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L-Carnitine and Pyruvate Are Prosurvival Factors During the Storage of Stallion Spermatozoa at Room Temperature[1]

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ABSTRACT

The spermatozoa of many stallions do not tolerate being cooled, restricting the commercial viability of these animals and necessitating the development of a chemically defined room temperature (RT) storage medium. This study examined the impact of two major modulators of oxidative phosphorylation, pyruvate (Pyr) and L-carnitine (L-C), on the storage of stallion spermatozoa at RT. Optimal concentrations of Pyr (10 mM) and L-C (50 mM) were first identified and these concentrations were then used to investigate the effects of these compounds on sperm functionality and oxidative stress at RT. Mitochondrial and cytosolic reactive oxygen species, along with lipid peroxidation, were all significantly suppressed by the addition of L-C (48 h MitoSOX Red negative: 46.2% vs. 26.1%; 48 and 72 h dihydroethidium negative: 61.6% vs. 43.1% and 64.4% vs. 46.9%, respectively; 48 and 72 h 4-hydroxynonenal negative: 37.1% vs. 23.8% and 41.6% vs. 25.7%, respectively), while the Pyr + L-C combination resulted in significantly higher motility compared to the control at 72 h (total motility: 64.2% vs. 39.4%; progressive motility: 34.2% vs. 15.2%). In addition, supplementation with L-C significantly reduced oxidative DNA damage at 72 h (9.0% vs. 15.6%). To investigate the effects of L-C as an osmolyte, comparisons were made between media that were osmotically balanced with NaCl, choline chloride, or L-C. This analysis demonstrated that spermatozoa stored in the L-C balanced medium had significantly higher total motility (55.0% vs. 39.0%), rapid motility (44.0% vs. 25.7%), and ATP levels (70.9 vs. 12.8 ng/ml) following storage compared with the NaCl treatment, while choline chloride did not significantly improve these parameters compared to the control. Finally, mass spectrometry was used to demonstrate that a combination of Pyr and L-C produced significantly higher acetyl-L-carnitine production than any other treatment (6.7 pg/10^6 spermatozoa vs. control at 4.0 pg/10^6 spermatozoa). These findings suggest that Pyr and L-C could form the basis of a novel, effective RT storage medium for equine spermatozoa.

artificial insemination, assisted reproductive technology, ATP, equids (donkeys, horses, zebras), fertility, L-carnitine, oxidative stress, pyruvate, reactive oxygen species, sperm, spermatozoa, sperm motility and transport, stallion

INTRODUCTION

Assisted reproductive technologies have revolutionized horse breeding practices in recent years, with over 90% of Standardbred horses being produced via artificial insemination (AI) in countries such as Australia. By reducing the need to transport horses between farms, AI has radically improved both biosecurity and welfare while reducing the economic costs associated with horse breeding practices. However, with this change, a new and previously unimportant limiting factor has been revealed. Despite the relatively low heritability of reproductive traits [1], a lack of selection for fertility has led to a situation in which spermatozoa from many stallions are of insufficient quality to tolerate the stresses associated with the chilling and cryopreservation of semen [2–4]. Such stallions do not achieve the same level of fertility with chilled semen as with fresh semen, reducing their commercial value. An obvious solution to such a problem would be to store spermatozoa at room temperature (RT) for transportation and insemination, thereby avoiding deleterious temperature-dependent, phase transition changes to the sperm plasma membrane.

The concept of avoiding this damage, frequently coined cold-shock, by storing spermatozoa above 15°C during shipping is not novel [5–10]. While one study found that fertility was not adversely affected by storage at 15°C compared to 5°C in INRA96 (a medium containing a skim milk derivative) over 72 h [9], the majority of studies showed that sperm motility and fertility decreased substantially between 12 h [5, 7] and 24 h [6, 8] of storage, potentially due to the toxic effects of milk components on spermatozoa stored at higher temperatures [11]. It should be noted that all of these studies utilized media derived from chemically undefined dairy products, which may pose biosecurity concerns during semen importation, and will be subject to a degree of biological variability. For this reason, a medium devoid of animal products and toxic components must be developed.

The major advantage of chilling semen is a reduction in sperm metabolic rate that results in improved longevity during transport and storage. This is of particular importance in the case of stallion spermatozoa, which are almost entirely dependent on oxidative phosphorylation (OXPHOS) for ATP production for motility [12]. As a result, if sperm metabolism is not curtailed by temperature reduction, OXPHOS will produce significant quantities of reactive oxygen species [13], which are known to compromise sperm function both in vivo and in vitro [14, 15]. Secondly, depletion of ATP is known to compromise a wide range of ATP-dependent functions in spermatozoa, disrupting homeostasis, and precipitating premature cell death [16]. Therefore, it is clear that in a RT storage medium,

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mitochondrial energy production must be supported while minimizing unnecessary ATP depletion as a result of pressure placed on ATP-dependent pathways such as the regulation of ionic or osmotic flux [17]. Two molecules that would potentially address all of these concerns are pyruvate (Pyr), the primary energy source utilized for OXPHOS, and L-carnitine (L-C), the biologically active free form of carnitine that plays an essential role in mitochondrial ATP synthesis while being a powerful antioxidant and an organic, nonionic osmolyte. A recent study has revealed that stallion spermatozoa contain a number of proteins involved in the major mitochondrial fatty acid metabolism pathway of beta-oxidation and that inhibition of beta-oxidation resulted in reduced sperm motility [18]. Because L-C plays an essential role in beta-oxidation, it was hypothesized that in addition to its role as an antioxidant and osmolyte, L-C may be assisting with mitochondrial ATP production through the transportation of acetyl groups from Pyr into the mitochondrial matrix and the buffering of free coenzyme-A (CoA). The acetylation of carnitine producing acetyl-L-carnitine (ALCAR) by spermatozoa occurs across the outer mitochondrial membrane to facilitate the provision of acetyl groups for β-oxidation and entry into the citric acid cycle for ATP production. The in vivo importance of L-C in sperm quality is well recognized [19–24]. Androgen regulated epithelial cells actively secrete L-C into the epididymal lumen [25, 26] resulting in concentrations of up to 2000-fold higher than that of blood, with spermatozoa containing the highest intracellular concentrations of L-C in the body [22], suggesting that this molecule is of extreme importance in fertility. In addition, oral supplementation of L-C results in increased uptake of Pyr by spermatozoa [27], demonstrating an important interactive role between these compounds in the support of sperm metabolism. The aim of this study was to investigate the potential application of L-C and Pyr on stallion spermatozoa in generating a RT storage medium for equine spermatozoa.

