L-carnitine and acetyl-L-carnitine enhance stallion sperm quality during semen storage at 5°C

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Abstract

Carnitine, a powerful antioxidant, has an essential role in sperm energy metabolism. Among carnitines, only L-carnitine's effect on stallion semen has been tested and not acetyl-L-Carnitine. Therefore, we aimed to determine the ideal concentrations of L-carnitine (LC) and acetyl-L-carnitine (AC) and their effects on stallion semen cooled at 5°C for up to 48 hours. Semen was extended to 50×10^6 sperm/ml in commercial extender (Control), and concentrations of 5, 10, and 15 mmol/l of LC and AC were evaluated in Experiment 1. Sperm motility and plasma membrane integrity were assessed by CASA and epifluorescence microscopy, respectively. In Experiment 2, the combination of the intermediate doses of LC (10 mmol/l) and AC (10 mmol/l) was tested. Sperm parameters were evaluated as in Experiment 1 and in addition, DNA fragmentation index (DFI), production of reactive oxygen species (ROS), and lipid peroxidation (PEROX) were evaluated by flow cytometry. All analyses were performed at 0, 24, and 48 hours after semen collection, processing, and cooled-stored at 5°C. In Experiments 1 and 2, the groups supplemented with LC and AC or LC+AC had higher plasma membrane integrity and motility parameters compared to Control group (p < 0.05). The LC and AC combination did not change sperm parameters compared to LC or AC alone (p > 0.05). No differences (p > 0.05) were observed for DFI, ROS, and PEROX. In conclusion, LC and AC's addition, alone or in combination, enhanced sperm motility and plasma membrane integrity of stallion sperm after cooled-storage at 5°C for up to 48 hours.

Keywords: Stallion, carnitine, sperm metabolism, cooled semen, sperm viability

Introduction

L-carnitine use as a nutraceutical drug is consolidated in equine reproduction since various reports have demonstrated positive results after oral supplementation.[1-3] However, only a few reports have documented the effects of carnitines inculsion in stallion semen.[4-6] Carnitines modulate several metabolic functions in sperm (e.g., beta-oxidation of fatty acids, acetyl-CoA, and free CoA ratio, using pyruvate and lactate as energetic substrates to produce adenosine triphosphate).[7] Only the L-isomer of carnitines is biologically active and is concentrated in tissues that require high amounts of energy, such as skeletal, cardiac muscles, and specialized organs of the reproductive tract (e.g., epididymis).[8] L-carnitine is carried from blood to epididymis through active transport and is accumulated into sperm through passive transport.[7] These interactions are androgen dependent.[1,9] For this reason, concentrations of carnitine in the epidymal fluid are 2,000 fold higher than in the blood, suggesting that this molecule is highly related to fertility.[4]

L-carnitine is a carrier of fatty acids in energy metabolism since it can cross the internal mitochondrial membrane it has an essential role in the oxidative pathways and ATP synthesis.[10,11]

L-carnitine needs to be acetylated through the carnitine acetyltransferase enzyme to cross the mitochondrial membrane, forming acetyl-L-carnitine. After crossing the mitochondrial membrane, acetyl-L-carnitine is dissociated into acetyl-CoA to synthesize ATP.[7] In summary, the acetylated form of carnitine is responsible for providing acetyl groups for sperm motility.[12,13]

The entry of L-carnitine and its conversion into acetyl-L-carnitine in sperm is evidence of good epididymal function.[1] Furthermore, there is a correlation between L-carnitine/acetyl-L-carnitine ratio in semen and sperm progressive motility, suggesting that carnitines might contribute to sperm viability during the cooling process.[14] Additionally, carnitines have a powerful antioxidant action by reducing the availability of phospholipids for lipid peroxidation and increasing concentrations and activity of antioxidant enzymes (e.g., superoxide dismutase and glutathione peroxidase).[15]

We hypothesized that adding L-carnitine and acetyl-L-carnitine could improve equine sperm quality during cooled storage, either by carrying acetyl groups available in the extender to the inside mitochondria or by providing them dircty by its acetylated form. We aimed to evaluate the isolated and asso-

ciated inclusion of L-carnitine and acetyl-L-carnitine in semen extender on sperm motility and viability parameters of cooled equine semen stored at 5°C for up to 48 hours.

Materials and methods

Reagents used were purchased from Sigma-Aldrich (St. Louis, MO). Experimental protocols were approved by the ethics committee for animal use (São Paulo State University [UNESP] protocol #74/2012). This study was carried out from March to May of 2013.

Animals

Twelve stallions (6 of each Quarter Horse and Mangalarga Marchador breed), aged between 5 and 15 years, were enrolled. Animals were kept in stalls, fed 3 kg of balanced grain and with free access to hay, water, and trace minerals. Stallions were in reproductive management and had been collected 3 times a week. Therefore, no washout semen collections were needed before the study.

