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Supplementing a skimmed milk–egg yolk-based extender with L-carnitine helps maintain the motility, membrane integrity and fertilizing capacity of chilled ram sperm

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Abstract

This study examines the effect of L-carnitine (LC) on chilled ram semen stored for up to 96 hr. Semen samples were collected, placed in a skimmed milk + 6% egg yolk extender, pooled, aliquoted and diluted with the same extender supplemented with different LC concentration: 0 (control), 1 mM (LC1), 2.5 mM (LC2.5), 5 mM (LC5), 7.5 mM (LC7.5) or10 mM (LC10). Sperm kinetics and membranes (plasma, acrosome and mitochondrial) were examined using the CASA system and triple fluorescence staining (PI/ PNA-FITC/Mitotracker). The progressive motility was greater ($p < .05$) with LC7.5 treatment than the control sperm at 96 hr. The curvilinear velocity ($p < .01$) and the percentage of sperm with intact membranes (plasma/acrosome/mitochondria) ($p < .01$) were greater with all LC treatments than the control group at all times. Straight line velocity was greater ($p < .01$) with LC5 and LC7.5 treatments than the control group after 48 hr. The LC5 group also returned lower ALH values ($p < .05$) than these seen for the control groups after 48 hr. The fertilizing capacity of LC5 samples stored at 15°C for 2 hr (LC5-15°C-2h) and at 5°C for 24 hr (LC5-5°C-24h) was tested in three ewe groups via cervical fixed-time artificial insemination. In two groups, the fertilizing capacity of the LC5-5°C-24h was reduced ($p < .001$). In the remaining group, however, no significant difference was seen between the LC5-15°C-2h and LC5-5°C-24h sperm in this respect (pregnancy rates 52.4% versus 42.8%; $p > .05$). Overall, the present results suggest that supplementing skimmed milk–egg yolk-based extenders with LC has a positive effect on chilled sperm variables and fertilizing capacity.

KEYWORDS

chilled, fertility, L-carnitine, ram sperm, skimmed milk

1 | INTRODUCTION

Cervical fixed-time artificial insemination (FTAI) using sperm diluted with a skimmed milk extender (e.g. egg yolk-free INRA-96®), and maintained at 15°C for 1–6 hr, is the most common method of assisted fertilization used in genetic improvement programmes for European sheep. It is known that chilling at 5°C reduces the metabolic activity of the sperm and helps preserve plasma membrane integrity and fertilizing capacity for some time. Indeed, high in vitro fertility rates have been recorded with semen preserved for up to 10 days (Maxwell & Salamon, 1993). However, sperm kinetic vigour and membrane integrity are inversely related to the duration of cold storage (Gil, Fierro, Bentancur, & Olivera-Muzante, 2011), and falling pregnancy rates are seen in ewes cervically inseminated at

synchronized (O'Hara et al., 2010) or spontaneous oestrus (Olivera-Muzante, Fierro, & Gil, 2011) when using chilled (5°C) semen diluted with a skimmed milk–egg yolk-based extender after storage for ≥24 hr. The same is true for semen diluted with egg yolk-based extender stored at 5°C for 24–48 hr (Maxwell & Salamon, 1993).

Two main factors affect sperm preserved under chilled conditions. The first, cold shock, is associated with irreversible changes in capacitation, damage to the plasma membrane, the induction of the caspase cascade involved in apoptosis, DNA hypomethylation and fragmentation, and low-fertilizing capacity (Budai, Egerszegi, Olah, Javor, & Kovacs, 2014; Salamon & Maxwell, 2000). The second, oxidative stress, is associated with lipid peroxidation (LPO), which leads to reduced sperm quality (Budai et al., 2014). The high concentration of polyunsaturated fatty acids (PUFA) in the membranes of small ruminant spermatozoa render them highly vulnerable to oxidative damage (Bucak, Tuncer, et al., 2010). Ram sperm cells are also vulnerable to attack by free radicals (e.g. H_2O_2, O_2- and OH-) since they are rich in PUFA; reactive oxygen species (ROS) readily combine with PUFA leading to the production of LPO (Alvarez, Touchstone, Blasco, & Storey, 1987). The increase in ROS during chilled storage is known to disrupt the mitochondrial and plasma membranes of ram sperm cells, thus impairing their motility (Amidi, Pazhohan, Shabani Nashtaei, Khodarahmian, & Nekoonam, 2016). They also cause DNA fragmentation (Kasimanickam, Pelzer, Kasimanickam, Swecker, & Thatcher, 2006).