MATERIALS AND METHODS

Materials

Unless otherwise stated, all the chemicals and reagents were purchased from Sigma-Aldrich. A modified Biggers, Whitten, and Whittingham (BWW) medium [28] containing 95 mM NaCl, 4.7 mM KCl, 1.7 mM $CaCl_2.2H_2O$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4 \cdot 7H_2O$, 25 mM $NaHCO_3$, 5.6 mM D-glucose, 275 μM sodium Pyr, 3.7 μl/ml 60% sodium lactate syrup, 50 units/ml penicillin, 50 μg/ml streptomycin, 0.25 mg/ml gentamicin to prevent growth of *Pseudomonas aeruginosa* [29], 20 mM HEPES, and 0.1% (w/v) polyvinyl alcohol, with an osmolarity of approximately 310 mOsm/kg, was utilized as the control medium throughout this study.

Preparation of Spermatozoa

Institutional and New South Wales State Government ethical approval was secured for the use of animal material in this study. This research was based on multiple ejaculates from three normozoospermic Shetland and Miniature crossbred pony stallions (between 6 and 9 yr of age) of proven fertility, held on institutionally approved premises. The stallions had access to native pasture all day and were supplementary fed with grass and lucerne hay once daily. Semen was collected using a pony-sized Missouri artificial vagina (Minitube Australia) with an in-line semen filter. The ejaculate was immediately diluted (extender:-semen [2:1]) with Kenney's extender consisting of 272 mM glucose, 24 mg/ml skim milk powder, 1500 units/ml penicillin, and 1.5 mg/ml streptomycin [30]. This initial dilution was performed to reduce the damage to spermatozoa caused by toxic seminal plasma proteins during transport [31]. Equipment and extender were maintained at temperatures between 30 and 37°C for the duration of semen collection and dilution. The tubes of extended semen were then transported to the laboratory in a polystyrene box at RT (approximately 20°C to 25°C). On arrival at the laboratory (approximately 1 h after collection), 10 ml aliquots of extended semen were centrifuged in 15 ml conical-bottomed tubes at $500 \times g$ for 15 min to concentrate the spermatozoa. Following centrifugation, the supernatant was aspirated and the sperm pellets were resuspended to a concentration of 20×10^6 spermatozoa/ml in BWW or experimental media under aerobic conditions.

Motility Analysis

Sperm motility was objectively determined with computer-assisted sperm analysis (CASA) (IVOS, Hamilton Thorne) using the following settings: negative phase-contrast optics, recording rate of 60 frames/sec, minimum contrast of 70, minimum cell size of 4 pixels, low size gate of 0.17, high size gate of 2.9, low intensity gate of 0.6, high intensity gate of 1.74, nonmotile head size of 10 pixels, nonmotile head intensity of 135, progressive average path velocity (VAP) threshold of 50 μm/sec, slow (static) cells VAP threshold of 20 μm/sec, slow (static) cells straight-line velocity (VSL) threshold of 0 μm/sec, and threshold straightness (STR) of 75%. Cells exhibiting a VAP of ≥50 μm/sec and a STR of ≥75 were considered progressive. Cells with a VAP greater than that of the mean VAP of progressive cells were considered rapid. A minimum of 200 spermatozoa in a minimum of five fields were assessed using 20 μm Leja standard count slides (Gytech) and a stage temperature of 37°C.

Flow Cytometry

All the flow cytometry was performed using a FACSCalibur flow cytometer (Becton Dickinson) with a 488 nm argon-ion laser. Emission measurements were made using 530/30 band pass (green/FL-1), 585/42 band pass (red/FL-2), 661/16 band pass (red/FL-4), and >670 long pass (far red/FL-3) filters. Debris was gated out using a forward scatter/side scatter dot plot, and a minimum of 5000 cells were analyzed per sample. All the data was analyzed using CellQuest Pro software (Becton Dickinson).

Acrosome Integrity

The acrosome integrity assay was performed as previously described [32] with some modifications. Briefly, sperm samples were incubated at 37°C for 20 min with reconstituted LIVE/DEAD far red fixable stain (Molecular Probes) as per the manufacturer's instructions at a concentration of 1 μl/ml. Following staining, spermatozoa were washed in BWW, fixed in 2% paraformaldehyde for 10 min at 4°C, washed in PBS, and stored for up to 1 wk at 4°C in 0.1 M glycine in PBS. Following storage, cells were permeabilized in a solution of PBS containing 0.1% Triton X-100 and 3.4 mM sodium citrate for 5 min at 4°C, after which they were pelleted via centrifugation and the pellet resuspended in a PBS solution containing 0.8 μg/ml fluorescein isothiocyanate-peanut agglutinin. Samples were incubated for 30 min at 37°C, after which they were washed and resuspended in PBS for flow cytometric analysis. Spermatozoa were classified as being either live and acrosome intact (green fluorescence only), live and acrosome damaged (no fluorescence), dead and acrosome intact (red and green fluorescence), or dead and acrosome damaged (red fluorescence only).

Mitochondrial and Cellular Superoxide Production

Mitochondrial and cellular superoxide production were measured by incubating spermatozoa with 2 μM MitoSOX Red (MSR) (Molecular Probes) or dihydroethidium (DHE) (Molecular Probes) and 5 nM Sytox Green vitality stain (Molecular Probes) for 15 min at 37°C. Samples were assessed via flow cytometry and classified as either MSR or DHE positive (live or dead) or MSR or DHE negative (live or dead). All dead cells stain positive for MSR and DHE due to the contamination of commercial preparations of these dyes with traces of ethidium bromide that can directly stain the nuclei of cells nonviable cells lacking membrane integrity. As a consequence, only live cell data were used for statistical analyses. A positive control treatment of 100 μM arachidonic acid (added during staining) [33] was utilized for gating purposes.

Oxidative DNA Damage

Oxidative guanine adducts—8-hydroxy-2′-deoxyguanosine (8OHdG)—were measured using the OxyDNA assay kit (Calbiochem) in conjunction with LIVE/DEAD far red fixable vitality stain as previously described [34] with some modifications. Briefly, spermatozoa were stained with LIVE/DEAD as described above for acrosome integrity assessment, washed with BWW, and the chromatin relaxed to facilitate probe access by incubation with 2 mM dithiothreitol for 30 min at RT. Following chromatin relaxation, spermatozoa were washed in BWW, fixed in 2% paraformaldehyde, washed in PBS, and stored in 0.1 M glycine for up to 2 wk. On the day of assessment, cells were permeabilized as described above for the acrosome integrity assay, after which they were pelleted via centrifugation and resuspended in a 1:50 dilution of the fluorescein isothiocyanate-conjugate solution in PBS. The cells were incubated for 1 h at 37°C, after which they were pelleted via centrifugation, resuspended in PBS and analyzed via flow cytometry. Spermatozoa were classified according to their vitality and 8OHdG positivity.

