BIOPRESERVATION AND BIOBANKING
Volume 18, Number 5, 2020
© Mary Ann Liebert, Inc.
DOI: 10.1089/bio.2020.0025

Effects of L-Carnitine on Equine Semen Quality During Liquid Storage

Igor H.A.V. Nery,[1] Robespierre A.J. Araújo Silva,[1] Helder M. Souza,[1] Lúcia C.P. Arruda,[1] Millena M. Monteiro,[1] Desirée C.M. Seal,[1] Girliane R. Silva,[2] Tânia M.S. Silva,[2] Gustavo F. Carneiro,[1] André M. Batista,[1] Diogo R. Câmara,[3] and Maria Madalena Pessoa Guerra[1]

L-Carnitine (LC) plays a key role in sperm metabolism, easily providing energy through β-oxidation, which positively affects motility. The objective of this study was to investigate the association between blood plasma and seminal plasma LC levels, as well as the effect of LC as an additive in a skimmed milk-based extender during sperm storage at 5°C. In the first experiment, semen and blood samples from 14 Quarter Horse stallions were used. The LC content in blood plasma and seminal plasma was determined by spectrophotometry and their relationships with seminal parameters were evaluated. In the second experiment, ejaculates ($n = 16$) from four Quarter Horses were used. Each ejaculate was split into four treatment groups with different LC concentrations: 0 (control), 0.5, 1.0, and 2.0 mM. Sperm motility, integrity of plasma and acrosomal membranes, intracellular reactive oxygen species content, and plasma membrane stability were evaluated immediately after samples reached 5°C (0 hour) and after 24, 48, and 72 hours. There was a positive correlation ($p < 0.05$) between LC levels in seminal plasma with both sperm concentration and plasma and acrosomal membrane integrity. Furthermore, the addition of LC (1 and 2 mM) preserved the motility of equine sperm stored at 5°C. It was concluded that the concentrations of LC with seminal plasma present correlate to semen parameters and the addition of LC to skimmed milk-based extender preserves the motility of equine sperm stored at 5°C for up to 48 hours.

Keywords: antioxidant, cooled semen, flow cytometry, seminal plasma, quarter horse

Downloaded by Eth Bibliothek from www.liebertpub.com at 09/30/20. For personal use only.

Introduction

Artificial insemination with cooled semen is a reproductive biotechnology often used in the horse industry.[1] However, not all stallions provide ejaculates that maintain acceptable quality after cooling, influencing pregnancy rates.[2,3] However, the exact causes of decreased sperm motility after cryopreservation remain unclear.[4,5] This variation might be related to oxidative stress, when an imbalance between the concentrations of reactive oxygen species (ROS) and antioxidants is observed,[6] or differences in seminal plasma composition between sires or even among ejaculates within a sire, such as the seminal levels of LC.[4,7]

LC plays a key role in sperm metabolism, providing energy readily available through β-oxidation, which positively affects sperm motility.[8] This beneficial effect is mediated by the transport of long-chain fatty acids into the mitochondria, which may reduce lipid availability for peroxidation, acting also as an antioxidant[9] and osmolyte.[10] It has been shown that dietary supplementation with LC improves the sperm progressive motility (PM) in oligoasthenospermic stallions over time.[11] It is worth noting that LC addition to semen extender medium improved the sperm parameters of men,[12,13] rabbits,[14,15] cats,[16] and rats.[17] However, studies investigating the effect of supplementation *in vitro* with LC during equine sperm cooling are limited.[18,19]

These features suggest that LC plays an important role in sperm fertility. Therefore, the objectives of this study were to investigate the relatioship of seminal and serum levels of LC on sperm function, as well as to evaluate the effects of LC added to a skimmed milk-based extender on horse sperm during storage at 5°C.

Materials and Methods

All procedures were approved by the Commission of Ethics in Animal Experimentation of the Universidade

[1]Andrology Laboratory (ANDROLAB), Department Veterinary Medicine, and [2]Bioprospecting Laboratory of Phytochemistry (BIOFITO), Department of Molecular Science, Universidade Federal Rural de Pernambuco, Recife, Brazil.
[3]Department of Veterinary Medicine, Animal Reproduction Laboratory, Federal University of Alagoas, Viçosa, Brazil.

Federal Rural de Pernambuco (UFRPE), license No. 057/2015 CEUA/UFRPE.

