Aa}	18.3 ± 4.6 ^{Aa}
VSL (μm/s)***	IMV	15.1 ± 4.2 ^{Aa}	32.1 ± 23.2 ^{Aa}	15.1 ± 2.9 ^{Aa}	13.9 ± 6.8 ^{Aa}	27.8 ± 14.6 ^{Aa}	23.0 ± 8.9 ^{Aa}	23.4 ± 17.2 ^{Aa}	27.0 ± 23.0 ^{Aa}	16.1 ± 10.0 ^{Aa}
	TRIS	26.6 ± 5.5 ^{Ba}	21.6 ± 8.9 ^{Aa}	16.4 ± 7.7 ^{Aa}	26.9 ± 13.0 ^{Aa}	30.5 ± 8.0 ^{Aa}	21.9 ± 5.3 ^{Aa}	27.7 ± 17.1 ^{Aa}	19.6 ± 10.5 ^{Aa}	13.8 ± 4.5 ^{Aa}
ALH (μm/s)***	IMV	1.7 ± 0.3 ^{Aa}	2.0 ± 0.5 ^{Aa}	2.1 ± 0.4 ^{Ba}	1.7 ± 0.6 ^{Aa}	1.8 ± 0.2 ^{Aa}	1.8 ± 0.1 ^{Aa}	1.5 ± 0.2 ^{Aa}	1.5 ± 0.1 ^{Aa}	1.2 ± 0.4 ^{Aa}
	TRIS	6.5 ± 9.0 ^{Ab}	1.9 ± 0.3 ^{Aab}	1.4 ± 0.2 ^{Aa}	1.5 ± 0.6 ^{Aa}	1.7 ± 0.2 ^{Aa}	1.6 ± 0.3 ^{Aa}	1.7 ± 0.1 ^{Aa}	1.7 ± 0.1 ^{Aa}	1.5 ± 0.2 ^{Aa}
STR (%)***	IMV	77.1 ± 5.9 ^{Aa}	76.5 ± 5.9 ^{Aa}	74.0 ± 3.3 ^{Aa}	72.8 ± 10.8 ^{Aa}	77.3 ± 12.9 ^{Aa}	77.2 ± 2.5 ^{Aa}	85.8 ± 5.4 ^{Aa}	77.9 ± 10.1 ^{Aa}	73.5 ± 15.7 ^{Aa}
	TRIS	85.1 ± 2.5 ^{Aa}	82.5 ± 8.0 ^{Aa}	84.1 ± 6.5 ^{Aa}	85.2 ± 1.4 ^{Aa}	86.4 ± 4.8 ^{Aa}	87.6 ± 1.6 ^{Ba}	84.1 ± 6.4 ^{Aa}	88.0 ± 0.9 ^{Aa}	74.5 ± 10.0 ^{Aa}
BCF (Hz)***	IMV	6.2 ± 1.2 ^{Aa}	7.4 ± 0.9 ^{Aa}	6.3 ± 1.0 ^{Aa}	5.2 ± 1.1 ^{Aa}	6.2 ± 0.5 ^{Aa}	6.7 ± 0.1 ^{Aa}	6.0 ± 1.7 ^{Aa}	4.7 ± 2.3 ^{Aa}	4.7 ± 2.4 ^{Aa}