Experimental design

Semen collections were performed using a Botucatu artificial vagina (Botupharma, Botucatu, São Paulo, Brazil) at 72 hour intervals off a dummy mount in the presence of a teaser mare. After semen collection, each ejaculate was filtered, and the sperm concentration was determined with a hemocytometer chamber. Thereafter, semen samples were equally split into groups and extended to 50 x 10⁶ sperm/ml using a commercially available Kenney-type (skim milk-based, SKM) extender (Botu-Semen, Botupharma), containing 20 g/l of SKM, glucose, bicarbonate buffer, 1 g/l of gentamicin sulfate, and 1 g/l of sodium penicillin supplemented or not with L-carnitine or acetyl-L-carnitine. All extenders had pH fixed between 6.8 - 7.0 and osmolarity at 340 - 360 mOsm. All semen samples were extended at a minimum dilution of 2:1 (v:v; extender:semen).[16]

After extension, samples were stabilized for 15 minutes at room temperature (22°C) and then stored in a passive semen cooling container (BotuFlex, Botupharma) at 5°C for 24 hours. Container was maintained in the laboratory at room temperature to avoid any additional interference. After this period, samples were transferred to a temperature-controlled refrigerator (Minitube do Brasil®, Porto Alegre, Rio Grande do Sul, Brazil) at 5°C out to 48 hours postcooling.

Experiment 1

Inclusion of various concentrations of L-carnitine and acetyl-L-carnitine into semen extender on sperm parameters of cooled sperm

Two ejaculates of each stallion (n = 24) were divided into: Control group, semen was extended in Botu-Semen; LC1, Botu-Semen supplemented with 5 mmol/l of L-carnitine; LC2, Botu-Semen supplemented with 10 mmol/l of L-carnitine; LC3, Botu-Semen supplemented with 15 mmol/l of L-carnitine; AC1, Botu-Semen supplemented with 5 mmol/l of acetyl-L-carnitine; AC2, Botu-Semen supplemented with 10

mmol/l of acetyl-L-carnitine; AC3, Botu-Semen supplemented with 15 mmol/l of acetyl-L-carnitine. Sperm motility parameters and PMI were evaluated immediately before cooling (0 hour), 24, and 48 hours.

Experiment 2

Effects of the combination of L-carnitine and acetyl-L-carnitine on sperm parameters in cooled semen

As there were no differences among treated groups in Experiment 1, intermediate doses of L-carnitine and acetyl-L-carnitine were chosen to test the combination of these molecules on sperm parameters. Two ejaculates (n = 24) of each stallion were divided into: Control, semen extended in Botu-Semen; LC, Botu-Semen supplemented with 10 mmol/l of L-carnitine; AC, Botu-Semen supplemented with 10 mmol/l of acetyl-L-Carnitine; LC/AC, Botu-Semen supplemented with 10 mmol/l of L-carnitine and 10 mmol/l of acetyl-L-carnitine. Sperm motility parameters, PMI, DNA fragmentation (DNA), lipid peroxidation (PEROX), and reactive oxygen species (ROS) were evaluated immediately before cooling before cooling (0 hour), 24, and 48 hours.

Sperm analyses

Sperm kinetics

Sperm motility parameters were evaluated using computer-assisted sperm analysis (IVOS 12, Hamilton Thorne Inc., Beverly, MA) using customized settings for equine sperm.[17] For each sample, the percentages of total motility (TM), progressive motility (PM), average path velocity (VAP, μm/s), straight-line velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s), and rapid sperm (RAP) were evaluated. Each sample was incubated in a dry bath at 37°C for 10 minutes before each evaluation. For each sample, 5 random fields were assessed.

Plasma membrane integrity

Plasma membrane integrity was performed by epifluorescence microscopy (Leica Microsystem-DMLB, Germany) based on the association of the fluorescent probes propidium iodide and 6-carboxyfluorescein diacetate.[18] Carboxifluorescein diacetate–positive cells were considered sperm with intact plasma membrane and PI-positive sperm with damaged plasma membrane.

Flow cytometry analyses

Flow cytometric analyses were carried out with Fortessa LSR equipment (Becton Dickinson, Mountain View, CA) equipped with blue (488-nm, 100 mW), red (640-nm, 40 mW), and violet (405-μm, 100 mW) lasers that were quality-controlled daily using CS&T beads and FACS DiVA software (BD Biosciences). The filter configurations for the PMTs (Photomultiplier tubes) measuring fluorescence emission of the applied fluorochromes were 450/50 nm (H342), 530/30 nm (FITC); 660/20 nm (APC); and 694/50 nm (PI). Auto-fluorescence and single-color controls were acquired to perform spectral overlap compensation using the automated compensation matrix feature in FACS DiVA software. Fluorescence minus the

controls was used to identify staining regions. Flow cytometry data were plotted using bi-exponential plots with axes < 0 to ensure that all data were visible and properly compensated. Thereafter, histograms were generated for analysis. Auto-fluorescence and controls of each fluorochrome were acquired to adjust wave overlap and compensation using the matrix compensation of the manufacturer's software. The percentage of positive sperm was considered for each assay.