Antioxidants can ameliorate the detrimental effect of ROS and improve the quality of frozen–thawed (Silva et al., 2011) and chilled sperm (Bucak & Tekin, 2007). A number of enzymatic antioxidants, for example reduced glutathione (GSH), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and catalase (CAT), protect against the formation of LPO in frozen–thawed ram sperm, allowing for acceptable motility values and percentages of cells showing plasma membrane integrity (Câmara, Mello-Pinto, et al., 2011; Câmara, Silva, Almeida, Nunes, & Guerra, 2011). Other antioxidants, such as resveratrol, vitamin E and their combination, have been shown to help maintain the motility of chilled ram semen stored for up to 10 days (Sarlós, Molnár, Kókai, Gábor, & Rátky, 2002).

L-carnitine (LC) is a non-essential amino acid, synthesized from the essential amino acids lysine and methionine that plays an important role in transporting short-, medium- and long-chain fatty acids into the mitochondria for β-oxidation; this increases the production of ATP (Jeulin, Soufir, Marson, Paquignon, & Dacheux, 1987). It therefore has a positive effect on sperm motility, maturation and indeed the entire spermatogenic process (Ramsay, Gandour, & Van Der Leij, 2001). The antioxidant properties of LC also help protect against ROS. In vitro studies have shown that carnitines also enhance human sperm motility and may have a cryoprotectant effect (Agarwal & Said, 2004). The effects of supplementing extenders with LC have been examined in bull (Hufana-Duran, Duran, Monson, & Parrish, 2017), buffalo (El-Raey, Badr, Assi, & Rawash, 2016), goat (Bucak, Tuncer, et al., 2010) and rabbit sperm (Sariözkan, Özdamar, Türk, Cantürk, & Yay, 2014), with certain beneficial effects being noted. However, a recent study has reported that, for frozen–thawed ram semen, adding LC to a synthetic-based extender has no consistent benefit (de Souza et al., 2019). The effect of LC on the sperm variables and fertility of chilled ram semen, however, have remained unknown.

The literature indicates that extenders based on skimmed milk plus egg yolk help maintain ram sperm quality during chilling for up to 96 hr (Galarza et al., 2019; Gil et al., 2011). Given the antioxidant properties and beneficial effects of LC on mitochondrial function, it is here hypothesized that supplementing a skimmed milk–egg yolk-based extender with LC might help maintain sperm survival and functionality for 48–96 hr, and prolong the fertilizing capacity of chilled ram sperm for up to 24 hr.

2 | MATERIALS AND METHODS

LC (Sigma 8400920025) was obtained from the Sigma Chemical Co. The extender and media were prepared at the INIA Department of Animal Reproduction Research Laboratory using reagent-grade chemicals purchased from Panreac Chemistry S.A. and the Sigma Chemical Co.

2.1 | Extender and L-carnitine supplementation

The basic extender employed used at initial sperm collection—ultra-high temperature-treated commercial skimmed milk plus antibiotics (100,000 IU penicillin sodium and 100 mg dihydrostreptomycin/100 ml) and 6% (v/v) egg yolk—was prepared according to Galarza et al. (2019) (osmolarity 298–310 mOsm/kg, pH adjusted to 7.2). This was centrifuged for 30 min at 4,000 × *g* to remove any large particles, and the supernatant filtered through a sterile Minisart® NML Syringe Filter (16555, Sartorius; pore size 0.45 μm).

For the later dilution of sperm samples, this same extender was supplemented with different LC concentrations: 0 (control), 1, 2.5, 5, 7.5, or 10 and stored at −20°C until use.

2.2 | Animals and semen collection and preparation

For laboratory experiments, sperm was provided by 4- to 6-year-old Merino rams (*n* = 12) housed at the INIA Department of Animal Reproduction (Madrid, Spain; 40° 25′N). All were fed a diet of grain, barley straw and dry alfalfa supplements. Water, vitamins and mineral blocks were freely available. All animals were handled according to procedures approved by the INIA Ethics Committee, and all work was performed in accordance with the Spanish Policy for Animal Protection (RD53/2013), which conforms to European Union Directive 86/609 regarding the protection of animals used in scientific experiments.

