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

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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 solution to such a problem would be to store spermatozoa above 15°C during 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 toxic components such as reactive oxygen species at a rate that could be detrimental to sperm function both in vivo and in vitro [14, 15]. Secondly, depletion of ATP is known to compromise a wide range of ATP-dependent functionalities in spermatozoa, disrupting homeostasis, and precipitating premature cell death [16]. Therefore, it is clear that in a RT storage medium,
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 beta-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 Whittington (BWW) medium [28] containing 95 mM NaCl, 4.7 mM KCl, 1.7 mM CaCl2·2H2O, 1.2 mM KH2PO4, 1.2 mM MgSO4, 7H2O, 25 mM NaHCO3, 5.6 mM D-glucose, 275 mM sodium Pyr, 3.7 μl/ml 60% sodium lactate syrup, 50 units/ml penicillin, 0.25 mg/ml streptomycin, 0.25 mg/ml gentamicin to prevent growth of Pseudomonas aeruginosa (P.a.), or dihydroethidium (DHE) (Molecular Probes) and 5 nM Sytox Green vitality stain (Molecular Probes) for 30 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 stain (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 with the use of 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.
L-CARNITINE ENHANCES STALLION SPERM LONGEVITY

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 × 10⁶ 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 × 10⁶ cells/ml. Sample was snap frozen in liquid nitrogen and stored at −80°C until analysis. 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 Na₂PO₄, 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 stained or double stained 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 20000 × g for 15 min at 4°C. The supernatant was retained and utilized for the assay. The ATP standard solution supplied with the kit was serially diluted to obtain concentrations of 10⁻⁷ g/ml to 10⁻³ 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 a 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 Ion 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/inch² (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 further 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. Standards 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/10⁶ 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 the term) with Bonferroni correction (SAS Institute Inc.). Where significant treatment effects were identified by ANOVA (α = 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 test for mean comparison (α = 0.05).

Experimental Design

Pyrurate 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 × 10⁶ 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 osmolalities of 310 mOsm/kg for all treatments. Prosurvival effects of Pyr and L-C 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 × 10⁶/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 pans (Sarstedt). The concentration of NaCl was varied between treatments to achieve a final osmolality 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 was replaced with Pyr (Pyr50) or Pyr50 + L-C (Pyr50L-C). The concentration of NaCl was reduced 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 used to index 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 × 10⁶/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 × 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...
motility of the L-C (63.6% ± 2.7%) treatment being significantly higher than that of the control (48.8% ± 4.0%, P ≤ 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 ≤ 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 ≤ 0.01) and progressive (P ≤ 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 ≤ 0.01; progressive motility: 26.3% ± 1.8% and 34.2% ± 2.3% vs. 15.2% ± 2.4%, respectively, P ≤ 0.05; Fig. 2, A and B). Similarly, significant treatment effects on percent rapid (P ≤ 0.01), VSL (P ≤ 0.01), and STR (P ≤ 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 ≤ 0.01; VSL: 74.2 ± 3.2 μm/sec and 81 ± 1.8 μm/sec vs. 60.4 ± 4.1 μm/sec, P ≤ 0.05 and P ≤ 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 ≤ 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 ≤ 0.05). In addition, there were significant treatment effects on cellular superoxide production at 24 h (P ≤ 0.01), 48 h (P ≤ 0.01), and 72 h (P ≤ 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 ≤ 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 ≤ 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 ≤ 0.05). In addition, there was a significant treatment effect on total oxidative DNA damage (P ≤ 0.01), which was significantly higher in the control than the L-C treatment after 72 h of storage (80HdG-positive cells: 15.6% ± 1.4% vs. 9.0% ± 1.0%, P ≤ 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 ≤ 0.05) and 72 h of storage (P ≤ 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 ≤ 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 ≤ 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 ≤ 0.05). These results are graphically displayed in the representative histogram shown in Figure 3B.

**RESULTS**

**Pyruvate and L-C Dose Response**

There were significant effects of both Pyr and L-C concentrations on total motility (P ≤ 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 ≤ 0.05; Fig. 1A). L-Carnitine supplementation at 50 and 100 mM resulted in significantly higher total motilities (33.0% ± 1.7%, P ≤ 0.01 and 31.3% ± 2.0%, P ≤ 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 ≤ 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 ≤ 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 ≤ 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 ≤ 0.05), and progressive (P ≤ 0.05) motilities, with the total
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 ≤ 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 ≤ 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 ≤ 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 ≤ 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 ≤ 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 conditions, spermatozoa treated with L-C alone or in combination with Pyr were able to maintain higher levels of motility and ATP concentrations compared to the control, suggesting a beneficial effect of L-C on sperm longevity.
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 transport 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. 1A and B, and 2A and B), and a reduction in oxidative stress parameters (Figs. 2C and 3A 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
L-CARNITINE ENHANCES STALLION SPERM LONGEVITY

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 necessitated the partial removal of NaCl to maintain isotonicity. 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.

L-CARNITINE ENHANCES STALLION SPERM LONGEVITY

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 ≤ 0.01.
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