For each assay, at least 10,000 cells per sample were analyzed, and the data were extracted using the manufacturer's software (BD FACSDiva™ v6.1). All samples were extended in modified TALP-PVA[19] at 5×10^6 sperm/ml concentration. Composition of the TALP-PVA medium was: 100 mM NaCl, 3.1 mM KCl, 25.0 mM NaHCO$_3$, 0.3 mM NaH$_2$PO$_4$, 21.6 mM DL 60% sodium lactate, 2.0 mM CaCl$_2$, 0.4 mM MgCl$_2$, 10.0 mM Hepes-free acid, 1.0 mM sodium pyruvate, 1.0 mg/ml polyvinyl alcohol-PVA, and 25 μg/ml gentamicin.

DNA fragmentation index

This assay was performed as described.[20,21] First, semen was diluted in a buffer solution (0.186 g of disodium EDTA, 0.790 g of Tris-HCl, 4.380 g NaCl in 500 ml deionized water, pH 7.4) to 1×10^6 sperm/ml. Then, 400 μl of acid detergent solution (2.19 g NaCI, HCI 1.0 ml of 2N solution, 0.25 ml Triton-X, in quality standards program 250 ml of deionized water was added, pH 1.8) was added, incubated for 30 seconds and then added 1 ml of acridine orange dye solution (AO; 3.8869 g of citric acid monohydrate, 8.9429 g Na$_2$HPO$_4$, 4.3850 g NaCI, 0.1700 g of disodium EDTA, 4 μg/ml of acridine orange solution - 1 mg/ml, in quality standards program 500 ml of water, pH 6.0). Samples were then analyzed by flow cytometry in 5 minutes, and the data generated were analyzed using the Win-List 6.0 software (Verify software house). The DNA fragmentation index (DFI, %) was generated from the analysis of 10,000 cells marked with acridine orange. The IDF is the proportion, expressed as a percentage of sperm DNA and fragmented DNA (red fluorescence) divided by total fluorescence.

ROS production

Intracellular hydrogen peroxide production was assessed using dihydrorhodamine 123 (DHR, D23806, Life Technologies, São Paulo, São Paulo, Brazil). After reacting with ROS such as hydrogen peroxide or peroxide nitrite, dihydrorhodamine 123DHR is oxidized into a fluorescent compound (rhodamine 123) that is retained within the cell. Each 500 μl aliquot of extended semen in TALP-PVA had 1.5 μM propidium iodide (diluted in TALP-PVA), and 1 μM DHR (diluted in dimethyl sulfoxide) added before being incubated at 37°C for 20 minutes under the light.

Lipid peroxidation

Lipid peroxidation assessments were carried out with the fluorescent probe C11-BODYPY (D-3861; Molecular Probes, Carlsbad, CA), a lipophilic fluorophore sensitive that reacts with oxygen and peroxynitrite and, once oxidized, converts its fluorescence from red to green. Lipid peroxidation was accessed in the total sperm population of each sample. Each aliquot of semen (2×10^6 sperm/ml TALP-PVA extended in

489.5 μl) was added C11BODIPY581/591 (0.5 μl, solution 1 mg/ml), propidium iodide (5 μl, solution 50 μg/ml), Hoescht 33,342 (5 μl, solution 100 μg/ml) and then the mixture was incubated for at 37°C for 30 minutes. After incubation, the samples were washed twice at 300 x g for 5 minutes, and the pellet was resuspended in 500 μl of TALP-PVA and then analyzed by flow cytometry.[22]

Data analyses

GraphPad Prism 8.0.1. (GraphPad Software, San Diego, CA) was used. The Gaussian distribution of the semen parameters was evaluated using the Shapiro-Wilk normality test. Semen parameters were assessed with a one-way ANOVA and Tukey's as a posthoc test. Stallion was accounted as a random effect. Ejaculate order, time of storage, and the treatment groups as fixed effects. Data are presented as mean ± SD.

Results

There were no effects ($p > 0.05$) of ejaculate order and stallions displayed minimal variation ($p > 0.05$), in raw semen parameters. The overall gel-free volume, sperm concentration/ml, and total sperm ejaculated were 36 ± 16 ml, 264 ± 164 x 10^6, and 8 ± 3.9 × 10^9, respectively.

Experiment 1

Motility parameters were enhanced in fresh semen in LC1 group compared to Control group ($p < 0.05$; Figure 1A,D,G). Progressive motility of fresh semen was higher ($p > 0.05$) in all groups supplemented with L-carnitine (LC1, LC2, and LC3) compared to Control group (Figure 1D). Inclusion of AC in semen did not change ($p > 0.05$) sperm motility in fresh semen samples (Figure 1A,D,G). Plasma membrane integrity was higher ($p > 0.05$) in fresh semen samples in LC2 and L3 groups than Control (Figure 1J). Semen in the other groups had intermediate PMI ($p > 0.05$, Figure 1J).