Thirty-six ejaculates, three per ram (*n* = 12), were collected weekly for three consecutive weeks from June to August (non-breeding season) using an artificial vagina (pre-warmed at

37°C). Ejaculate volumes were measured (in ml) using a graduated conical glass tube. At collection, each ejaculate was initially diluted (1:1 v/v) with the unsupplemented extender described above at 37°C and transported to the laboratory for initial assessment. Those ejaculates with a volume of 0.75–2 ml, a sperm motility value of >70%, a score of >3 on a mass motility scale of 0–5 and a sperm concentration of >3.5 × 10^9 sperm/ml were selected for subsequent experimental work. Twelve pools of semen were then formed, each from three different, randomly selected ejaculates. Each pool was divided into six aliquots in 15 ml Falcon tubes and then diluted with the extender supplemented with different LC concentrations to reach a final sperm concentration of each sample 200 × 10^6 sperm/ml. These aliquots were then chilled at 5°C in a refrigerator for either 0, 48 or 96 hr.

2.3 | Analysis of sperm quality

2.3.1 | Sperm kinetics

Sperm motility was assessed using a CASA system running SCA v.4.0 software (Sperm Class Analyzer, SCA® 1999, Microptic S.L.), coupled to a model 50i Nikon Eclipse phase-contrast microscope (negative contrast). Sperm samples (5 μl) were placed on slides warmed at 37°C and covered with a coverslip. A minimum of three fields and 200 sperm tracks were examined at 100× (image acquisition rate 25 frames/s). The percentage of total sperm motility (SM), the percentage of progressive sperm motility (PSM), the curvilinear velocity (VCL, μm/s), straight line velocity (VSL, μm/s), beat–cross frequency (BCF, Hz) and amplitude of lateral head displacement (ALH, μm) were assessed as described by Galarza, López-Sebastián, Woelders, Blesbois, and Santiago-Moreno (2018).

2.3.2 | Status of sperm membranes

Sperm membrane (plasma, acrosome and mitochondrial) status was assessed using propidium iodide (PI, Sigma P4170), fluorescein isothiocyanate-conjugated peanut (Arachis hypogaea) agglutinin (PNA-FITC, Sigma L7381) and Mitotracker Green FM® (MITO, Invitrogen M7514) fluorescent probes according to Galarza et al. (2019). A total of 200 sperm cells per slide were examined, and eight cell subpopulations quantified (as percentages): those showing (a) an intact plasma membrane/intact acrosome/intact mitochondrial membrane (IPIAIM); (b) an intact plasma membrane/intact acrosome/damaged mitochondrial membrane (IPIADM); (c) an intact plasma membrane/damaged acrosome/intact mitochondrial membrane (IPDAIM); (d) an intact plasma membrane/damaged acrosome/damaged mitochondrial membrane (IPDADM); (e) a damaged plasma membrane/intact acrosome/intact mitochondrial membrane (DPIAIM); (f) a damaged plasma membrane/intact acrosome/damaged mitochondrial membrane (DPIADM); (g) a damaged plasma membrane/damaged acrosome/intact mitochondrial membrane (DPDAIM); and (h) a damaged plasma membrane/damaged acrosome/damaged mitochondrial membrane (DPDADM).

2.4 | In vivo fertilizing capacity

Fertility experiments were performed using Assaf breed sheep, employing sperm from five donor rams housed at the Ovigen (Centre for Selection and Genetic Improvement of Sheep and Goats, Toro, Zamora, Spain); this centre performs most of the sheep artificial insemination procedures undertaken in the north of central Spain. The males, which also belonged to the Assaf breed, were selected based on their fertility recorded data. All semen was collected using artificial vaginas and diluted with LC5 (the concentration that provided the best results in the previous experiment) extender at 28°C to a concentration of 1,600 × 10^6 sperm/ml. These samples were then cooled to 5°C for 24 hr or 15°C for 2 hr (to produce LC5-5°C-24h or LC5-15°C-2h semen samples, respectively) at a cooling rate of 0.3°C/min, and loaded into 0.25 ml French straws (IMV).

These straws were used in cervical FTAI performed in three ewe groups (A, B and C) from one private farm. All ewes of the farm were under same conditions of feeding, handling and reproductive management. The ewes were randomly divided into these three groups. Multiparous Assaf breed ewes in synchronized oestrus (achieved via the insertion of an intravaginal fluorogestone acetate [20 mg] sponge [Chronogest® CR, Intervet] for 14 days, followed by the intramuscular administration of 480 IU equine chorionic gonadotropin [Folligon®, Intervet] at sponge removal) were inseminated with LC5-15°C-2h (group A n = 27; group B n = 25; and group C n = 25) or LC5-5°C-24h (group A n = 21; group B, n = 24; and group C, n = 25) sperm. In each flock, all inseminations were performed at the same time, by the same technician, 53–54 hr after sponge removal using a universal insemination syringe (Ref: 007071, IMV Technology). Artificial inseminations were done in May (i.e. non-breeding season): May 5th, May 20th and May 23rd in groups A, B and C, respectively. Pregnancy was checked by ultrasonography using a transabdominal lineal probe (5 Mz) at 35 days post-AI.