Lipid Peroxidation

Lipid membrane peroxidation was determined by the presence of 4-hydroxynonenal (4HNE) adducts using an anti-4HNE antibody (Jogmar Diagnostics). Approximately 2×10^6 cells were pelleted via centrifugation, resuspended in a solution containing a 1:50 dilution of antibody and 1:1000 dilution of LIVE/DEAD far red stain in BWW, and incubated for 30 min at 37°C. Following incubation, cells were centrifuged and sperm pellet resuspended in a 1:100 dilution of labeled secondary antibody, that is, Alexa Fluor 488 goat anti-rabbit immunoglobulin G (Molecular Probes), in BWW and incubated for 10 min at 37°C. Spermatozoa were then washed twice in BWW to remove any unbound antibody and resuspended in BWW for flow cytometric analysis. A technical control of secondary antibody only was used to set the gate between low (background fluorescence) and high levels of lipid peroxidation.

Sperm Chromatin Structure Assay

The sperm chromatin structure assay (SCSA) was performed as previously described by Evenson and Jost [35]. Briefly, aliquots of spermatozoa were further diluted to a concentration of 10×10^6 cells/ml, snap frozen in liquid nitrogen, and stored at −80°C until assessment. Immediately prior to assessment, samples were thawed at 37°C and stored on ice after which 200 μl of acid detergent solution (0.08 N HCl, 0.15 M NaCl, 0.1% Triton X-100, pH 1.2) was added to 100 μl of sperm suspension and exactly 30 sec later 600 μl of acridine orange staining solution (0.1 M citric acid, 0.2 M Na_2PO_4, 1 mM ethylenediaminetetraacetic acid, 0.15 M NaCl, 22.6 μM acridine orange, pH 6.0) was added. Samples were run on a FACScan flow cytometer (BD) with a standard argon laser (488 nm) using CellQuest software (BD) for 3 min prior to acquiring data. Debris was gated out using a forward scatter/side scatter dot plot with a region drawn around sperm cells. Green fluorescence was detected in FL-1, and red fluorescence was detected in FL-3. The percentage of cells outside the main population (detectable differential fluorescence index [%DFI]), the ratio of red fluorescence to total fluorescence (DFI = ratio of single stranded or denatured DNA to total DNA following the acid challenge, whereby poorly compacted chromatin will succumb to acidic denaturation), and the percentage of cells with high green fluorescence (considered to be poorly protaminated) were calculated from the output of CellQuest software as previously described [35].

ATP Concentration

ATP levels were measured using an ATP bioluminescence assay kit (Sigma Aldrich) following the manufacturer's instructions. Briefly, 200 μl aliquots of spermatozoa were snap frozen in liquid nitrogen following treatment and stored at −80°C until analysis. On the day of analysis, samples were thawed on ice and centrifuged at $20\,000 \times g$ for 15 min at 4°C. The supernatant was retained and was utilized for the assay. The ATP standard solution supplied with the kit was serially diluted to obtain concentrations of 10^{-6} g/ml to 10^{-9} g/ml. The luciferin-luciferase reagent (100 μl) was then run for 5 min at 37°C in a Berthold AutoLumat luminometer LB-953 (Berthold) to stabilize the chemiluminescent system. Samples of standards (100 μl) were then added and the resulting chemiluminescence was monitored for further 5 min; the results were expressed as integrated counts. For this assay, media blanks were also run for every treatment in order to ensure that the signals recorded were not due to the spontaneous activation of the probe.

Mass Spectrometry

For quantitative liquid chromatography-tandem mass spectrometry (LC/MS/MS) analysis, samples were diluted 1:100 in mobile phase A (20 mM ammonium acetate, pH 4.5) and 1 μl was loaded onto an polar reversed-phase column (4 μm Polar-RP, 150 × 4.6 mm; Phenomenex Synergy) at 0.9 ml/min. High-performance liquid chromatography separation was carried out on a Shimadzu Nexera UPLC system using isocratic elution for 6 min at 90% mobile phase A/10% mobile phase B (5% ammonium acetate, 95% acetonitrile). Following separation, the column was washed by running a gradient up to 99% mobile phase B over 3 min after which the column was reequilibrated for 3 min at initial conditions. The LC system was directly coupled to an AB Sciex (Chromos) 6500 QTRAP equipped with a Turbo V Ions source scanning in multiple reaction monitoring-information-dependent acquisition-enhanced product ion (MRM-IDA-EPI) mode. The electrospray ionization source settings were optimized using an ALCAR standard: positive polarity, curtain gas 30 pounds/inch^2 (psi), ions source gas 1 set at 40 psi, ions source gas 2 set at 50 psi at a temperature of 400°C, declustering potential of 90 V, entrance potential of 10 V, and a collision cell exit potential of 12 V. MRM transitions were again optimized from the commercial ALCAR standard, and two transitions were selected for monitoring, 204 → 85 and 204 → 145, with collision energies set at 30 and 40 eV, respectively. MRM transition which exceeded counts of 10,000 automatically triggered an enhanced product ion full linear ion trap MS/MS scan for confirmation of the transitions identity. Standard curves were prepared in triplicate and 1–1000 pg of ALCAR was loaded on the column and analyzed under the same conditions as the experimental samples. MS acquisition files for the standard, 100 pg quality controls, and samples were loaded into AB Sciex MultiQuant 3.0 software. Briefly, smoothed and baseline-subtracted extracted ion chromatograms were created for the targeted MRM transitions, the integrated area under this peak was submitted for quantitation, and the results were exported to Microsoft Excel (version 14.0.7140.5002) for further analysis and reported as pg ALCAR/10^6 spermatozoa.

Statistical Analyses

All the data used in this study were found to be normally distributed prior to further analyses. Data for all the experiments were analyzed by one-way ANOVA (using stallion as a blocking term) with JMP version 11.0 software (SAS Institute Inc.). Where significant treatment effects were identified by ANOVA ($\alpha = 0.05$), means comparisons were performed. Differences between the parameters of spermatozoa stored in control (BWW or NaCl-BWW) and various treatment media in all the experiments were identified using Dunnett method for mean comparison ($\alpha = 0.05$).