Reagents and chemicals

With the exception of those specified, all chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO). An extender containing 2.5 g/L of glucose, 0.5 g/L of skimmed milk powder, 0.33 g/L of sucrose, 0.025 g/L of sodium citrate, 50 IU/mL of penicillin, 50 μg/mL of streptomycin, 0.25 mg/mL of gentamicin, and 2 mM/L of HEPES, with an osmolarity of 310 mOsm/kg and a pH of 7.1, was used as a control in this study.

Experiment 1

The association between seminal and blood plasma levels of LC and sperm traits were investigated. This study was conducted with ejaculates from 14 Quarter Horse stallions (4–8 years old), with proven fertility from three different farms located in the municipality of Camaragibe, Brazil (7° 57′ 04.7″ S and 35° 00′ 41.7″ W), during the reproductive season. All animals were kept in individual pens, with free access to water and hay, being supplemented with 5 kg/day of concentrate.

Blood samples were collected by jugular venipuncture using vacutainer heparinized tubes before the collection of semen. The samples were centrifuged at 1500 *g* for 10 minutes and the plasma was harvested and stored at −20°C until analysis.

One ejaculate from each stallion ($n = 14$) was obtained using an artificial vagina (Botucatu model) and filtered to remove the gel fraction. Immediately after the collection, the sperm motility was subjectively evaluated using a phase contrast microscope (Olympus, Tokyo, Japan; 100×), followed by determination of sperm concentration (Neubauer chamber). Subsequently, the total volume of each ejaculate was split into two parts. The first part was processed according with the method described by Stradaioli.[20] The semen was centrifuged at 600 *g* for 15 minutes and, after filtration through a 0.45 μm disposable syringe filter (Cellulose Ester Filter, HAWG04700; Merck-Millipore), 1 mL aliquots of seminal plasma were stored at −20°C until analysis. The second part was diluted to achieve a concentration of 50×10^6 sperm/mL using a skimmed milk-based extender and chilled to 5°C, using an isothermal container, and transported to the laboratory for analysis of sperm kinematics, plasma, and acrosomal membrane integrity (iPAM), as depicted for Experiment 2.

Experiment 2

Four ejaculates were collected from four Quarter Horse stallions once a week. Semen collection, evaluation, and initial processing were performed as described for *Experiment 1*. After semen dilution in skimmed milk-based extender (final concentration of 50×10^6 sperm/mL), the samples were transported (1 hour) to the laboratory in containers at room temperature. In the laboratory, the semen was split into four treatment groups comprised 0, 0.5, 1.0, and 2.0 mM of LC. The different preparations were stored in microtubes (0.5 mL) that were sealed and packed in a 5°C cooler. After 0, 24, 48, and 72 hours of storage at 5°C, sperm quality was evaluated as described below.

Sperm kinematic evaluation

The evaluation of sperm motility was performed using computer-assisted semen analysis (SCA TM; Microptics, S.L., Version 5.1, Barcelona, Spain) using the following settings: magnification 100×; number of images 25; images per second 24; head area 4 to 75 μm^2; average path velocity (VAP): slow 10 μm/s < medium 45 μm/s < fast 90 μm/s; progressivity 75% straightness; and circular 50% linearity (LIN).

An aliquot (5.0 μL) of the sample was placed on a preheated (37°C) slide, covered with a cover slip and evaluated in phase contrast microscopy (Eclipse 50i, Nikon, Japan). For each sample, at least 500 sperm were recorded in five random consecutive fields by the same operator.

The parameters evaluated were total motility (TM, %), PM (%), rapid sperm (RAP%), LIN (%), curvilinear velocity (VCL, μm/s), straight line velocity (VSL, μm/s), and VAP (μm/s).

Flow cytometry sperm analysis

The microtubes containing chilled samples were immersed in a water bath (37°C) for 5 minutes. An aliquot (300 μL) of each sample was harvested, placed into microtubes, and 1 mL of phosphate-buffered saline (PBS) was added prior centrifugation (600 *g* for 10 minutes). The supernatant was removed, the pellet was resuspended in 90 μL of PBS and split into three aliquots of 30 μL each, which were used for analysis of the sperm membranes integrity, intracellular ROS production, and plasma membrane stability. All flow cytometric analyses were conducted according to Souza et al.[21]

For analysis of the iPAM, each sperm aliquot was incubated (37°C, 10 minutes) in 1 μL of FITC-conjugated to Peanut agglutinin (FITC-PNA; 200 μg/mL) and 2 μL of propidium iodide (IP; 0.5 mg/mL) in PBS. After staining, the sperm were fixed with 5.0 μL of 4% paraformaldehyde, incubated for 10 minutes at room temperature and evaluated. The sperm was classified as presenting intact plasma and acrosomal membranes (IP-/PNA-), intact plasma membrane and damaged acrosomal membrane (IP-/PNA+), damaged plasma membrane and intact acrosomal membrane (IP+/PNA-), and damaged plasma and acrosomal membrane (IP+/PNA+).