2.5 | Statistical analysis

Results are presented as mean ± *SEM*. Values for sperm variables that showed non-normal distributions, as determined by the Shapiro–Wilk test, were arcsine- or *log*10-transformed (percentages and numeric values, respectively) before analysis. The effects of the six LC concentrations, chilling time and its interaction on in vitro sperm quality were compared using repeated-measures ANOVA and Tukey's multiple range test, employing the General Linear Model procedure. The pregnancy rate achieved with the LC5-15°C-2h and LC5-5°C-24h sperm types along with AI-time was compared using the chi-squared test. Significance was set at $p < .05$. All statistical analyses were performed using Statistica software for Windows v.12 (StatSoft Inc.).

3 | RESULTS

3.1 | Sperm kinetics

Figure 1 shows the differences in the laboratory-tested sperm motility variables between the different LC preparations over the examined cold storage times. The interaction between LC concentration and chilling time was only registered for the ALH variable ($p < .001$).

The PSM, velocities and ALH variables were affected ($p < .001$) by chilling time. The percentage of PSM was higher ($p < .05$) for the LC7.5 group at 96 hr (only) than the control group. VCL was improved in all LC groups compared to the control group at 0 hr ($p < .01$) and 48 hr ($p < .001$); at 96 hr, VCL values were greater ($p < .01$) for the LC2.5, LC5, LC7.5 and LC10 groups than the control group. LC5 and LC7.5 groups returned higher VSL values ($p < .01$) at 48 and 96 hr compared to the control group. At 48 and 96 hr, the LC5 group also returned lower ALH values than for the control groups ($p < .05$; Figure 1).

3.2 | Status of sperm membranes

The percentage of IPIAIM sperms was greater ($p < .001$) in all LC groups than in the control group at 48 and 96 hr of storage. Lower percentages of IPDAIM sperms were recorded after cold storage at 96 hr in the LC1, LC7.5 and LC10 groups than in the control group ($p < .05$). Lower ($p < .05$) percentages of DPIAIM sperms were observed after 96 hr in the LC7.5 and LC10 groups than in the control group (Table 1). Overall, these results show that the percentage of sperm with an intact plasma membrane, an intact acrosome and an intact mitochondrial membrane decreases as cold storage time increases.

3.3 | In vivo fertilization: pregnancy rates

In group A, no significant differences were seen in pregnancy rate between ewes inseminated with the LC5-15°C-2h and LC5-5°C-24h sperm (52.4% versus 42.8%). However, in group B and group C, the pregnancy rate was lower among the LC5-5°C-24h-inseminated ewes than the LC5-15°C-2h-inseminated ewes (12.5% versus 56.0%, $p < .001$, and 20.0 versus 52.0%, $p < .01$, respectively; Figure 2).