Experimental Design

Pyruvate and L-C dose-response. To ascertain the optimal concentrations of Pyr and L-C to use in the following prosurvival experiments, separate dose responses were conducted at 37°C for 24 h. Spermatozoa (n = 3 ejaculates) were extended to a concentration of 20×10^6 spermatozoa/ml in BWW medium supplemented with sodium Pyr at final concentrations of 275 μM (BWW control) and 1.25, 2.5, 5, 10, and 20 mM or L-C inner salt at 0 (BWW control), 1, 12.5, 25, 50, and 100 mM, with motility assessment at 24 h. It should be noted that due to the osmotic pressure exerted by the various concentrations of Pyr and L-C, the amount of NaCl in the base BWW medium was reduced to provide final osmolarities of 310 mOsm/kg for all treatments.

Prosurvival effects of L-C and Pyr in RT storage medium. To investigate the prosurvival effects of Pyr and L-C at their optimal doses (both separately and in combination) at RT, three ejaculates from each of the three pony stallions (n = 9) were processed as described above and resuspended to a final sperm concentration of 20×10^6/ml in either standard BWW (control), BWW supplemented with 10 mM (final concentration) Pyr, BWW supplemented with 50 mM L-C or BWW supplemented with both 10 mM Pyr and 50 mM L-C in 5 ml flat-bottomed specimen jars (Sarstedt). The concentration of NaCl was varied between treatments to achieve a final osmolarity of approximately 310 mOsm/kg. Once resuspended, samples were stored at RT in the dark for 72 h; during these experiments, the RT was recorded using a temperature logger every 5 min over 100 h with an average of 23.28°C ± 0.01°C. Every 24 h, each sample was measured for motility (CASA), acrosome integrity, superoxide production (MSR and DHE), and lipid peroxidation (4HNE). Oxidative DNA damage and SCSA measurements were performed at 72 h of storage.

Effect of L-C as an osmolyte. In order to ascertain whether the prosurvival effects of L-C could be attributed to a reduced rate of ATP depletion following the removal of a proportion of NaCl from the medium, three media were compared: standard BWW (containing 95 mM NaCl) and two modified BWW media in which the NaCl component was removed and the osmolarity balanced to 310 mOsm/kg by addition of either choline chloride (95 mM) or L-C (170 mM). Stallion spermatozoa (n = 3 ejaculates) were prepared as described above and incubated at RT in the dark for 72 h, after which motility (CASA) and ATP levels (luminometry) were measured.

Metabolic effects of Pyr and L-C. The measurement of ALCAR was utilized to indicate the direct involvement of Pyr and L-C in ATP synthesis. Stallion spermatozoa (n = 3 ejaculates) were collected and prepared as described above, after which they were extended to a final concentration of 20×10^6/ml in either standard BWW (control) or BWW supplemented with 2.5, 5, or 10 mM Pyr, 12.5, 25 or 50 mM L-C, or both 10 mM Pyr and 50 mM L-C (as used for the combined treatment during the prosurvival study). Spermatozoa were incubated at RT in the dark for 72 h after which 1 ml aliquots were centrifuged at $500 \times g$ for 5 min, the supernatant removed, the pellet resuspended in 250 μl of ice cold Milli-Q water, snap frozen in liquid nitrogen, thawed in an ice bath, and sonicated using a Bandelin Sonopuls (Bandelin

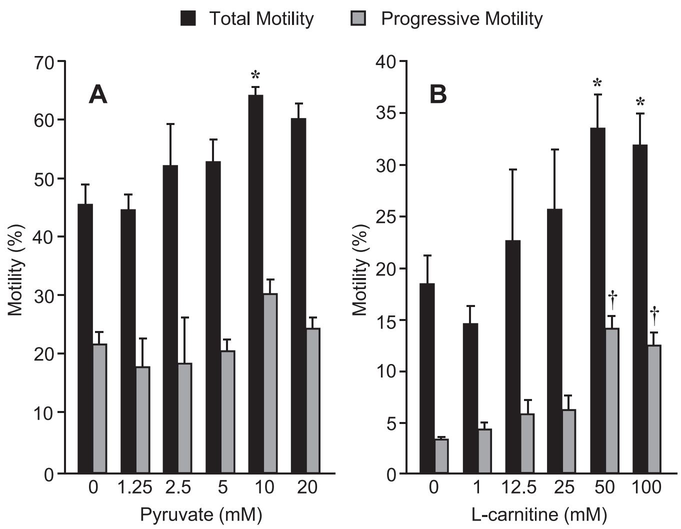

FIG. 1. Pyruvate (**A**) and L-C (**B**) dose response. Total and progressive motilities of stallion spermatozoa (n = 3) incubated for 24 h at 37°C in the presence of either Pyr at 0, 1.25, 2.5, 5, 10, and 20 mM or L-C at 0, 1, 12.5, 25, 50, and 100 mM. Significant differences ($P \leq 0.05$) between the control (0 mM) and Pyr or L-C dosages are denoted by * or † for total and progressive motilities, respectively.

Electric) sonicator with a MS73 microtip at an amplitude of 37% to ensure complete liberation of intracellular ALCAR. The sonication protocol involved 5 sec pulses with 5 sec rest between pulses for 30 sec, the tip was then iced for 20 sec to prevent overheating and the regime was repeated. Samples were kept in an ice bath for the duration of the sonication protocol. Following sonication, the samples were centrifuged at 10 000 × *g* for 10 min (4°C), the pellet was discarded and the supernatant was retained and stored at −80°C for up to 3 mo until analysis.

RESULTS

Pyruvate and L-C Dose Response

There were significant effects of both Pyr and L-C concentrations on total motility ($P \leq 0.05$ and 0.01, respectively; Fig. 1). After 24 h at 37°C, the total motility of spermatozoa incubated with 10 mM Pyr (63.7% ± 1.7%) was significantly higher than that of the control (45.7% ± 3.5%, $P \leq 0.05$; Fig. 1A). L-Carnitine supplementation at 50 and 100 mM resulted in significantly higher total motilities (33.0% ± 1.7%, $P \leq 0.01$ and 31.3% ± 2.0%, $P \leq 0.05$, respectively) than the control (18.3% ± 1.2%; Fig. 1B). While there was no effect of Pyr concentration on progressive motility, supplementation with L-C significantly improved progressive motility ($P \leq 0.0001$). Incubation of spermatozoa with 50 and 100 mM L-C resulted in progressive motilities of 13.7% ± 0.6% and 12.3% ± 0.3% compared to 3.3% ± 1.0% for the control spermatozoa (Fig. 1B). On the basis of these results, the doses of 10 mM Pyr and 50 mM L-C were selected for use in the following experiments.