	TRIS	7.8 ± 0.3 ^{Aa}	6.9 ± 0.4 ^{Aa}	7.0 ± 0.7 ^{Aa}	6.4 ± 1.5 ^{Aa}	6.5 ± 1.0 ^{Aa}	6.4 ± 0.9 ^{Aa}	6.6 ± 0.6 ^{Aa}	6.6 ± 0.3 ^{Aa}	6.2 ± 1.4 ^{Aa}
LIN (%)***	IMV	49.7 ± 6.4 ^{Aa}	54.2 ± 9.7 ^{Aa}	53.3 ± 10.6 ^{Aa}	47.4 ± 12.6 ^{Aa}	60.9 ± 16.0 ^{Aa}	59.1 ± 12.4 ^{Aa}	61.5 ± 18.6 ^{Aa}	55.4 ± 22.3 ^{Aa}	50.6 ± 25.0 ^{Aa}
	TRIS	72.6 ± 6.4 ^{Ba}	66.0 ± 17.9 ^{Aa}	70.0 ± 12.7 ^{Aa}	73.6 ± 7.9 ^{Ba}	74.7 ± 8.6 ^{Aa}	74.1 ± 1.8 ^{Ba}	69.3 ± 10.6 ^{Aab}	76.7 ± 3.7 ^{Ab}	53.6 ± 14.5 ^{Aa}
WOB (%)***	IMV	65.7 ± 5.5 ^{Aa}	75.0 ± 12.7 ^{Aa}	69.6 ± 9.6 ^{Aa}	64.4 ± 9.5 ^{Aa}	77.9 ± 8.2 ^{Aa}	74.4 ± 11.8 ^{Aa}	75.0 ± 13.7 ^{Aa}	69.4 ± 19.5 ^{Aa}	65.0 ± 23.1 ^{Aa}
	TRIS	85.3 ± 5.7 ^{Ba}	79.0 ± 14.1 ^{Aa}	82.8 ± 8.2 ^{Aa}	85.1 ± 7.0 ^{Ba}	88.6 ± 2.0 ^{Aa}	84.6 ± 2.4 ^{Aa}	82.0 ± 7.2 ^{Aab}	87.2 ± 4.4 ^{Ab}	71.0 ± 10.1 ^{Aa}
PM integrity (%)***	IMV	4.7 ± 5.4 ^{Aa}	4.9 ± 4.1 ^{Aa}	2.7 ± 1.1 ^{Aa}	1.9 ± 1.7 ^{Aa}	6.4 ± 5.6 ^{Aa}	8.1 ± 6.4 ^{Aa}	3.9 ± 4.1 ^{Aa}	3.2 ± 3.1 ^{Aa}	2.8 ± 2.1 ^{Aa}
	TRIS	1.5 ± 0.7 ^{Aa}	1.8 ± 1.8 ^{Aa}	3.2 ± 2.6 ^{Aa}	2.0 ± 1.2 ^{Aa}	1.7 ± 0.6 ^{Aa}	2.6 ± 2.5 ^{Aa}	3.4 ± 3.3 ^{Aa}	2.8 ± 1.7 ^{Aa}	2.9 ± 2.1 ^{Aa}