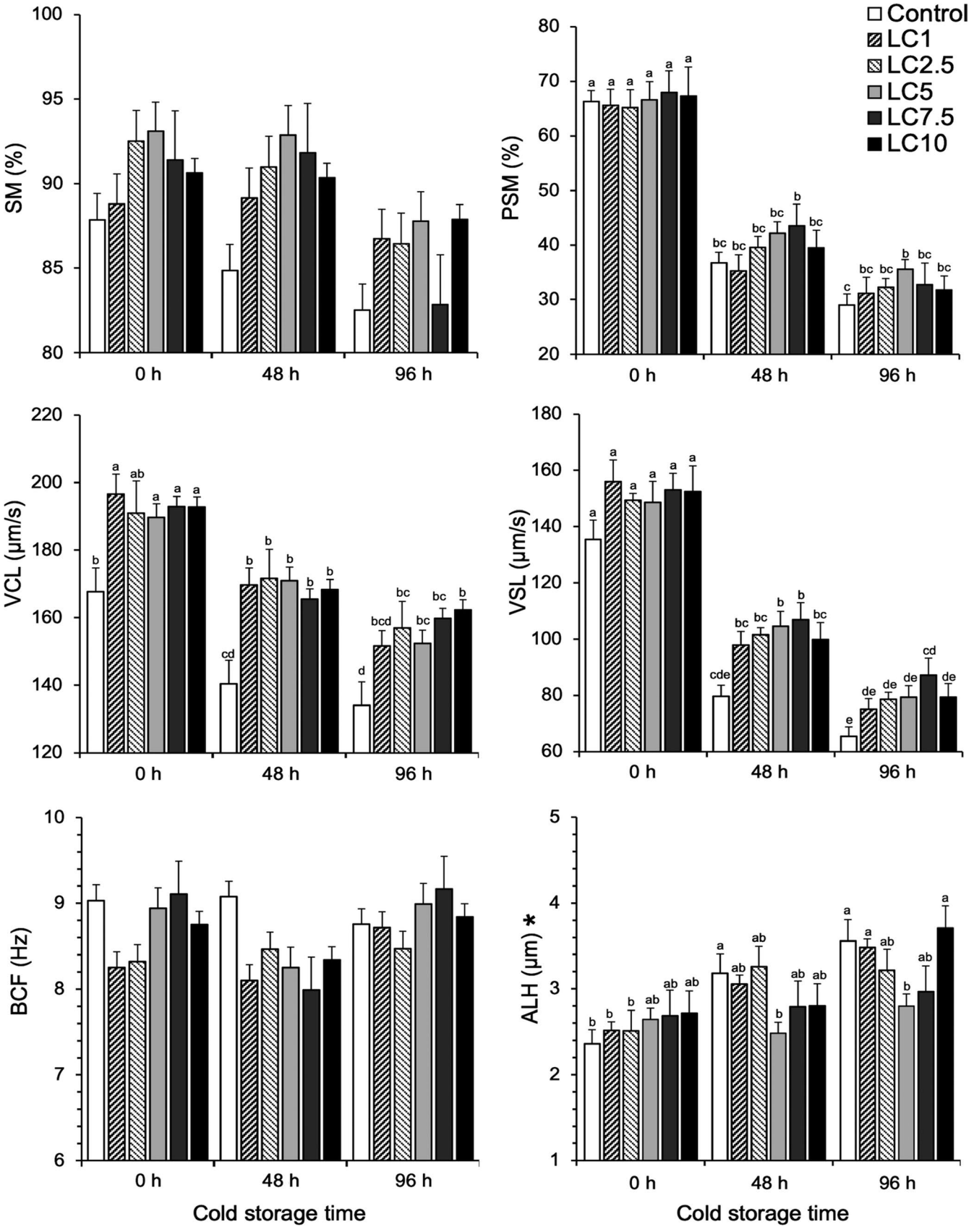
4 | DISCUSSION

The present study is the first to report the effect of supplementing a skimmed milk–egg yolk extender with LC on chilled ram sperm quality and fertilizing capacity. The results indicate that such supplementation can help maintain sperm kinetics during cold storage. In addition, no difference was seen in terms of the pregnancy rate associated with the use of the LC5-5°C-24h or LC5-15°C-2h sperm in group (A), although in groups B and C the fertility rate was reduced when the LC5-5°C-24h was used. It should be noted that, in order to simplify the technique, the semen previously diluted 1:1 (v/v) without LC was again diluted in the laboratory using extenders with fixed concentrations of LC (1, 2.5, 5, 7.5 and 10 mM) to reach a final sperm concentration of each sample 200×10^6 sperm/ml. Obviously, the final concentrations of LC, to which sperm were exposed, were therefore lower.

Previous work has suggested that supplementing ram semen with antioxidants such as CAT, GSH, taurine, trehalose, quercetin, palmitoleate, butylated hydroxytoluene and kinetin helps maintain the motility variables and integrity of sperm membranes (plasma and acrosome) during storage at 4–5°C (Banday, Lone, Rasool, Rashid, & Shikari, 2017; Eslami, Ghasemiyan, & Zadeh Hashem, 2017; Rather, Islam, Malik, & Lone, 2016). The present results indicate that LC at a final concentration of 1–10 mM helps preserve motility and sperm velocity variables, and can help protect the integrity of ram sperm membranes during storage at 5°C for up to 96 hr. Desirable PSM and VCL and VSL values were achieved with 5 and 7.5 mM LC compared to controls after 48 hr of chilling storage. The ALH values recorded were lower with the LC5 treatment compared to the other LC groups and controls at 48 and 96 hr. Appropriate ALH values are needed if the cervical mucus and the oocyte barriers to fertilization (i.e. the cumulus cell layers and the zona pellucida) are to be overcome (Aitken, Sutton, Warner, & Richardson, 1985). Previous reports have suggested that lower ALH values are compatible with effective progressive movement by cold-stored ram sperm (Galarza et al., 2018, 2019); the ALH is known to increase greatly in hyperactive sperm undergoing capacitation (Santiago-Moreno et al., 2017).