Prosurvival Effects of Pyr and L-C in a RT Storage Medium

Although there was no effect of treatment on total motility after 24 h (Fig. 2A), there was a significant treatment effect on progressive motility ($P \leq 0.05$), with spermatozoa stored in the Pyr + L-C treatment (26.1% ± 1.7%) having significantly higher progressive motility than the control (18.9% ± 1.4%, $P \leq 0.05$; Fig. 2B). There were no effects of treatment on total motility, percent rapid, or any other kinematic parameter at 24 h. After 48 h, there was a significant treatment effect on total ($P \leq 0.05$), and progressive ($P \leq 0.05$) motilities, with the total motility of the L-C (63.6% ± 2.7%) treatment being significantly higher than that of the control (48.8% ± 4.0%, $P \leq 0.05$; Fig. 2A), and the progressive motility of the Pyr + L-C treatment (28.7% ± 2.3%) was significantly higher than the control (18.3% ± 2.6%, $P \leq 0.05$; Fig. 2B). There were no effects of treatment on percent rapid or any other kinematic parameters at 48 h. After 72 h, significant treatment effects on total ($P \leq 0.01$) and progressive ($P \leq 0.0001$) motilities were observed, with the total and progressive motilities of L-C and Pyr + L-C treatments being significantly higher than the control (total motility: 60.4% ± 3.3% and 64.2% ± 2.9% vs. 39.4% ± 5.1%, respectively, $P \leq 0.01$; progressive motility: 26.3% ± 1.8% and 34.2% ± 2.3% vs. 15.2% ± 2.4%, respectively, $P \leq 0.05$; Fig. 2, A and B). Similarly, significant treatment effects on percent rapid ($P \leq 0.01$), VSL ($P \leq 0.01$), and STR ($P \leq 0.01$) were observed at 72 h, with the percent rapid and VSL of L-C and Pyr + L-C treated spermatozoa being significantly higher than the control (percent rapid: 50.3% ± 3.7% and 53.4% ± 3.0% vs. 29.9% ± 4.7%, respectively, both $P \leq 0.01$; VSL: 74.2 ± 3.2 μm/sec and 81 ± 1.8 μm/sec vs. 60.4 ± 4.1 μm/sec, $P \leq 0.05$ and $P \leq 0.001$, respectively), and the STR of spermatozoa stored in the Pyr + L-C treatment was significantly higher than the control (75.4 ± 1.5 vs. 65.9 ± 2.0, respectively, $P \leq 0.01$).

There were no significant differences between the percentages of viable, acrosome intact cells in any treatment at any time point (24 h: 52.4% ± 2.7%, 53.0% ± 2.8%, 53.8% ± 2.7%, and 53.5% ± 3.3%; 48 h: 39.6% ± 3.9%, 39.3% ± 4.4%, 45.2% ± 3.3%, and 43.7% ± 3.5%; 72 h: 38.6% ± 4.5%, 39.3% ± 5.0%, 45.8% ± 3.8%, and 44.6% ± 4.4% for the control, Pyr, L-C and Pyr + L-C treatments, respectively). However, there was a significant treatment effect on mitochondrial superoxide production at 48 h, with the L-C treatment having a higher percentage of MSR negative cells than the control (46.2% ± 5.0% vs. 26.1% ± 4.5%, $P \leq 0.05$). In addition, there were significant treatment effects on cellular superoxide production at 24 h ($P \leq 0.01$), 48 h ($P \leq 0.01$), and 72 h ($P \leq 0.05$; Fig. 2C). After 24 h, there were significantly more DHE negative cells in the L-C treatment compared to the control (68.4% ± 1.6% vs. 58.8% ± 1.5%, $P \leq 0.01$), and after 48 h, there were significantly more DHE negative cells in the L-C and Pyr + L-C treatments compared to the control (61.6% ± 2.2% and 58.5% ± 2.8% vs. 43.1% ± 3.8%, $P \leq 0.01$ and 0.05, respectively). By 72 h, only the L-C treated spermatozoa had more DHE negative cells than the control (64.4% ± 3.2% vs. 46.9% ± 5.2%, $P \leq 0.05$). In addition, there was a significant treatment effect on total oxidative DNA damage ($P \leq 0.01$), which was significantly higher in the control than the L-C treatment after 72 h of storage (8OHdG-positive cells: 15.6% ± 1.4% vs. 9.0% ± 1.0%, $P \leq 0.01$; Fig. 2D).

While there was an effect of treatment on the proportion of live spermatozoa with minimal peroxidation (live, 4HNE negative) after 24 h, treatment effects were observed after 48 h ($P \leq 0.05$) and 72 h of storage ($P \leq 0.01$; Fig. 3A). After 48 h, the L-C treatment contained significantly more 4HNE negative, live cells than the control (37.1% ± 4.7% vs. 23.8% ± 2.0%, $P \leq 0.05$), and by 72 h the percentage of 4HNE negative cells was significantly higher in both the L-C and Pyr + L-C treatments compared to the control (41.6% ± 3.3% and 39.2% ± 4.5% vs. 25.7% ± 2.6%, respectively, $P \leq 0.05$ for both). L-Carnitine supplementation alone also significantly reduced the percentage of spermatozoa with high levels of lipid peroxidation by 48 h compared to the control (61.3% ± 4.8% vs. 75.1% ± 2.2%, $P \leq 0.05$). These results are graphically displayed in the representative histogram shown in Figure 3B.