The production of intracellular ROS was measured by incubating the sperm with 2 μL of CM-H_2DCFDA (0.5 mM in DMSO; Molecular Probes, Life Technologies, Eugene, OR) for 30 minutes at 37°C. Then, the samples were centrifuged (600 *g* for 10 minutes) to remove the unbound CM-H_2DCFDA, the supernatant was discarded and the sperm aliquots were resuspended in 30 μL of PBS, followed by the addition of 1 μ L of IP (0.5 mg/mL in PBS). Once stained, the samples were fixed in 4% paraformaldehyde, incubated for 5 minutes at room temperature, and evaluated. Populations were separated in accordance with the fluorescence intensity and classified as sperm with low levels of ROS and intact plasma membrane (DCFDA-/IP-) and cells with high levels of ROS and intact plasma membrane (DCFDA+/IP-).

For sperm membrane stability analysis, aliquots (30 μL) of semen were incubated (37°C for 15 minutes) with 1 μL of YO-PRO®-1 iodide (YP, 1 mM in DMSO; Molecular Probes, Life Technologies). After incubation, 1 μL of merocyanine

Downloaded by Eth Bibliothek from www.liebertpub.com at 09/30/20. For personal use only.

540 (M540; 54 mM in DMSO) was added and the samples were fixed in 4% paraformaldehyde, incubated for 5 minutes at room temperature and evaluated. Samples were classified as viable cells with stable membrane (YP-/M540-), viable cells with destabilized membrane (YP-/M540+), nonviable cells with stable membrane (YP+/M540-), and nonviable cells with destabilized membrane (YP+/M540+).

All analyses were performed using an AmnisImage-Stream® x Mark II flow cytometer (EMD Millipore Corp.), equipped with a microscope with a 60×lens with an image rate of 5000 cells/s. The size of the cells and the flow velocity were 7.0 μm and 44 mm/s, respectively. The acquisition of the raw images was obtained through INSPIRE® software. The analysis of the raw images was performed using IDEAS® software (version 6.0).

A 488 nm excitation laser with intensity set to 55.0 mV (PNA/IP), 80 mV (DCFDA/IP), and 100 mV (YP/M540) was used. Approximately 5000 events were collected per sample. FITC-PNA, CM-H_2DCFDA, and YP were collected on channel 2 (505–560 nm), and IP was collected on channel 5 (640–745 nm). The images of M540 were collected on channel 3 (560–595 nm).

Downloaded by Eth Bibliothek from www.liebertpub.com at 09/30/20. For personal use only.

LC in blood and seminal plasma

The concentrations of LC in blood and seminal plasma were determined using an LC Enzymatic UV test (Roche, Diagnostics GmBH, Mannheim, Germany). After a stage of deproteinization, an aliquot (300 μL) from each sample was evaluated according to the manufacturer's instructions. The assay was analyzed on a UV-spectrophotometer Visible (UVM AsysHiTech 340, Biochrom, USA) at a wavelength of 340 nm in the beginning, middle, and end of the reaction. All samples were prepared and analyzed in triplicate. The LC concentrations were expressed as nmol/mL.

Statistical analysis

Data analyses were performed using GraphPadInStat software version 3.10. The data were tested for a normal distribution using the Kolmogorov–Smirnov test. When necessary, the data were transformed (log or square root) to obtain a normal distribution. The variables expressed in percentages were transformed by arcsine (arcsine $\sqrt{P}/100$).

For the Experiment I, Pearson correlation analysis was performed to investigate the relationship between the LC concentration in both seminal and blood plasma and the parameters of quality sperm. For the Experiment II, possible interaction between LC concentration in the extender and storage time were evaluated using two-way analysis of variance (ANOVA), followed by Tukey's *post hoc* test. The influence of LC and storage time on the seminal quality were tested by an ANOVA for repeated measurements, followed by the Tukey-Kramer multiple comparison test. The differences were considered significant when $p < 0.05$.

Results

Levels of LC on blood and seminal plasma, sperm concentration, iPAM, and kinetic parameters of semen are shown in Table 1.

There was no correlation between seminal plasma and blood plasma LC concentration ($p = 0.74$). Correlations between seminal plasma or blood plasma LC concentration and sperm parameters are shown in Table 2. There were positive correlations between seminal plasma LC and sperm concentration ($r = 0.6$, $p = 0.02$), iPAM ($r = 0.69$, $p < 0.01$), and progressive motilty ($r = 0.69$, $p < 0.01$).