Supplementing synthetic-based extenders with LC has been reported to help maintain frozen–thawed sperm motility and membrane integrity (plasma and acrosome) in human (Banihani, Agarwal, Sharma, & Bayachou, 2014) and ruminant (bull, buffalo and goat) (Bucak, Sariözkan, et al., 2010; Hufana-Duran et al., 2017) sperm. A similar effect of LC on motility has also been demonstrated in mouse sperm when using Ham's F10 medium as an extender compared to pentoxifylline (Aliabadi et al., 2012). Gibb, Lambourne, Quadrelli, Smith, and Aitken (2015) report better total and progressive motilities in stallion sperm maintained in skimmed milk extender supplemented with pyruvate and LC after 72 hr of storage at 20°C. However, a recent publication reports the supplementation of Tris-based extenders (Tris or optiXcell™) with LC to have no consistently beneficial effect on frozen–thawed ram sperm (de Souza et al., 2019).

FIGURE 1 CASA system-obtained values for kinetic variables for ram semen diluted with a skimmed milk–egg yolk-based extender supplemented to a final concentration of either 0 (control), 1 (LC1), 2.5 (LC2.5), 5 (LC5), 7.5 (LC7.5), and 10 mM (LC10) L-carnitine, and cold-stored at 5°C for up to 96 hr. SM (%) sperm motility; PSM (%) progressive sperm motility; VCL (μm/s) curvilinear velocity; VSL (μm/s) straight line velocity; BCF (Hz) beat–cross frequency; and ALH (μm) amplitude of lateral head displacement. * interaction between LC concentration and chilling time ($p < .001$). Different letters (a-e) at each evaluation time indicate significant differences between values for LC concentrations and cold storage times ($p < .01$ for a–b, b–c, c–d, c–e and d–e; $p < .001$ for a–c, a–d, a–e, b–d and b–e)



During the storage of chilled semen, LPO accumulates in a time-dependent manner (Eslami et al., 2017). High ROS production increases sperm sensitivity, resulting in damage to the structure, morphology and function of the cells, manifesting as reduced sperm motility, viability and fertilizing capacity (Bucak, Tuncer, et al., 2010). Free oxygen radical species can impair sperm motility

by interrupting ATP production or flagellar axoneme phosphorylation (Aitken & Baker, 2006). LC supplementation might therefore enhance sperm locomotion via its facilitation of the transport of activated fatty acid into the mitochondrial matrix for β-oxidation (Agarwal & Said, 2004). Further, as an antioxidant, LC may protect the sperm plasma membrane, which has a high unsaturated fatty acid content (Aitken & Clarkson, 1987; Aliabadi et al., 2012). This protection occurs as a result of a repair mechanism by which elevated intracellular toxic acetyl-coenzyme A is removed and/or fatty acids in membrane phospholipids are replaced (Kalaiselvi & Panneerselvam, 1998; Vicari & Calogero, 2001). It may be that the present LC-supplemented extender promotes mitochondrial ATP production while minimizing both ATP depletion and the damaging effects of metabolic by-products such as free radicals. Overall, the in vitro results of the present study suggest that supplementing the extender used with LC provided protection against cold shock by better preserving membrane integrity and functionality.

The pregnancy rate achieved by cervical FTAI using the LC5-15°C-2h ram sperm was 52%–56%. No difference in pregnancy rates was found in one of the ewe groups inseminated with LC5-5°C-24h and LC5-15°C-2h sperm. However, in the other groups—and despite our earlier reporting that the in vitro fertilizing capacity of ram semen diluted in skimmed milk extender cold-stored for up to 48 hr did not diminish (fertilizing rates 43.3%–48.8%) Galarza et al. (2019)—reduced pregnancy rates were seen. The desirable pregnancy rates obtained in flock A with LC5-5°C-24h

TABLE 1 Percentage of sperm (mean ± *SEM*) in the various categories of membrane integrity (assessed using fluorescent markers PI/PNA-FITC/MITO) for ram semen diluted with the present extender supplemented with different concentrations of LC under chilled conditions