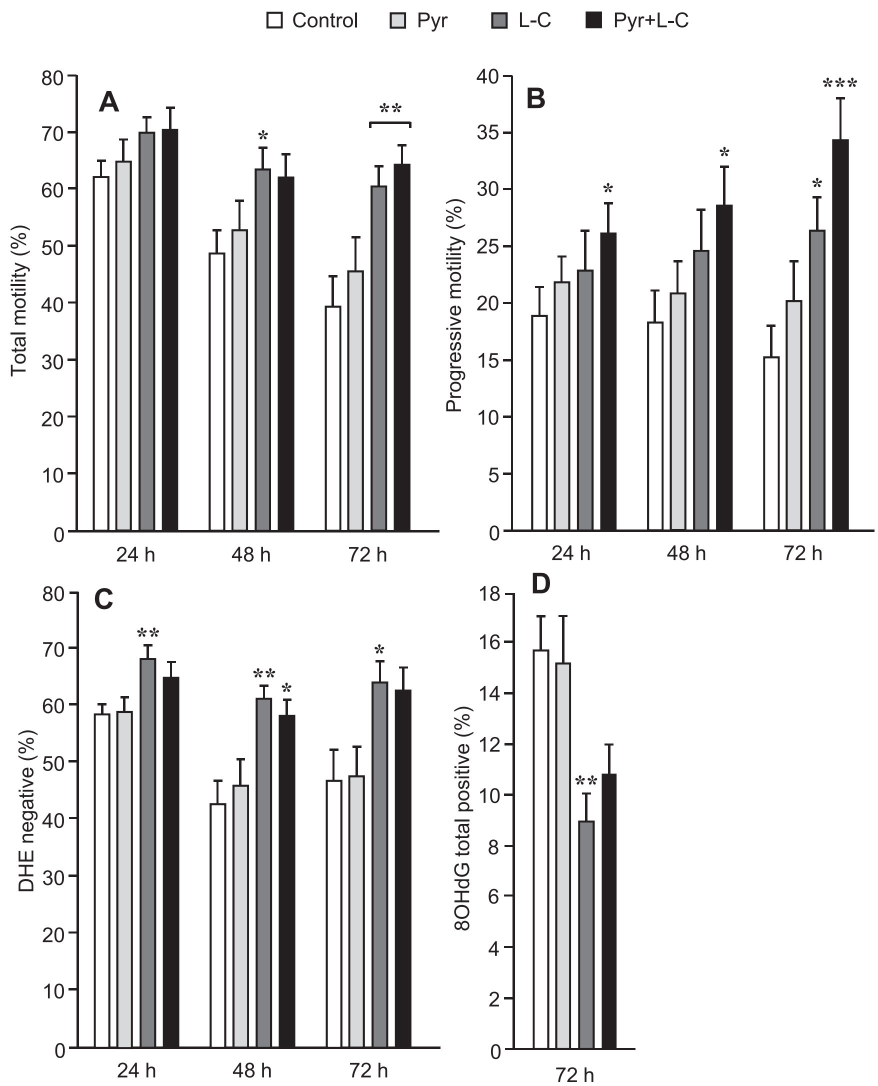

FIG. 2. Total motility (**A**), progressive motility (**B**), DHE negative cells (**C**) and total 8OHdG (**D**) of stallion spermatozoa (n = 9) following storage at RT in control (BWW), Pyr (BWW supplemented with 10 mM Pyr), L-C (BWW supplemented with 50 mM L-C), or Pyr + L-C (BWW supplemented with both 10 mM Pyr and 50 mM L-C) media over a 72 h period. Significant differences between the control (BWW) and treatments denoted by *$P \leq 0.05$, **$P \leq 0.01$, or ***$P \leq 0.001$.

There was no effect of treatments on DFI (chromatin stability; SCSA) after 72 h (23.8% ± 0.4%, 23.3% ± 0.4%, 23.6% ± 0.4%, and 23.4% ± 0.4% for control, Pyr, L-C, and Pyr + L-C, respectively).

Effect of L-C as an Osmolyte

Significant osmolyte treatment effects were observed for total and percent rapid motility as well as ATP concentration (all $P \leq 0.05$; Fig. 4). The total and percent rapid motility of spermatozoa stored in LC-BWW was significantly higher (55.0% ± 1.3% and 44.0% ± 2.9%; both $P \leq 0.05$) than the control (NaCl-BWW: 39.0% ± 2.6% and 25.7% ± 1.1% for total and rapid motility, respectively), though neither the total or rapid motilities of spermatozoa in the Choline Cl-BWW treatment were significantly higher than the control (45.3% ± 1.9% and 33.7% ± 2.1% for total and rapid motility, respectively). The intracellular ATP levels of spermatozoa stored in LC-BWW were also significantly higher than that of spermatozoa stored in the NaCl-BWW control medium (70.9 ± 10.5 vs. 12.8 ± 8.6 ng/ml, respectively, $P \leq 0.05$), while storage in Choline Cl-BWW did not significantly increase ATP concentrations compared to the control (30.3 ± 1.9 ng/ml).

Metabolic Effects of Pyr and L-C

A significant effect of treatment on intracellular ALCAR levels was observed ($P \leq 0.01$, Fig. 5). While supplementation with Pyr or L-C alone did not significantly increase ALCAR formation (4.1 ± 0.4, 4.2 ± 0.1, and 4.5 ± 0.2 pg/10^6 spermatozoa for 2.5, 5, and 10 mM Pyr; and 4.8 ± 0.2, 5.0 ± 0.2, and 5.2 ± 0.3 pg/10^6 spermatozoa for 12.5, 25, and 50 mM L-C alone compared to 4.0 ± 0.4 pg/10^6 spermatozoa for the control), supplementation with a combination of 10 mM Pyr and 50 mM L-C resulted in significantly higher ALCAR formation (6.7 ± 0.7 pg/10^6 spermatozoa) than the control ($P \leq 0.001$, Fig. 5).

DISCUSSION

By supplementing stallion spermatozoa with both Pyr and L-C, sperm cells stored at RT over a 72 h period maintained acceptable motility for use in an AI regime [36]. Under these