No interaction was detected between LC concentration in the extender and storage time for all sperm parameters evaluated ($p > 0.05$). The storage time had a negative effect on all motility traits evaluated (Table 3). However, supplementation with LC mitigated some of these effects. At time 0 hour, TM did not differ between groups ($p > 0.05$), but a significant reduction in the control group was observed after 24 hours of storage, whereas LC-treated groups significantly reduced TM only after 48 hours, regardless of LC concentration. Moreover, between 24 and 72 hours of storage, samples treated with 1.0 mM LC showed higher values of TM ($p < 0.05$) than control group. There was no difference ($p > 0.05$) on PM among treatments at 0 or 24 hours. However, after 48 hours, samples supplemented with 2.0 mM LC had higher PM ($p < 0.05$) than the control, but no difference ($p > 0.05$) was observed after 72 hours of storage (Table 3).

There was no influence of LC supplementation on extender or the storage times in any parameter evaluated by flow cytometry (iPAM, intracellular ROS production or membrane stability (Fig. 1A–C).

Table 1. Blood and Seminal Plasma L-Carnitine Concentration, Sperm Concentration, Plasma and Acrosomal Membrane Integrity, and Kinematic Parameters of Sperm from Individual Ejaculates of Quarter Horse Stallions ($N = 14$)

Parameter	*Average*	*Standard deviation*	*Range*
LC in seminal plasma (nmol/mL)	763.22	371.52	466.10–1686.80
LC in blood plasma (nmol/mL)	140.16	11.32	126.00–159.00
Sperm concentration ($\times 10^9$)	2.38	1.19	0.51–4.75
Plasma and acrosomal membrane integrity (%)	56.36	16.96	33.00–92.00
Total motility (%)	78.64	15.12	49.50–92.20
Progressive motility (%)	32.32	12.44	14.50–47.70
Rapid sperm (%)	42.23	12.32	21.05–57.29
VCL (μm/s)	108.28	18.20	80.70–137.14
VSL (μm/s)	57.58	15.60	27.50–83.30
VAP (μm/s)	89.31	17.6	56.90–110.94
LIN (%)	47.00	7.38	34.45–55.61

LV, L-carnitine; LIN, linearity; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity.

Downloaded by Eth Bibliothek from www.liebertpub.com at 09/30/20. For personal use only.

Table 2. Correlation Analysis Between Seminal Plasma or Blood Plasma l-Carnitine Concentration and Sperm Parameters from Individual Ejaculates of Quarter Horse Stallions (N = 14)

Sperm parameter	*Seminal plasma LC (mmol/mL)*	*Blood plasma LC (mmol/mL)*
Sperm concentration ($\times 10^9$/mL)	r = 0.60 $\mathbf{p = 0.02}$	r = –0.28 p = 0.33
Plasma and acrosomal membrane integrity (%)	r = 0.69 $\mathbf{p < 0.01}$	r = 0.20 p = 0.48
Total motility (%)	r = 0.16 p = 0.56	r = 0.06 p = 0.82
Progressive motility (%)	r = 0.69 $\mathbf{p < 0.01}$	r = –0.14 p = 0.61
Rapid sperm (%)	r = 0.31 p = 0.27	r = –0.21 p = 0.46
VCL (μm/s)	r = 0.40 p = 0.15	r = –0.07 p = 0.81
VSL (μm/s)	r = 0.20 p = 0.49	r = –0.32 p = 0.26
VAP (μm/s)	r = 0.35 p = 0.21	r = –0.21 p = 0.46
LIN (%)	r = –0.02 p = 0.94	r = –0.36 p = 0.20

Bold indicates values with significant difference.

Discussion

Although it was reported that nutraceuticals affect horse seminal parameters,[10] the present study is the first to report the effects of LC on equine semen quality during liquid storage and to evaluate the relationship of LC levels between blood and seminal plasma.

A positive correlation was detected among the levels of LC in seminal plasma, sperm concentration, and iPAM.

These results are similar to those previously reported by Stradaioli,[20] who found that there was a positive correlation between levels of LC in seminal plasma with sperm concentration of Maremmano stallions, after dietetic supplementation with LC.

A positive correlation between TM and sperm LC was found in this study. This interaction could be associated with a direct effect of LC on spermatogenesis and sperm quality; therefore, it can be suggested that this compound might be a marker of semen quality.