Membrane integrity category	Time (hr)		L-Carnitine groups				
		Control	LC1	LC2.5	LC5	LC7.5	LC10
IPIAIM (%)	0	74.8 ± 2.7^{ab}	80.5 ± 1.4^{ab}	82.1 ± 2.0^{ab}	83.2 ± 1.4^{a}	82.8 ± 1.6^{a}	84.0 ± 1.4^{a}
	48	58.8 ± 3.2^{de}	69.3 ± 2.0^{c}	70.5 ± 2.0^{c}	73.8 ± 1.3^{bc}	74.0 ± 1.4^{bc}	73.3 ± 1.7^{bc}
	96	49.2 ± 3.0^{e}	62.3 ± 2.0^{cd}	63.3 ± 1.7^{cd}	63.3 ± 2.8^{cd}	66.5 ± 2.4^{cd}	66.3 ± 1.9^{cd}
IPIADM (%)	0	0	0.08 ± 0.08	0	0	0	0
	48	0	0.08 ± 0.08	0.17 ± 0.17	0	0.08 ± 0.08	0
	96	0.4 ± 0.26	0.08 ± 0.08	0.55 ± 0.37	0	0.17 ± 0.17	0.08 ± 0.08
IPDAIM (%)	0	0.7 ± 0.2^{b}	0.5 ± 0.3^{b}	0^{b}	0.2 ± 0.1^{b}	0.3 ± 0.2^{b}	0.8 ± 0.4^{b}
	48	2,7 ± 0.7^{ab}	1.4 ± 0.4^{b}	1.9 ± 0.6^{b}	1.3 ± 0.5^{b}	1.3 ± 0.5^{b}	1.1 ± 0.4^{b}
	96	4.3 ± 1.0^{a}	1.7 ± 0.4^{b}	2.5 ± 0.7^{ab}	2.1 ± 0.5^{ab}	1.6 ± 0.5^{b}	0.9 ± 0.4^{b}
IPDADM (%)	0	0	0	0	0	0	0
	48	0.08 ± 0.08	0	0	0.08 ± 0.08	0	0
	96	0	0	0	0	0	0
DPIAIM (%)	0	10.8 ± 1.8^{c}	9.5 ± 0.7^{c}	10.0 ± 1.2^{c}	9.8 ± 1.1^{c}	10.2 ± 0.9^{c}	8.8 ± 0.7^{c}
	48	18.3 ± 2.2^{ab}	13.8 ± 1.2^{b}	12.8 ± 1.1^{b}	12.2 ± 1.0^{b}	11.9 ± 0.8^{bc}	12.5 ± 1.2^{bc}
	96	20.0 ± 1.7^{a}	16.7 ± 1.2^{ab}	15.0 ± 1.0^{abc}	16.9 ± 1.3^{ab}	13.7 ± 1.2^{bc}	12.6 ± 1.4^{bc}
DPIADM (%)	0	4.9 ± 1.3^{ab}	4.4 ± 1.1^{b}	2.9 ± 0.8^{b}	2.7 ± 0.6^{b}	2.5 ± 0.9^{b}	2.3 ± 0.8^{b}
	48	6.9 ± 0.9^{ab}	5.0 ± 1.0^{ab}	5.3 ± 1.1^{ab}	4.5 ± 0.9^{ab}	3.9 ± 0.6^{b}	4.3 ± 0.6^{b}
	96	9.3 ± 1.0^{a}	4.8 ± 1.1^{ab}	6.6 ± 1.7^{ab}	6.5 ± 1.2^{ab}	6.5 ± 1.0^{ab}	6.4 ± 0.9^{ab}
DPDAIM (%)	0	8.2 ± 1.4^{bc}	4.8 ± 0.7^{c}	4.7 ± 1.0^{c}	4.3 ± 0.8^{c}	3.8 ± 0.6^{c}	4.2 ± 0.8^{c}
	48	12.0 ± 0.9^{ab}	10.0 ± 1.1^{ab}	8.8 ± 1.1^{bc}	7.2 ± 1.2^{bc}	9.5 ± 0.9^{b}	8.3 ± 0.8^{bc}
	96	15.7 ± 1.9^{a}	14.2 ± 1.5^{a}	11.9 ± 1.6^{ab}	11.0 ± 1.8^{ab}	11.3 ± 1.8^{ab}	12.6 ± 1.3^{ab}
DPDADM (%)	0	0.6 ± 0.4	0.3 ± 0.2	0.3 ± 0.3	0	0.5 ± 0.3	0
	48	1.3 ± 0.6	0.4 ± 0.3	0.7 ± 0.4	0.9 ± 0.6	0.1 ± 0.1	0.5 ± 0.3
	96	1.1 ± 0.6	0.3 ± 0.2	0.2 ± 0.1	0.3 ± 0.2	0.3 ± 0.2	1.1 ± 0.5

Note: Different superscripts inside each membrane integrity category that include L-carnitine groups (rows) and chilling times (columns) reflect significant differences between experimental groups ($p < .05$ for a–b, b–c, c–d and d–e; $p < .01$ for a–c, b–d; and $p < .001$ for a–d, a–e and b–e).