Sperm motility parameters and PMI decreased ($p > 0.05$) over time in all groups (Figure 1). After 24 hours of storage at 5°C, higher ($p > 0.05$) TM was observed in all groups supplemented with LC and AC (Figure 1B). Semen in all LC groups and AC3 had higher ($p > 0.05$) PM and RAP than the Control group, but not different ($p > 0.05$), than AC1 and AC3 (Figure 1E). At 48 hours, TM, PM, and RAP were higher ($p > 0.05$) in all groups supplemented with LC or AC compared to the Control group (Figure 1F). However, PM was higher ($p > 0.05$) in the LC2 group compared to the others, except LC3 (Figure 1F). Higher ($p > 0.05$) PMI was observed in all LC groups at 24 and 48 hours after cooling compared to Control (Figure 1K,L). The AC groups had intermediate ($p > 0.05$) PMI values at the same time points (Figure 1K,L). Values for VAP, VCL, and VSL are highlighted (Table 1). Sperm velocity parameters were reduced ($p > 0.05$) over time in all groups, and the addition of L-carnitine change VAP and VSL in fresh semen samples compared to Control.
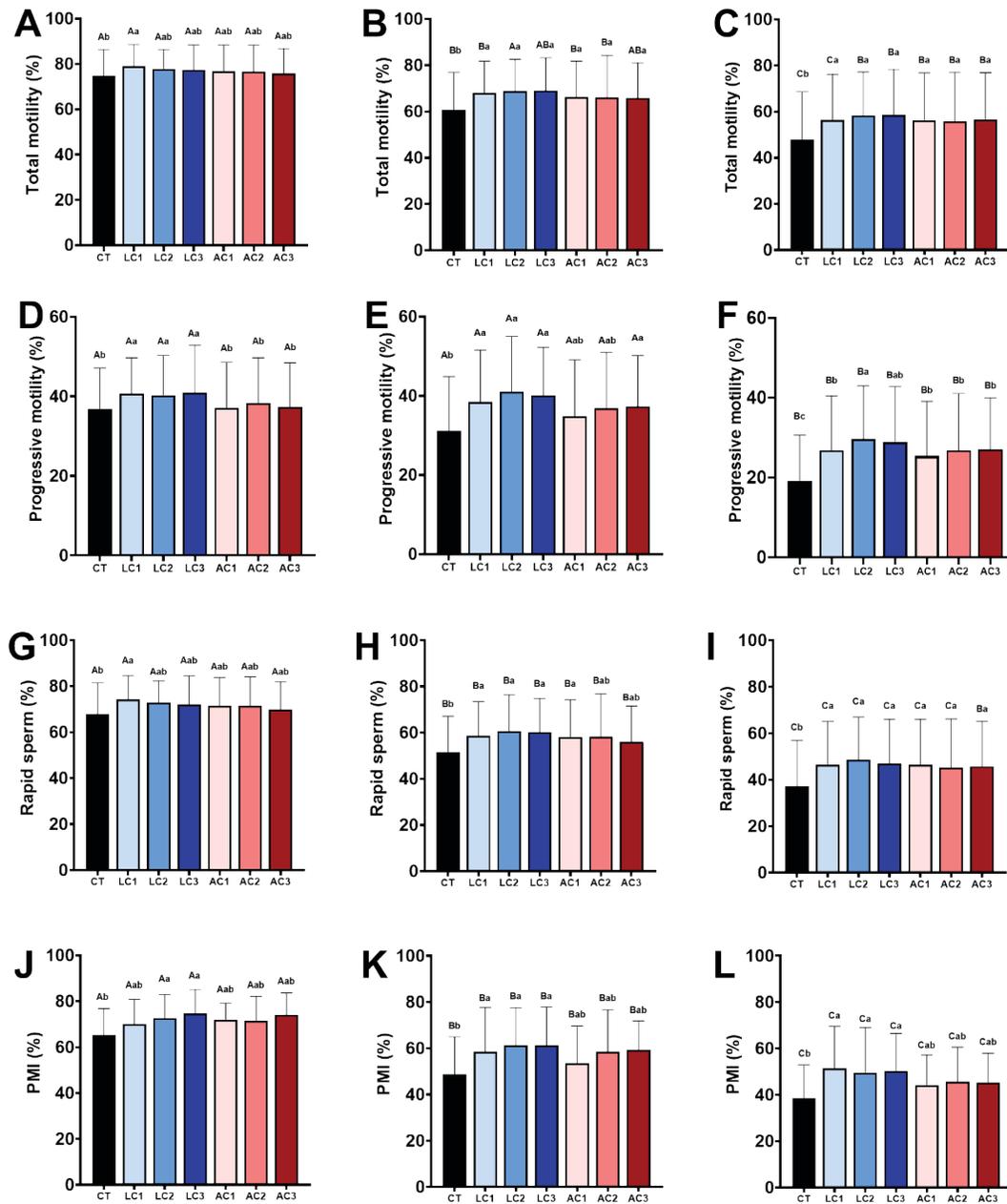
Figure 1. Mean ± SD of sperm motility parameters and plasma membrane integrity (PMI) of stallion semen (n = 24 ejaculates) in vitro supplemented with various concentrations of L-carnitine (LC) or acetyl-L-carnitine (AC) at 0 (A,D,G,J), 24 (B,E,H,K), and 48 hours (C,F,I,L). CT, semen extended in milk-based extender (Botu-Semen) without supplementation; LC1, Botu-Semen supplemented with 5 mmol/l of L-carnitine; LC2, Botu-Semen supplemented with 10 mmol/l of L-carnitine; LC3, Botu-Semen supplemented with 15 mmol/l of L-carnitine; AC1, Botu-Semen supplemented with 5 mmol/l of acetyl-L-Carnitine; AC2, Botu-Semen supplemented with 10 mmol/l of acetyl-L-Carnitine; AC3, Botu-Semen supplemented with 15 mmol/l of acetyl-L-carnitine. Different superscripts denote effects of time (^{A,B,C}) and differences ($p > 0.05$) among groups within each time point (^{a,b,c}).