There are many studies that have shown that the supplementation of an extender using different concentrations of LC have improved the sperm motility and membrane integrity in ruminants[22] and in humans.[23] In a study using LC and Pyruvate during horse sperm storage at 37°C for 72 hours, it was observed that the semen treated with LC presented higher total and PM, when compared to the control group for up to 72 hours of storage.[10]

The results of this study are in agreement with earlier reports of the beneficial effects of LC and Acetyl-l-Carnitine during horse sperm storage for up to 48 hours at 5°C.[18] It is noteworthy that the first contact of spermatozoa with LC occurs in the epididymal lumen, where they develop the capacity for PM, which might explain the positive relationship between the initiation of PM and higher levels of LC.[24]

Table 3. Mean and Standard Deviation of Kinematic Parameters of Equine Sperm Stored at 5°C for 72 Hours, in the Presence of Different l-Carnitine Concentrations

l-Carnitine (mM)	*Time (hours)*	*TM (%)*	*PM (%)*	*RAP (%)*	*VCL (μm/s)*	*VSL (μm/s)*	*VAP (μm/s)*	*LIN (%)*
0.0	0	68.2 ± 14.5^{aA}	26.4 ± 10.4^{aA}	47.9 ± 24.0^{aA}	128.8 ± 29.4^{aA}	67.0 ± 18.4^{aA}	106.8 ± 29.9^{aA}	52.0 ± 9.1^{aAB}
	24	56.0 ± 15.2^{bB}	22.5 ± 10.4^{aA}	36.0 ± 18.7^{aAB}	116.7 ± 31.3^{aA}	64.7 ± 17.0^{aA}	99.4 ± 29.6^{aAB}	56.2 ± 9.7^{aA}
	48	44.6 ± 16.6^{bC}	13.2 ± 8.1^{bB}	25.0 ± 17.6^{aBC}	109.7 ± 29.5^{aAB}	47.6 ± 12.6^{aB}	86.7 ± 24.7^{aB}	44.7 ± 11.0^{aB}
	72	32.5 ± 19.3^{bD}	8.2 ± 4.8^{aB}	16.5 ± 19.0^{aC}	94.8 ± 32.3^{aB}	42.7 ± 11.9^{aB}	66.6 ± 24.5^{aC}	47.7 ± 13.9^{aAB}
0.5	0	71.3 ± 12.1^{aA}	28.4 ± 10.8^{aA}	47.6 ± 23.9^{aAB}	127.6 ± 30.3^{aA}	67.1 ± 19.2^{aA}	104.8 ± 30.5^{aA}	52.8 ± 9.3^{aA}
	24	63.8 ± 15.2^{abAB}	25.8 ± 10.0^{aA}	43.5 ± 19.3^{aAB}	119.0 ± 19.7^{aAB}	64.3 ± 14.7^{aAB}	100.0 ± 18.6^{aA}	54.3 ± 10.9^{aA}
	48	53.1 ± 20.0^{abB}	17.9 ± 10.8^{abB}	33.9 ± 22.6^{aB}	113.5 ± 27.9^{aAB}	53.4 ± 16.3^{aB}	92.3 ± 26.1^{aA}	47.3 ± 11.1^{aAB}
	72	39.7 ± 21.8^{abC}	8.1 ± 5.1^{aC}	21.5 ± 22.0^{aC}	99.9 ± 37.9^{aB}	37.8 ± 11.6^{aC}	73.8 ± 30.2^{aB}	41.0 ± 15.0^{aB}
1.0	0	76.3 ± 11.5^{aA}	28.4 ± 9.8^{aA}	53.0 ± 25.7^{aA}	133.7 ± 29.0^{aA}	67.3 ± 18.9^{aA}	111.4 ± 30.9^{aA}	50.6 ± 10.4^{aA}
	24	67.1 ± 14.1^{aA}	24.8 ± 10.3^{aAB}	43.7 ± 16.6^{aAB}	121.4 ± 22.4^{aABC}	61.8 ± 16.4^{aAB}	101.8 ± 21.9^{aAB}	50.9 ± 9.5^{aA}
	48	56.5 ± 16.4^{aB}	17.6 ± 10.1^{abB}	34.3 ± 20.9^{aBC}	111.9 ± 27.1^{aBC}	50.2 ± 17.4^{aBC}	89.4 ± 25.1^{aBC}	45.7 ± 14.9^{aAB}
	72	45.5 ± 19.5^{aC}	9.3 ± 5.6^{aC}	24.9 ± 21.8^{aC}	102.6 ± 30.7^{aC}	39.3 ± 11.4^{aC}	77.1 ± 25.2^{aC}	40.1 ± 13.7^{aB}
2.0	0	72.7 ± 13.9^{aA}	28.2 ± 12.8^{aA}	48.7 ± 23.1^{aA}	124.4 ± 26.6^{aA}	64.8 ± 18.0^{aA}	102.8 ± 25.6^{aA}	52.2 ± 10.0^{aAB}
	24	63.7 ± 15.3^{abAB}	28.0 ± 11.1^{aA}	41.8 ± 18.4^{aA}	115.7 ± 19.6^{aAB}	67.7 ± 17.1^{aA}	99.4 ± 19.6^{aA}	58.5 ± 11.4^{aA}
	48	56.5 ± 18.5^{aB}	21.2 ± 10.4^{aA}	37.0 ± 22.5^{aA}	113.4 ± 26.2^{aAB}	59.2 ± 17.0^{aA}	94.9 ± 24.5^{aA}	53.3 ± 15.1^{aAB}
	72	40.7 ± 19.5^{abC}	11.4 ± 7.4^{aB}	20.3 ± 18.3^{aB}	99.7 ± 28.8^{aB}	43.0 ± 13.4^{aB}	74.8 ± 23.8^{aB}	44.2 ± 12.3^{aB}