Within a column, values with different superscripts differ significantly ($P < 0.05$).
a,b differ among groups treated with different concentrations of LC (0, 5, 10 mM) and at the same moment (during incubation).
A,B differ between extenders (IMV *vs*. TRIS) at the same moment (during incubation) and at the same concentration of LC (0, 5, 10 mM).
*Concentrations of L-carnitine (Sigma C0158) used (either 0, 5 or 10 mM) in the semen extender for cryopreservation.
**Extenders: IMV: OptiXcell™; TRIS egg yolk.
***VCL: curvilinear velocity; VAP: average path velocity; VSL: straight-line velocity; ALH: amplitude of lateral head displacement; STR: straightness; BCF: beat/cross frequency; LIN: linearity; WOB: wobble; PM integrity: plasma membrane integrity.

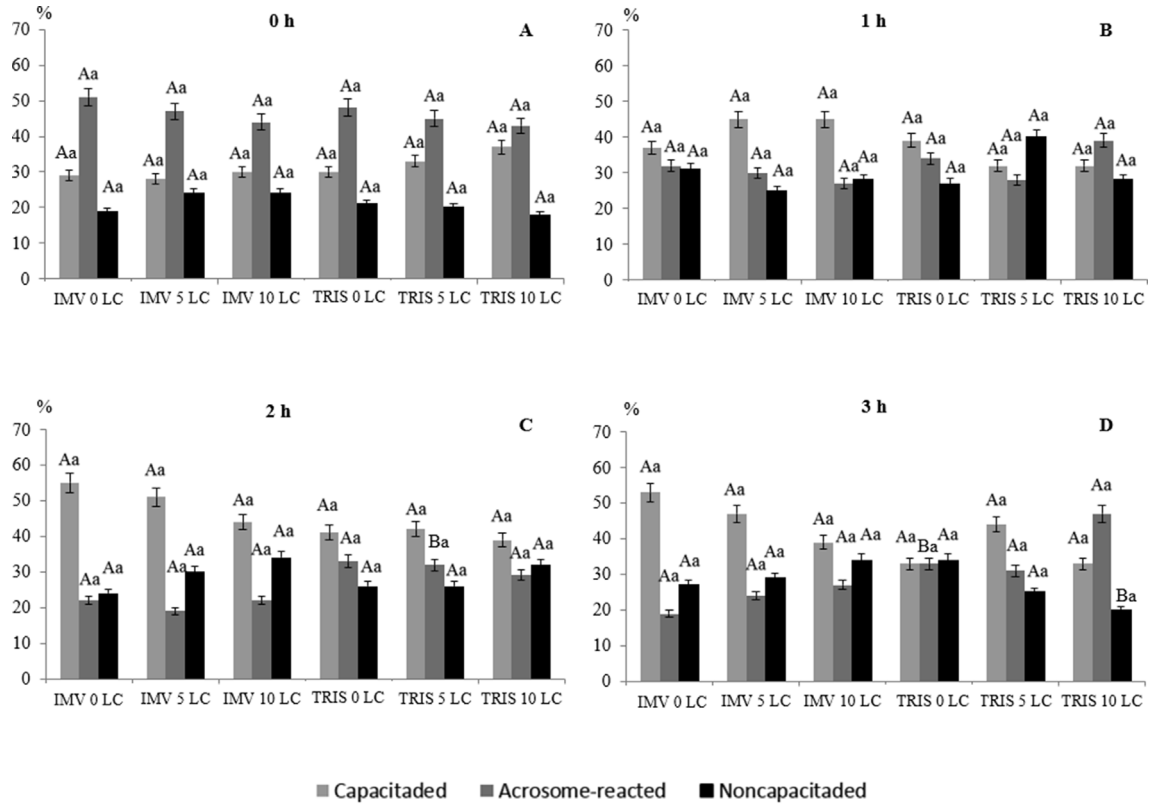

Fig. 1. Capacitation status of ram sperm: A) immediately after freezing-thawing (0 h) and during incubation moments, B) 1 h, C) 2 h and D) 3 h. Sperm was frozen using different extenders (IMV or TRIS) and concentrations (0, 5 and 10 mM) of L-carnitine (LC) (Sigma C0158). A,B differ significantly between extenders (IMV *vs.* TRIS) at the same moment (during incubation) and at the same concentration of LC. a,b differ significantly among groups treated with different concentrations of LC and at the same moment (during incubation).

excitation. At least 200 sperm cells were assessed.

The plasma membrane integrity was performed by the association of two fluorescent probes: acridine orange [10.000x] and propidium iodide (0.5 mg/mL). The analysis was performed in the CASA system connected to a microscope under epifluorescence illumination (100x) equipped with appropriate filter set (465–495 nm excitation and 515–555 nm emission). At least 300 sperm per slide were analyzed [1]. For the hypoosmotic test, aliquots of 30 µL of fresh semen were collected and placed in tubes with 1 mL of hypoosmotic solution of 100 mOsm/kg, composed of sodium citrate (0.49 g), fructose (0.9 g) and MilliQ water (100 mL). Then, these aliquots were incubated at 37 °C for 60 min and evaluated through wet preparation between slide and cover slip, by means of phase contrast microscopy in a 1000x fold increase and immersion. At least 200 sperm cells were counted.

The method to quantify lipoperoxidation was based on the reaction between the molecules of thiobarbituric acid and malondialdehyde, producing a rose color that is quantified by spectrophotometry at a wavelength of 532 nm (nm). Reactions occur at a temperature between 90 °C and 100 °C, at acidic pH. Aliquots of 500 µL of each treatment, and 1000 µL of 10% trichloroacetic acid solution (10% TCA) were centrifuged at 1800 *g* for 15 min and at 15 °C for precipitation of proteins. Aliquots of 500 µL of the supernatant were placed in test tubes along with 500 µL of 1% thiobarbituric acid, dissolved in 0.05 N sodium hydroxide, freshly prepared. The tubes containing this mixture were incubated in a boiling bath at 100 °C for 10 min and then cooled in an ice bath at 0 °C. The Thiobarbituric Acid Reactive Species (TBARs) were quantified in a spectrophotometer, at a length of 532 nm and was expressed in nanograms of TBARs/mL of semen.