Table 1. Mean ± SD of sperm kinetic parameters evaluated by CASA of cooled equine semen (n = 24 ejaculates) supplemented or not with various concentrations of L-carnitine (LC) or acetyl-L-carnitine (AC) at moments 0, 24, and 48 hours (n = 24).

TIME (hour)	Group	VAP (µm/s)	VSL (µm/s)	VCL (µm/s)
T0	CT	123 ± 14[Ac]	94 ± 12[Ab]	229 ± 23[Aa]
	LC1	135 ± 9[Aa]	103 ± 7[Aa]	239 ± 47[Aa]
	LC2	134 ± 9[Aab]	103 ± 7[Aa]	237 ± 49[Aa]
	LC3	131 ± 12[Ab]	102 ± 10[Aa]	240 ± 22[Aa]
	AC1	133 ± 9[Aab]	99 ± 6[Aab]	246 ± 21[Aa]
	AC2	132 ± 10[Aab]	100 ± 7[Aab]	245 ± 23[Aa]
	AC3	129 ± 10[Aab]	98 ± 7[Aab]	238 ± 22[Aa]
T24	CT	111 ± 13[Ba]	87 ± 11[Aa]	211 ± 22[ABa]
	LC1	115 ± 15[Ba]	91 ± 12[Ba]	207 ± 46[Ba]
	LC2	116 ± 14[Ba]	90 ± 20[Ba]	207 ± 46[Ba]
	LC3	113 ± 16[Ba]	91 ± 12[Ba]	210 ± 31[Ba]
	AC1	115 ± 15[Ba]	91 ± 11[Ba]	208 ± 46[Ba]
	AC2	114 ± 13[Ba]	91 ± 10[Ba]	214 ± 25[Ba]
	AC3	112 ± 13[Ba]	90 ± 11[Ba]	209 ± 23[Ba]
T48	CT	103 ± 19[Ba]	75 ± 13[Ba]	205 ± 33[Ba]
	LC1	108 ± 15[Ba]	83 ± 12[Cb]	212 ± 29[Ba]
	LC2	108 ± 18[Ba]	84 ± 13[Bb]	207 ± 33[Ba]
	LC3	103 ± 17[Ca]	81 ± 13[Cab]	199 ± 29[Ba]
	AC1	105 ± 16[Ca]	81 ± 11[Ca]	206 ± 29[Ba]
	AC2	104 ± 17[Ca]	80 ± 12[Ca]	204 ± 31[Ba]
	AC3	102 ± 17[Ca]	80 ± 12[Ca]	200 ± 30[Ba]

VAP, average path velocity; VSL, straight line velocity; VCL, Curvilinear velocity. Group CT (Botu-Semen®); LC1 group (Botu-Semen® + 5 mmol/l of LC); LC2 group (Botu-Semen® + 10 mmol/l of LC); LC3 group (Botu-Semen® + 15 mmol/l of LC); AC1 group (Botu-Semen® + 5 mmol/l of AC); group AC2 (Botu-Semen® + 10 mmol/l of AC); group AC3 (Botu-Semen® + 15 mmol/l of AC). T0 (15 minutes of incubation), T24 (24 hours of refrigeration) and T48 (48 hours of refrigeration). Different superscripts denotes effect of time in the same group ([A,B,C]) and among groups at the same moment ([a,b]) ($p < 0.05$).