Different lowercase letters within the same column indicate a significant difference ($p < 0.05$) among treatments at the same time. Different capital letters in the same column indicate significant differences ($p < 0.05$) between times within the same treatment.

PM, progressive motility; RAP, percentage of quick sperm; TM, total motility.

Downloaded by Eth Bibliothek from www.liebertpub.com at 09/30/20. For personal use only.

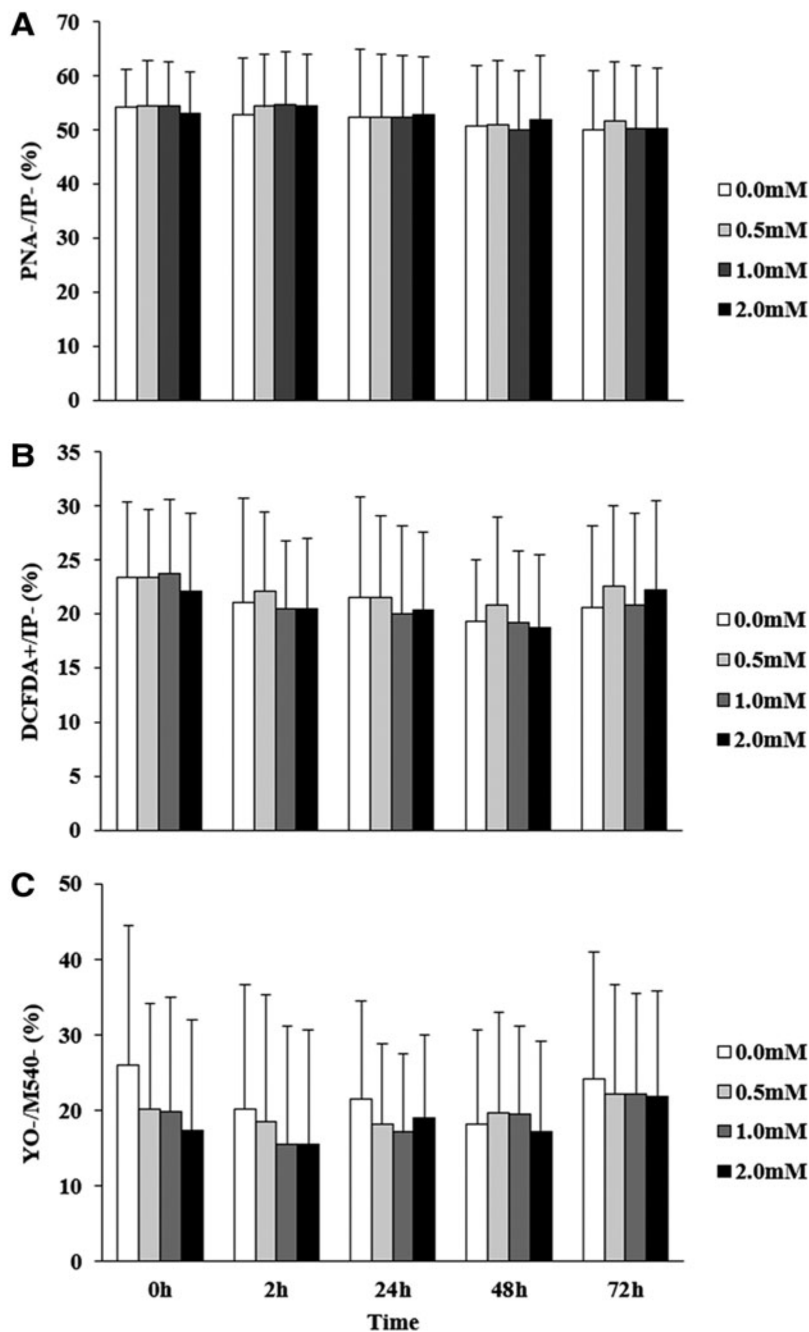

FIG. 1. Mean and standard deviation of plasma and acrosomal membrane integrity **(A)**, intracellular ROS **(B)** and membrane stability **(C)** of equine sperm subjected to cooling for 72 hours in the presence of different L-carnitine concentrations. ROS, reactive oxygen species.

Most experiments have shown that there is a significant reduction in the intracellular concentration of LC[25] and Acetyl-L-Carnitine[4] in human sperm after cryopreservation. In this way, it has been postulated that decreased sperm motility after semen cryopreservation can be associated with a decrease in concentration of sperm carnitine.[4] These findings suggest that the metabolic pathways for the production of adenosine triphosphate (ATP) from sperm refrigeration are compromised over time, negatively interfering with sperm kinetics.

A previous study showed that the addition of LC to a commercial extender was effective in improving the seminal quality, with a positive influence on plasma membrane integrity and the kinetics of equine sperm that was stored at 5°C for 48 hours.[19] Studies have shown that the addition of LC to a chemically defined extender prolonged the survival of equine sperm that was stored for 72 hours at room temperature. This result is in support of mitochondrial ATP production, while minimizing ATP depletion because of the harmful effects of metabolic by-products such as ROS.[10]

In this sense, it is reasonable to suggest that the exogenous supplementation of LC was able to maintain the intracellular concentration for a longer period, maintaining the metabolic pathways for the production of ATP for up to 48 hours of chilling, as was shown by the kinetic variables that were maintained in samples with added LC.