Data were submitted to the Shapiro Wilk normality test and Levene variance homogeneity. Parametric data were submitted to one-way ANOVA followed by Tukey test whilst non-parametric data were analyzed by either Mann Whitney or Kruskal Wallis followed by Dunn's test. The general linear model (GLM) was used to check the interaction between diluents and treatments. Values of $P < 0.05$ were considered significant.

All macroscopic analyses were within the standard for the ovine species. The sperm kinetics before and post-thawing are shown in Table 1. The supplementation of 10 mM LC positively affected the VAP parameter in TRIS and VSL in IMV extender. Indeed, 5 mM LC improved the STR parameter in IMV extender. The sperm kinetics values throughout incubation are listed in Table 2. The TRIS extender promoted better indices of VSL, LIN, WOB and STR than IMV extender along incubation, regardless the LC presence.

The plasma membrane integrity was similar ($P > 0.05$) among groups (Table 1; Table 2). For hypoosmotic test, the average number of intact cells was higher in TRIS supplemented with 10 mM LC at 1 h (31.0 ± 1.8) and 5 mM LC at 2 h (29.6 ± 5.1) compared to the IMV extender groups. The capacitation status in both extenders differed at 2 and 3 h (Fig. 1). IMV groups supplemented with 5 and 10 mM LC had greater ($P > 0.05$) oxidative stress than TRIS supplemented with 5 and 10 mM LC, respectively.

This study investigated the role of different concentrations of LC added in the semen extender for cryopreservation of ram sperm. Overall, our hypothesis could not be supported, since the LC effect was random i.e., it did not consistently affect any group in any concentration used. The post-thawing motility was similar among groups, corroborating to the reported for goat frozen-thawed sperm, where LC addition resulted in insignificant increase in sperm motilities [3]. Conversely, in human [2] and rabbit [9], sperm motility improved after LC supplementation. Perhaps this could be related to a species effect or to the concentration used in each study.

We wrongly expected that throughout incubation LC

supplementation would promote a higher sperm longevity, due to its function as energy supplier and being a facilitator for activated fatty acid transport into the mitochondrial matrix for β-oxidation. Perhaps, the concentrations used were not enough to improve ram sperm. LC supplementation in the semen extender has been previously tested in buffalo [7], goat [3], rabbit [9], humans [2], rooster [5], among others; but, in these studies, incubation times were not evaluated.

In the current study, LC did not affect lipoperoxidation, different from that reported in rooster semen [5]. However, in IMV groups, oxidative stress was higher than in TRIS, probably due the action of egg yolk providing an antioxidant support. Regarding plasma membrane integrity, hypoosmotic test and capacitation status, we did not find any protective effect of LC. The LC supplementation in the semen extender in goats [3] and bovine [4] improved sperm acrosomal integrity in freezing-thawing semen. This variance may be related to factors such as species, components of extenders, procedure of freezing and different concentrations of antioxidant used.

In conclusion, although LC affected some isolated parameters, its supplementation in semen extender had no consistently beneficial effect on freezing-thawing ram sperm and throughout incubation for up to 3 h. The commercial optiXcell™ IMV medium extender compromised sperm kinetics and it had greater oxidative stress compared to the TRIS extender. Our findings provide a platform to further investigate and design protocols to test the beneficial effects of LC supplementation on cryopreserved ram semen.

Conflicts of interest

The authors have no conflict to report.

Acknowledgments

The authors thank the Federal Fluminense University – InfraLabPesq/PROPPi and FAPERJ for funding the project. CVS was supported by FAPERJ, FZB is a CNPq fellow and JMGS-F is a FAPERJ fellow.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cryobiol.2019.05.009.

References

[1] V.A.P. Alfradique, R.I.T.P. Batista, J.M.G. Souza-Fabjan, L.R. Côrtes, G.M. Bragança, C.V. Souza, L.C. Costa, F.Z. Brandão, Supplementation of 17β-estradiol and progesterone in the co-culture medium of bovine oviductal epithelial cells and ovine spermatozoa reduces the sperm kinematics and capacitation, Reprod. Biol. 18 (2018) 368–379 https://doi.org/10.1016/j.repbio.2018.10.004.