Experiment 2

Supplementation did not change ($p > 0.05$) TM and RAP in fresh semen samples (Figure 2A,G). However, semen supplemented with LC had higher ($p > 0.05$) PM at 0 hour than semen in Control group, whereas AC and LC/AC had intermediate values not different ($p > 0.05$) than others (Figure 2D). Sperm motility parameters and PMI decreased ($p > 0.05$) over time in all groups (Figure 2). After cooled storage for 24 and 48 hours, TM, PM, and RAP were higher ($p > 0.05$) in all treated groups compared to Control (Figure 2). Velocity parameters (VAP, VCL, and VSL) are highlighted in Table 2. Sperm velocity parameters were reduced ($p > 0.05$), overtime in all groups. Additionally, canitines enhanced ($p > 0.05$) PMI at all time points (0, 24, and 48 hours) in compared to Control (Figure 2J,K,L). However, carnitines did not change ($p > 0.05$) the sperm DFI, production of ROS, or PEROX over cooling storage compared to unsupplemented samples (Figure 3).

Discussion

We determined whether the inclusion of L-carnitine and acetyl-L-carnitine, alone or in combination, in a milk-based extender, improved stallion semen quality after storage at 5°C for up to 48 hours. Although the benefits of L-carnitine as a nutraceutical have been suggested,[1-3] only a few studies have reported the effects of the inclusion of L-carnitine into semen extender on stallion sperm parameters.[4-6] Furthermore, to the best of the authors' knowledge, there is no study evaluating the inclusion of acetyl-L-carnitine into equine semen extender.

Sperm motility parameters improved by the inclusion of L-carnitine (5, 10, and 15 mmol/l) in fresh semen. It suggested a rapid effect of L-carnitine on sperm metabolism that increased the availability of ATP for sperm motility.[4] Furthermore, sperm parameters of oligospermic stallions improved by including carnitines in semen.[1] Additionally, inclusion of L-carnitine in chemically-defined medium for stallion semen kept at room temperature resulted in better TM and PM, and reduced oxidative DNA damage.[4] It is known that L-carnitine carries fatty acids through the internal mitochondrial membrane that

improved the β-oxidation of fatty acids and consequently increased the production of energy by the cell,[11] improving sperm kinetics.[4,6] Interestingly, a positive correlation between plasma concentrations of L-carnitine and sperm quality was reported in stallions[6,14] and humans.[7] Furthermore, nutraceutical supplementation of L-carnitine improved sperm parameters in both species.[1,3,23] Higher availability of L-carnitine might be associated with improved energy metabolism in sperm. Oral supplementation of L-carnitine has been suggested to improve spermatogenesis by reducing the percentage of abnormal sperm,

such as sperm with detached heads and tails.[1] Additionally, oral supplementation with 40 g/day of L-carnitine for over 6 weeks improved conception rates in Standardbred stallions.[24] However, in another report, oral supplementation with L-carnitine (10 mg/day) did not affect sperm parameters of an Arabian stallion.[25] Besides different doses in each study, the authors hypothesized that the different results may be associated with the individual seminal plasma concentrations of carnitines, which makes some stallions benefit more from L-carnitine supplementation.
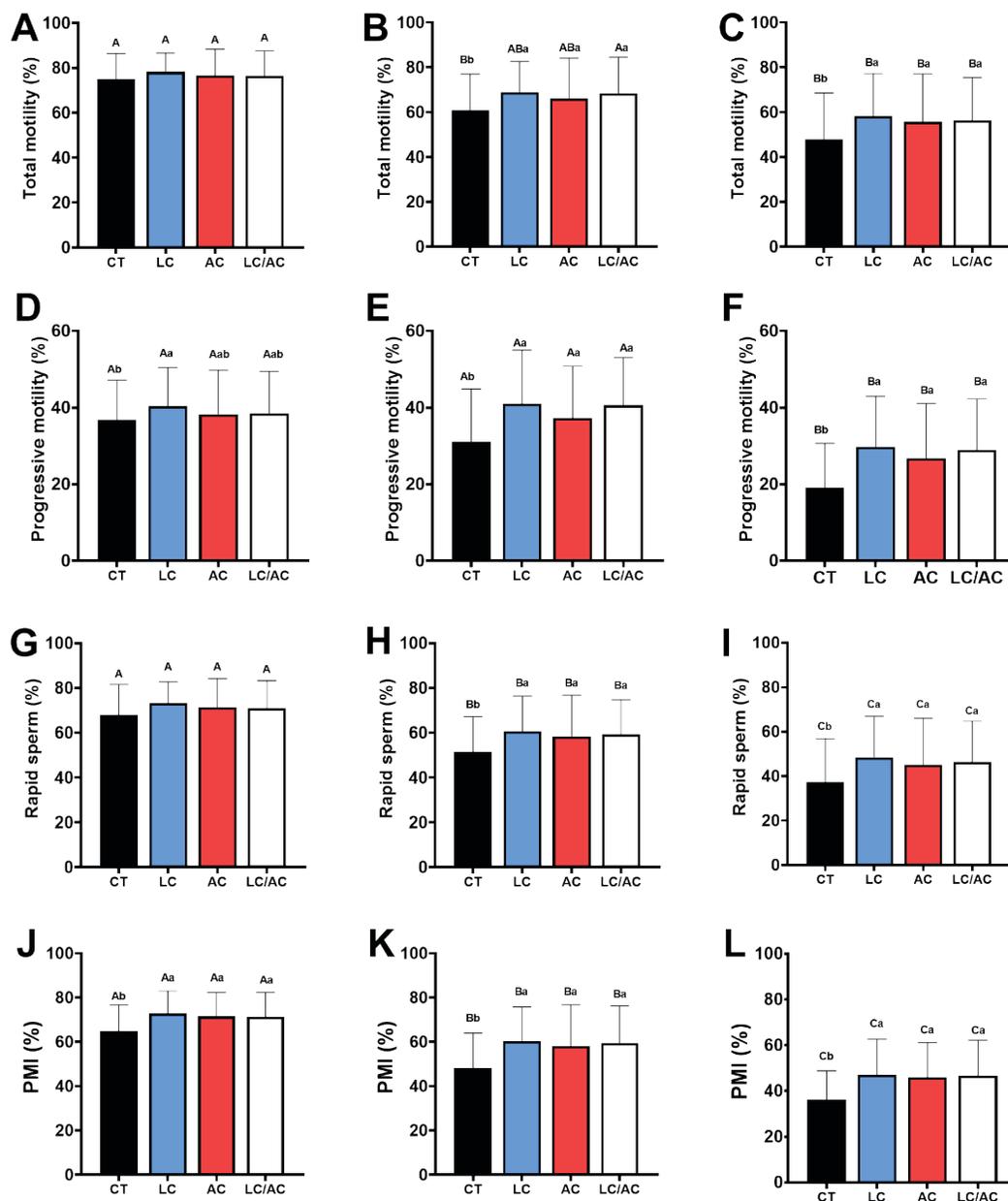