Abbreviations: DPDADM, damaged plasma membrane/damaged acrosome/damaged mitochondrial membrane; DPDAIM, damaged plasma membrane/damaged acrosome/intact mitochondrial membrane; DPIADM, damaged plasma membrane/intact acrosome/damaged mitochondrial membrane; DPIAIM, damaged plasma membrane/intact acrosome/intact mitochondrial membrane; IPDADM, intact plasma membrane/damaged acrosome/damage mitochondrial membrane; IPDAIM, intact plasma membrane/damaged acrosome/intact mitochondrial membrane; IPIAIM, intact plasma membrane/intact acrosome/intact mitochondrial membrane; IPIALM, intact plasma membrane/intact acrosome/damage mitochondrial membrane.

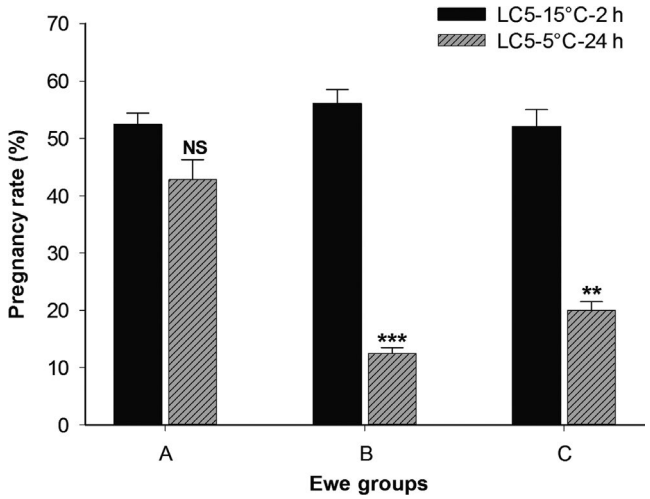


FIGURE 2 Pregnancy rates achieved in ewes (three groups) cervically inseminated with ram semen diluted with LC5-supplemented extender stored at 15°C for 2 hr (LC5-15°C-2h) or at 5°C for 24 hr (LC5-5°C-24h). $^{**}p < .01$; $^{***}p < .001$. NS, no significant difference

semen might be due to more efficient semen handling before FTAI (optimally pre-warmed straws [37°C]). Several authors indicate that pregnancy rates can be affected by the quality of semen handling, the farm system, environmental elements, animal health and physiological status of the ewes (Anel et al., 2005; Hill, Thompson, & Perkins, 1998).

The FTAI protocol most commonly used in many European countries requires semen (400×10^6 sperm/ewe) diluted with egg yolk-free INRA-96® extender maintained at 15°C until the moment of insemination (approximately 1–6 hr after sperm collection). The average pregnancy rate in the last ten years for Assaf breed in Spain when using the standard protocol was 42% (Pérez, Gutiérrez, Lavin, & Mantecón, 2019). O'Hara et al. (2010) report the pregnancy rate in ewes following cervical FTAI with fresh ram semen diluted with INRA-96® extender to reach 46.3%–72.7% in best-case scenarios (under very controlled conditions). Prior to the present experiment, the average pregnancy rate achieved where the AI was performed (average for all flocks) was 38%–40%. In the present work, the pregnancy rate after FTIA with the LC-15°C-2h sperm was always >50% and better than the mean fertility rate obtained for Assaf breed in Spain; it also superseded that reported by Casali et al. (2017), a pregnancy rate of 36% after cervical FTAI. In addition, it has been suggested that high CASA-obtained progressive motility and velocities values are the most consistent indicators of fertility (Yániz, Palacín, Vicente-Fiel, Sánchez-Nadal, & Santolaria, 2015). The present CASA-returned data indicate that LC-15°C-2h yields good results.

5 | CONCLUSION

The supplementation of a skimmed milk–egg yolk extender with LC helped preserve the quality of chilled ram semen at 5°C for 48–96 hr, at least, in part, by protecting the acrosome and plasma and mitochondrial membranes, and/or maintaining the fertilizing capacity for 24 hr. Ram semen chilled at 15°C and supplemented with LC shows satisfactory fertilizing capacity. LC may be useful supplement to extenders when storing ram sperm at 5 or 15°C for use in cervical FTAI.

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CONFLICTS OF INTEREST

None to declare.

AUTHORS' CONTRIBUTIONS

DAG, ALS and JSM designed the study. DAG collected and assessed the studied samples, performed the statistical analysis and wrote the draft of the article. ALS and JSM conceived the initial idea of the study, worked on the draft and approved the final version.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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