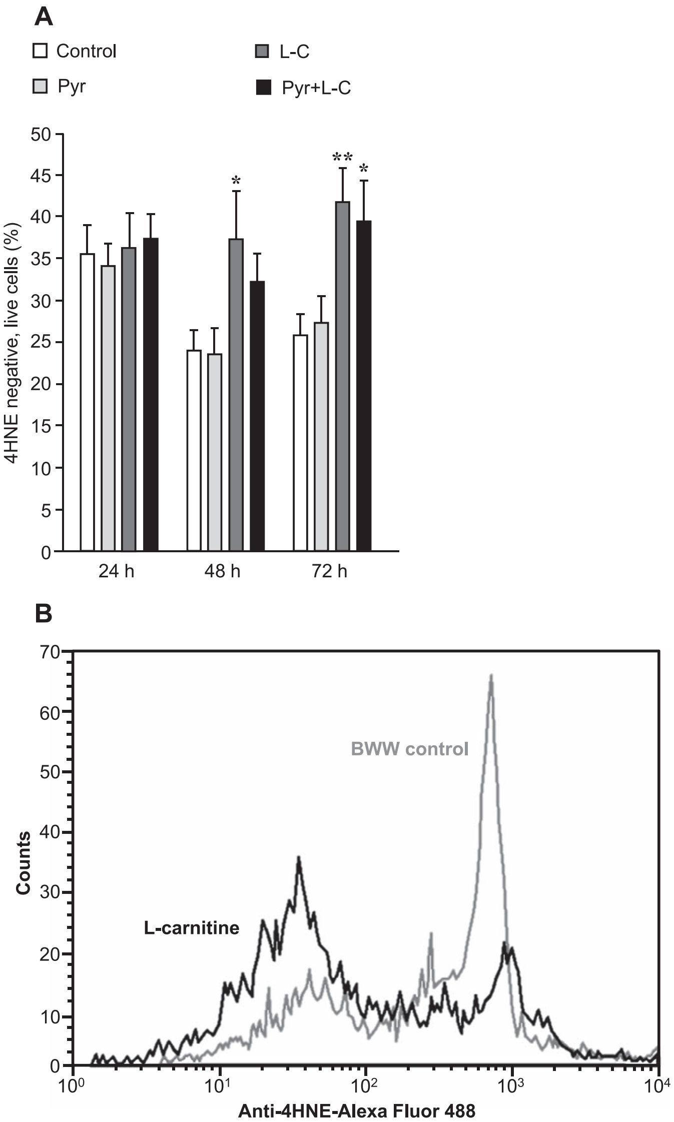

FIG. 3. Lipid peroxidation of stallion spermatozoa (n = 9) measured by flow cytometry using a 4-hydroxynonenal (4HNE) primary antibody and an Alexa Fluor 488 secondary antibody. **A**) 4HNE negative, live spermatozoa following storage at RT in control (BWW), Pyr (BWW supplemented with 10 mM Pyr), L-C (BWW supplemented with 50 mM L-C), or Pyr + L-C (BWW supplemented with both 10 mM Pyr and 50 mM L-C) media over a 72 h period. **B**) Representative histogram showing total lipid peroxidation of spermatozoa stored at RT in the control medium (BWW) and medium supplemented with 50 mM L-C after 72 h. Significant differences between the control (BWW) and treatments denoted by $*P \leq 0.05$ or $**P \leq 0.01$.

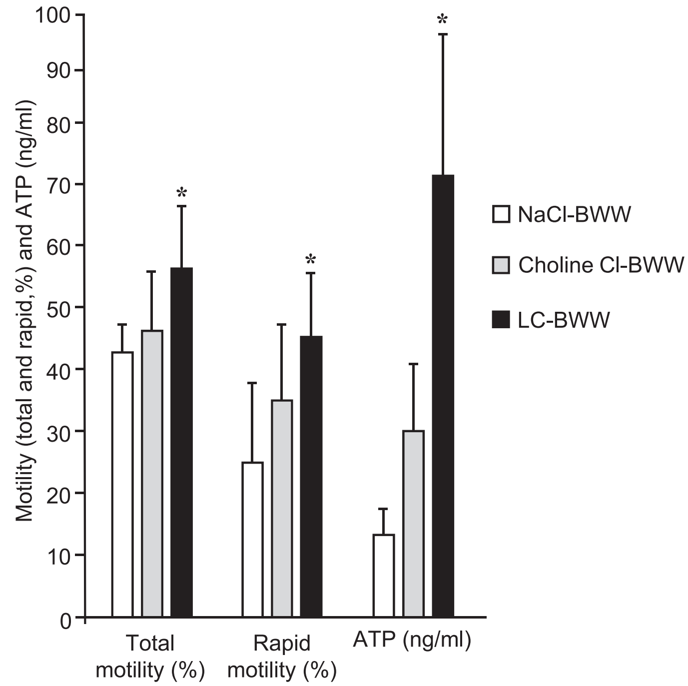

FIG. 4. Total motility, rapid motility, and ATP concentration (ng/ml) of stallion spermatozoa (n = 3) incubated at RT over 72 h in BWW media osmotically balanced to 310 mOsm/kg using either NaCl (NaCl-BWW), choline chloride (Choline Cl-BWW), or L-C (LC-BWW). Significant differences between the control (NaCl-BWW) and treatments denoted by $*P \leq 0.05$.

conditions, the progressive motility of these cells approached 35% (Fig. 2B), a figure that is well above the minimum preinsemination value of 25%–30% that is recommended by industry [37]. This development will facilitate the transportation of semen from stallions that have previously been identified as poor chillers, and as such the commercial viability of these stallions will be considerably enhanced. Supplementation with L-C resulted in significant improvements to motility parameters (Figs. 1, A and B, and 2, A and B), and a reduction in oxidative stress parameters (Figs. 2C and 3, A and B) and oxidative DNA damage (Fig. 2D). The addition of Pyr further enhanced the stimulation of motility without improving the suppression of oxidative stress, suggesting a metabolic role.

L-C is essential for normal in vivo sperm production and function. Low concentrations of L-C have been found in the semen of azoospermic, oligoasthenozoospermic, and infertile men [19, 20, 23, 37], and positive correlations between L-C, ALCAR, and sperm concentration as well as between ALCAR and total numbers of motile, morphologically normal stallion spermatozoa have been reported [24]. In addition, positive correlations between levels of free L-C in seminal plasma and both the concentration and motility of human spermatozoa have been described [38]. The role of L-C in the epididymis is not for the initiation of motility per se, but rather the stimulation of movement in previously motile spermatozoa that have been depleted of ATP [39], suggesting a role for L-C in energy production. While the mechanisms underpinning the beneficial effects of L-C are complex and difficult to isolate, they appear to be due to the combined roles of L-C as an antioxidant (Figs. 2 and 3), an osmolyte (Fig. 4), and in mitochondrial energy production (Fig. 5), while the role of Pyr appears to be the provision of acyl groups for the citric acid cycle (Fig. 5).

The antioxidant and antiradical properties of L-C are well reported and include the direct scavenging of free radicals, destruction of hydrogen peroxide, metal chelation, and reducing activity [40], along with inhibition of xanthine oxidase activity [41]. Of the antioxidant properties of carnitines, the suppression of lipid peroxidation is most widely reported and is routinely exploited in clinical settings to reduce the severity of damage caused by ischemia-reperfusion-induced lipid peroxidation following organ surgery [42, 43]. The results of this study agree with previous reports of beneficial effects of L-C on spermatozoa [44–46] and other cell types [41, 42, 47, 48]. Supplementation with L-C improved all the oxidative stress-related parameters looked at in this study, with