Figure 2. Mean ± SD of sperm motility parameters and plasma membrane integrity (PMI) of stallion semen (n = 24 ejaculates) in vitro supplemented with L-carnitine (LC) or acetyl-L-carnitine (AC) at moments 0 hour (A,D,G,J), 24 hours (B,E,H,K), and 48 hours (C,F,I,L). CT, semen extended in Botu-Semen; LC, Botu-Semen supplemented with 10 mmol/l of L-carnitine; AC, Botu-Semen supplemented with 10 mmol/l of acetyl-L-carnitine; LC/AC, Botu-Semen supplemented with 10 mmol/l of L-carnitine and 10 mmol/l of acetyl-L-carnitine. Different superscripts denote effects of time (^{A,B,C}) and differences (p > 0.05) among groups within each time point (^{a,b,c}).

Table 2. Mean ± SD of sperm kinetics parameters evaluated by CASA of cooled equine semen (n = 24 ejaculates) supplemented or not with L-carnitine (LC), acetyl-L-carnitine (AC), and their combination (LC/AC) at moments 0, 24, and 48 hours (n = 24).

Time (hour)	Group	VAP (µm/s)	VSL (µm/s)	VCL (µm/s)
T0	C	123 ± 15[Aa]	94 ± 12[Aa]	228 ± 23[Aa]
	LC	133 ± 12[Aa]	103 ± 7[Ab]	247 ± 18[Aa]
	AC	132 ± 10[Aa]	100 ± 7[Ab]	244 ± 23[Aa]
	LC/AC	130 ± 12[Aa]	100 ± 8[Ab]	241 ± 22[Aa]
T24	C	111 ± 13[Ba]	88 ± 10[Aa]	210 ± 22[Aba]
	LC	118 ± 17[Ba]	94 ± 12[Bb]	216 ± 25[Ba]
	AC	114 ± 13[Ba]	91 ± 10[Bab]	214 ± 24[Ba]
	LC/AC	113 ± 13[Ba]	92 ± 10[Bab]	208 ± 25[Ba]
T48	C	103 ± 19[Ba]	75 ± 13[Ba]	205 ± 33[Ba]
	LC	107 ± 18[Ca]	84 ± 13[Cb]	207 ± 33[Ba]
	AC	104 ± 17[Ba]	80 ± 12[Cb]	200 ± 41[Ba]
	LC/AC	105 ± 15[Ba]	83 ± 12[Cb]	205 ± 26[Ba]