[2] S. Banihani, A. Agarwal, R. Sharma, M. Bayachou, Cryoprotective effect of L-carnitine on motility, vitality and DNA oxidation of human spermatozoa, Andrology 46 (2013) 637–641 https://doi.org/10.1111/and.12130.

[3] M.N. Bucak, S. Sarıozkanb, P.B. Tuncera, F. Sakinc, A. Atessahin, R. Kulaksız, M. Cevik, The effect of antioxidants on post-thawed Angora goat (*Capra hircus ancryrensis*) sperm parameters, lipid peroxidation and antioxidant activities, Small Rumin. Res. 89 (2010) 24–30 https://doi.org/10.1016/j.smallrumres.2009.11.015.

[4] M.N. Bucak, P.B. Tuncer, S. Sarıozkan, N. Baspınar, M. Taspınar, K. Coyan, A. Bilgili, P.P. Akalın, S. Buyukleblebici, S. Aydos, S. Ilgaz, A. Sunguroglu, D. Oztuna, Effects of antioxidants on post-thawed bovine sperm and oxidative stress parameters: antioxidants protect DNA integrity against cryodamage, Cryobiology 61 (2010) 248–253 https://doi.org/10.1016/j.cryobiol.2010.09.001.

[5] A. Fattah, M. Sharafi, R. Masoudi, A. Shahverdi, V. Esmaeili, A. Najafi, L-Carnitine in rooster semen cryopreservation: flow cytometric, biochemical and motion findings for frozen-thawed, Cryobiology 74 (2016) 148–153 https://doi.org/10.1016/j.cryobiol.2016.10.009.

[6] D.M. Kozink, M.J. Estienne, A.F. Harper, J.W. Knight, Effects of dietary L-carnitine supplementation on semen characteristics in boars, Theriogenology 61 (2004) 1247–1258 https://doi.org/10.1016/j.theriogenology.2003.07.022.

[7] V. Longobardi, A. Salzano, G. Campanile, R. Marrone, F. Palumbo, M. Vitiello, G. Zullo, B. Gasparrini, Carnitine supplementation decreases capacitation-like changes of frozen-thawed buffalo spermatozoa, Theriogenology 88 (2016) 236–243 https://doi.org/10.1016/j.theriogenology.2016.09.031.

[8] M.S. Maia, S.D. Bicudo, H.C. Azevedo, C.C. Sicherle, D.B. Sousa, L. Rodello, Motility and viability of ram sperm cryopreserved in a Tris-egg yolk extender supplemented with anti-oxidants, Small Rumin. Res. 85 (2009) 85–90 https://doi.org/10.1016/j.smallrumres.2009.07.001.

[9] S. Sarıozkan, S. Ozdamar, G. Turk, F. Canturk, A. Yay, In vitro effects of L-carnitine and glutamine on motility, acrosomal abnormality, and plasma membrane integrity of rabbit sperm during liquid-storage, Cryobiology 68 (2014) 349–353 https://doi.org/10.1016/j.cryobiol.2014.04.006.

[10] P.P.N. Snoeck, L.C.O. Moura, M.C. Silva, M. Machado-Neves, M.I.V. Melo, L.G.D. Heneine, M. Henry, Effect of storage conditions on the LDL effectiveness in ovine sperm cryopreservation, Cryobiology 75 (2017) 88–90 https://doi.org/10.1016/j.cryobiol.2017.01.007.

[11] F.M. Vaz, R.J.A. Wanders, Carnitine biosynthesis in mammals, Biochem. J. 361 (2002) 417–429 https://doi.org/10.1042/bj3610417.

[12] L. Vichas, I.A. Tsakmakidis, D. Vafiadis, G. Tsousis, E. Malama, C.M. Boscos, The effect of antioxidant agents addition and freezing method on quality parameters of frozen thawed ram, Cell Tissue Bank. 19 (2017) 113–121 https://doi.org/10.1007/s10561-017-9633-6.