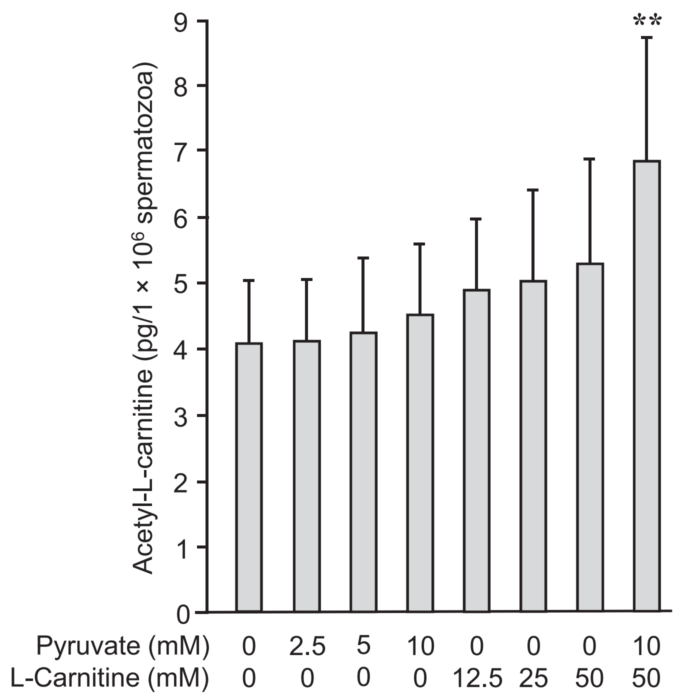

FIG. 5. Acetyl-L-carnitine (ALCAR) concentrations (pg/10^6 spermatozoa) of stallion spermatozoa (n = 3) following storage at RT for 72 h BWW medium either without supplementation (control), in the presence of Pyr at 2.5, 5, or 10 mM, in the presence of L-C at 12.5, 25 or 50 mM, or a combination of 10 mM Pyr and 50 mM L-C. Significant differences between the control and Pyr and L-C dosages denoted by $^{**}P \leq 0.01$.

significant reductions in lipid peroxidation (Fig. 3, A and B) and oxidative DNA damage (Fig. 2D) as well as increased numbers of cells without detectable mitochondrial or cellular free radical production (Fig. 2C).

While the present study reports a reduction in oxidative DNA damage (8OHdG adduct formation; Fig. 2D) with L-C supplementation, other reports have revealed no such effect [45]. It should be noted, however, that the present study utilized considerably higher concentrations of L-C along with a DNA decondensation (dithiothreitol treatment) step to allow the 8OHdG probe to have access to the highly condensed DNA and reveal the effect of treatment on this variable.

While there are numerous reports of beneficial effects of oral L-C administration on sperm parameters of horses [27] and other species [44, 46, 49–56], there are notably few publications investigating the effect of in vitro supplementation of L-C on stallion spermatozoa [57, 58]. L-C is powerful osmolyte, and therefore its addition to media in this study necessitated the partial removal of NaCl to maintain isotonicity. Because NaCl accelerates the depletion of ATP through the activation of Na-ATPase pumps [17], it was hypothesized that the beneficial effects of L-C may be partially or wholly due to the removal of NaCl for these incubations. While other studies have revealed beneficial effects of carnitines at concentrations akin to those used in the present study, the carnitines were added to a commercially prepared media [57] that is already osmotically balanced, thereby raising the osmolarity above the physiological range upon carnitine addition and inducing osmotic stress, which stimulates the production of reactive oxygen species [59]. This high osmolarity may have been the source of disappointing results and have led to a loss of confidence in the potential of this molecule. During the present study, the amount of NaCl was reduced with increasing L-C concentrations to maintain the isotonicity of the experimental media. The ATP-dependent Na^+/K^+ pump can consume up to 20% of the ATP produced by a cell in an attempt to maintain homeostasis [17]. By reducing the amount of Na^+ in solution, the energetic demands of the cell are reduced, the rate of ATP depletion is slowed, and cell vitality is maintained for a longer period. To isolate the effects of reducing NaCl concentrations from that of L-C supplementation, NaCl was substituted with either choline chloride or L-C and motility and ATP levels were measured following a 72 h incubation period. Though not statistically significant, a mildly beneficial effect of substituting NaCl with choline chloride was observed (Fig. 4), suggesting that a proportion of the prosurvival effects observed in the earlier experiment may be attributed to the reduction of NaCl in the storage medium. However, the magnitude of this effect was neither equal to that of L-C, nor sufficient to explain the improvements in longevity observed during the prosurvival study. The additional benefit observed when L-C was utilized to balance osmolarity (compared to choline chloride) suggests that it not only reduced ATP depletion through alleviating the pressure on the ATP-dependent Na^+/K^+ pumps, but that L-C may in fact be assisting with the production of ATP.

In addition to its antioxidant and osmoprotective effects, L-C plays an essential role in mitochondrial ATP production by facilitating the transport of activated fatty acids into the mitochondria for β-oxidation [60] and through the buffering of intracellular free CoA. The free L-C sequesters excess acetyl-CoA within the mitochondria and stores it as ALCAR, keeping the effective intracellular pool of acetyl-CoA:free CoA ratio low, an important role given that a high acetyl-CoA:free CoA ratio inhibits pyruvate dehydrogenase, preventing further ATP production via the citric acid cycle [22]. This exchange system is active in spermatozoa [61] and is integral for ATP production and the maintenance of motility, with failure of the system (which has been observed by high intracellular ALCAR:free L-C ratios) resulting in immotility [21, 62]. To investigate whether this action contributed to the prosurvival effects of L-C observed during this study, intracellular ALCAR levels were measured in spermatozoa supplemented with increasing concentrations of Pyr and L-C over a 72 h period. While Pyr addition resulted in a small but nonsignificant increase in ALCAR levels, supplementation with L-C resulted in a dose-dependent increase in intracellular ALCAR, with Pyr and L-C in combination producing significantly higher levels of ALCAR than all the other treatments (Fig. 5). The lack of an increase in ALCAR levels in the presence of Pyr alone, the dose-dependent increase in ALCAR in the presence of L-C, and the additive effect of Pyr and L-C demonstrate that the latter is the rate-limiting molecule for the production of ATP and that the presence of Pyr supports this system through the provision of acetyl groups to enter the citric acid cycle.

In conclusion, supplementation of stallion sperm storage medium with both Pyr and L-C facilitates the RT storage of spermatozoa over a 72 h period by supporting mitochondrial ATP production while minimizing both ATP depletion and the damaging effects of metabolic by-products such as free radicals. With the recent development of a cost-effective RT shipping method [10], this technical development removes the need to subject spermatozoa to low temperatures in order to promote their long-term storage and in so doing, avoids the deleterious temperature-dependent phase-transition changes to the sperm lipid membrane that reduce the fertility and thus the commercial value of many stallions.

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