VAP, average path velocity; VSL, straight line velocity; VCL, Curvilinear velocity. Group CT (Botu-Semen®); LC1 group (Botu-Semen® + 5 mmol/l of LC); LC2 group (Botu-Semen® + 10 mmol/l of LC); LC3 group (Botu-Semen® + 15 mmol/l of LC); AC1 group (Botu-Semen® + 5 mmol/l of AC); group AC2 (Botu-Semen® + 10 mmol/l of AC); group AC3 (Botu-Semen® + 15 mmol/l of AC). T0 (15 minutes of incubation), T24 (24 hours of refrigeration) and T48 (48 hours of refrigeration). Different superscripts denotes effect of time in the same group ([A,B,C]) and among groups at the same moment ([a,b]) (p < 0.05).
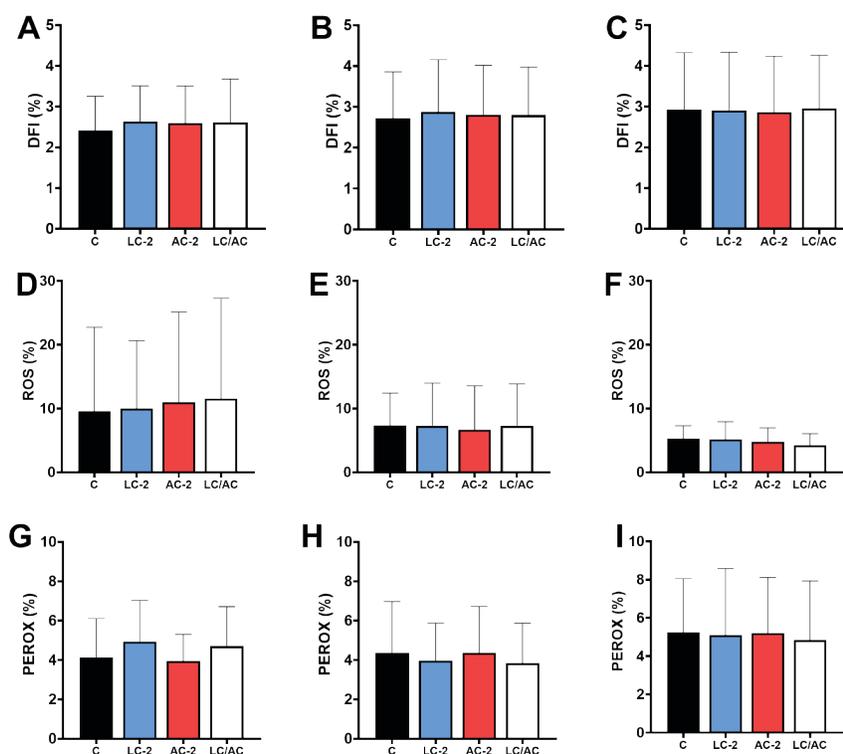

Figure 3. Mean ± SD of sperm with DNA fragmentation index (DFI), production of reactive oxygen species (ROS), and lipid peroxidation (PEROX) of stallion semen (n = 24 ejaculates) in vitro supplemented with L-carnitine (LC) or acetyl-L-carnitine (AC) at moments 0 (A,D,G), 24 (B,E,H), and 48 hours (C,F,I). CT, semen extended in Botu-Semen; LC, Botu-Semen supplemented with 10 mmol/l of L-carnitine; AC, Botu-Semen supplemented with 10 mmol/l of acetyl-L-carnitine; LC/AC, Botu-Semen supplemented with 10 mmol/l of L-carnitine and 10 mmol/l of acetyl-L-carnitine.

Besides improving the energy metabolism of sperm, carnitines have an antioxidant role.[15] Carnitines reduce the availability of free lipids, as carnitines transport fatty acids through the mitochondrial membrane for β-oxidation and the consequent production of ATP that may reduce lipid peroxidation and oxidative stress.[7] Furthermore, this substance increased the activity of antioxidant enzymes (e.g., superoxide desmutase and glutathione peroxidase) that reduced lipid peroxidation.[15] Interesingly, the inclusion of L-carnitine in semen extenders has been suggested to reduce oxidative DNA damage of equine sperm.[4] However, in the present study, the inclusion of either L-carnitine and acetyl-L-carnitine, or their combination, did not change sperm DNA fragmentation, ROS, or PEROX. These results, in part, may be associated with the group of stallions used in this study, as they were not selected based on semen cooling ability or fertility rates, or with the semen storage method. Also, reults might have been different if semen was stored at room temperature with no seminal plasma.[4] However, cooling semen at 5°C reduced sperm metabolism,[26] and ROS generation was enhanced when the metabolism was accelerated[27] also in abnormal sperm.[28] Furthermore, seminal plasma posses antioxidants mechanism that scavenge ROS to prevent cellular damage[15,27] that may have influenced the oxidative stress in the present study.

It is also important to note that the inclusion of acetyl-L-carnitine alone or in combination with L-carnitine resulted in greater sperm motility parameters and PMI than the control group. A correlation between the acetyl-L-carnitine/L-carnitine ratio and progressive motility postcooling has already been reported.[4,6,14] However, an imbalance in the acetyl-L-carnitine/L-carnitine ratio can cause an absence of sperm motility.[29] In fact, the carnitine acetylated form releases acetyl-CoA to the Krebs cycle for energy production; however, when in excess, it blocks the pyruvate dehydrogenase activity, interrupting energy production through glycolysis.[30]

In conclusion, inclusion of carnitines improved sperm parameters of stallion semen extended in a milk-based extender and stored at 5°C for up to 48 hours. The combination of L-carnitine and acetyl-L-carnitine did not prove to be more efficient than including 1 of them alone in equine semen extender. Further studies are needed to evaluate the effect of these substances in semen of subfertile or stallions with poor semen cooling ability.

Funding

Conflict of interest

None to declare.

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