In contrast, the addition of LC had no positive effect on the integrity of plasma and acrosomal membranes, membrane stability, or intracellular ROS concentrations during this experiment. This fact can be associated with the concentrations of LC, once some experiments have[10] used considerably larger concentrations of LC and an association

Downloaded by Eth Bibliothek from www.liebertpub.com at 09/30/20. For personal use only.

with Pyruvate, than the ones used in this experiment, finding results correlated with acrosomal membranes, membrane stability, and ROS concentration. In addition, differences between breeds and analytical procedures may make comparison between the studies difficult.

LC has been implicated as being responsible for the transport of polyunsaturated fatty acids into the mitochondria for ATP generation through β-oxidation in sperm metabolism, which may improve the sperm motility and resistance by affecting the motility, maturation, and sperm quality.[8] Therefore, these results reinforce the need of further studies using a larger set of animals to evaluate the implication of LC as a seminal quality marker.

Based on the results of the present study, the equine seminal plasma LC shows a relationship with sperm concentration, sperm membranes, and PM. Furthermore, LC added to a skimmed milk extender (1 or 2 mM) mitigates the negative effect of storage at 5°C on kinematic parameters of horse sperm, indicating the potential use of LC as an additive for equine semen extenders.

Author Disclosure Statement

No conflicting financial interests exist.

Funding Information

The authors thank for Coordination for the Improvement of Higher Education Personnel (CAPES), Funding Agency for Studies and Projects (FINEP), National Council for Scientific and Technological Development (CNPq), and Pernambuco State Science and Technology Support Foundation (FACEPE) for funding the research.

References

1. Lindahl J, Dalin A, Stuhtmann G, et al. Stallion spermatozoa selected by single layer centrifugation are capable of fertilization after storage for up to 96 h at 6°C prior to artificial insemination. Acta Vet Scand 2012;54:1–40.
2. Loomis PR. The equine frozen semen industry. Anim Reprod Sci 2001;68:191–200.
3. Ecot P, Vidament M, de Mornac A, et al. Freezing of stallion semen: Interactions among cooling treatments, semen extenders and stallions. J Reprod Fertil Suppl 2000;56:141–150
4. Grizard G, Vignon N, Boucher D. Changes in carnitine and acetylcarnitine in human semen during cryopreservation. Hum Reprod.1992;7:1245–1248.
5. Ugur MR, Abdelrhman AS, Evans HC, et al. Advances in Cryopreservation of bull sperm. Front Vet Sci 2019; 6:268.
6. Watson PF. The causes of reduced fertility with cryopreserved semen. Anim Reprod Sci 2000; 60–61:481–492.
7. Aurich JE, Ktibne JA, Hoppe H, et al. Seminal plasma affects membrane integrity and motility of equine spermatozoa after cryopreservation. Theriogenology 1996;46: 791–797.
8. Matalliotakis I, Koumantaki Y, Evageliou A, et al. L-Carnitine levels in the seminal plasma of fertile and infertile men: Correlation with sperm quality. Int J Fertil 2000;45:236–240.
9. Neuman SL, Lin TL, Heste PY. The effect of dietary carnitine on semen traits of white Leghorn roosters. Poult Sci 2002;81:495–503.
10. Gibb Z, Lambourne SR, Quadrelli J, et al. L-Carnitine and pyruvate are prosurvival factors during the storage of stallion spermatozoa at room temperature. Biol Reprod 2015;104:1–9.
11. Stradaioli G, Sylla L, Zelli R, et al. Effect of L-carnitine administration on the seminal characteristics of oligoasthenospermic stallions. Theriogenology 2004;62:761–777.
12. Banihani S, Agarwal A, Sharma R, et al. Cryoprotective effect of L-carnitine on motility, vitality and DNA oxidation of human spermatozoa. Andrologia 2014;46:637–641.
13. Zhang W, Li F, Cao H, et al. Protective effects of l-carnitine on astheno and normozoospermic human semen samples during cryopreservation. Zygote 2016;24:1–8.
14. El-Nattat WS, El-Sheshtawy RI, Mohamed AA. Effect of L-carnitine on semen characteristics of chilled rabbit semen. Global J Biotech Biochem 2011;6:8–12.
15. Sarıozkan S, Ozdamar S, Turk G, et al. In vitro effects of L-carnitine and glutamine on motility, acrosomal abnormality, and plasma membrane integrity of rabbit sperm during liquid-storage. Cryobiology 2014;68:349–353.
16. Manne-In S, Parmornsupornvichit S, Kraiprayoon S, et al. L-carnitine supplemented extender improves cryopreserved-thawed cat epididymal sperm motility. Asian Australas J Anim Sci 2014;27:791–796.
17. Aliabadi E, Karimi F, Talaei-Khozani T. Effects of L-carnitine and pentoxifylline on carbohydrate distribution of mouse testicular sperm membrane. Iran J Med Sci 2013; 38:107–115.
18. Lisboa FP, Hartwig FP, Maziero RRD, et al. Use of L-carnitine and acetyl-L-carnitine in cooled-stored stallion semen. J Equine Vet Sci 2012;32:493–494.
19. Lisboa FP, Hartwig FP, Freitas-Dell'aqua CP, et al. Improvement of cooled equine semen by addition of carnitines. J Equine Vet Sci 2014;34:48.
20. Stradaioli G, Sylla L, Zelli R, et al. Seminal carnitine and acetylcarnitine content and carnitineacetyltransferase activity in young *Maremmano stallions*. Anim Reprod Sci 2000;64:233–245.
21. Souza HM, Arruda LCP, Monteiro MM, et al. The effect of canthaxanthin on the quality of frozen ram spermatozoa. Biopreserv Biobank 2017;15:220–227.
22. Galarza DA, Lopez-Sebastian A, Santiago-Moreno J. Supplementing a skimmed milk-egg yolk-based extender with L-carnitine helps maintain the motility, membrane integrity and fertilizing capacity of chilled ram sperm. Reprod Domest Anim 2020;01–09.
23. Banihani S, Sharma R, Bayachou M, Sabanegh E, Agarwal A. Human sperm DNA oxidation, motility and viability in the presence of l-carnitine during in vitro incubation and centrifugation. Andrologia 2012;44:505–512.
24. Jeulin C, Soufir JC, Marson J, et al. The distribution of carnitine and acetylcarnitine in the epididymis and epididymal spermatozoa of the boar. J Reprod Fertil 1987;79:523–529.
25. Suter DAI, Holland MK. The concentrations of free L-carnitine and L-O-acetylcarnitine in spermatozoa and seminal plasma of normal, fresh and frozen human semen. Fertil Steril 1979;31:541–544.

Address correspondence to:
Maria Madalena Pessoa Guerra, PhD
Andrology Laboratory (ANDROLAB)
Department Veterinary Medicine
Universidade Federal Rural de Pernambuco
Rua Dom Manoel de Medeiros, s/n, Dois Irmãos
Recife-PE 52171-900
Brazil

E-mail: mmpguerra@gmail.com