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RESEARCH ARTICLE

Intramuscular L-carnitine supplementation and its effects on reproductive hormones and fresh and chilled semen quality in exercised Warmblood stallions: A preliminary randomized crossover study

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ABSTRACT

Background and Aim: Repeated high-intensity exercise may alter reproductive hormone concentrations and compromise semen quality in performance stallions. L-carnitine participates in mitochondrial energy metabolism and has been investigated for its potential reproductive benefits. However, evidence regarding systemic L-carnitine supplementation in exercised Warmblood stallions remains limited. This study aimed to evaluate the effects of intramuscular L-carnitine administration on reproductive hormone concentrations and the quality of fresh and chilled semen in Warmblood stallions subjected to repeated exercise.

Materials and Methods: Two clinically healthy, proven fertile Warmblood stallions were enrolled in a preliminary randomized-sequence crossover study. Each stallion underwent three experimental conditions: no exercise or supplementation (negative control), repeated exercise with saline placebo (positive control), and repeated exercise with intramuscular L-carnitine supplementation (6 mg/kg body weight, twice weekly). Each treatment period lasted 8 weeks, with an 8-week washout between periods. Serum luteinizing hormone, also termed interstitial cell-stimulating hormone, and testosterone concentrations were measured using enzyme-linked immunosorbent assays. Fresh semen was evaluated for macroscopic characteristics, progressive motility, viability, and plasma membrane integrity. Chilled semen was assessed at 0, 24, 48, and 72 h of storage.

Results: Exercise with placebo was associated with lower reproductive hormone concentrations and poorer fresh semen quality than the negative control. L-carnitine supplementation was associated with higher luteinizing hormone (1.79 ± 0.28 vs. 1.12 ± 0.14 ng/mL) and testosterone (1.91 ± 0.26 vs. 1.43 ± 0.12 ng/mL) concentrations than exercise with placebo. Fresh semen progressive motility (73.40 ± 4.68% vs. 60.40 ± 1.47%), viability (80.00 ± 3.74% vs. 68.50 ± 1.77%), and plasma membrane integrity (64.10 ± 3.97% vs. 55.30 ± 1.10%) were also higher during supplementation. At 72 h of chilled storage, progressive motility was 36.92 ± 1.01% with supplementation and 34.79 ± 0.95% with placebo. Semen quality declined during storage under all conditions, with broadly similar proportional reductions.

Conclusion: Intramuscular L-carnitine supplementation was associated with more favorable reproductive hormone concentrations and semen quality during repeated exercise. However, including only two stallions limits statistical inference and generalizability. Larger, controlled studies are needed before establishing practical breeding recommendations.

Keywords: chilled semen, exercise-induced stress, L-carnitine, luteinizing hormone, sperm motility, testosterone, Warmblood stallions.

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INTRODUCTION

Physical conditioning, including regular exercise, is essential for managing and optimizing the performance of Warmblood stallions. However, strenuous or prolonged exercise can induce physiological stress and disrupt metabolic homeostasis [1]. One potential consequence is oxidative stress, which occurs when reactive oxygen species (ROS) production exceeds the capacity of endogenous antioxidant defense systems [2]. Although physiological ROS concentrations are necessary for normal sperm function, including fertilization processes, excessive ROS production can impair sperm motility, viability, and plasma membrane integrity, potentially compromising reproductive capacity [3].

Stallion reproductive function also depends on coordinated endocrine regulation and the maintenance of testicular cellular integrity. Interstitial cell-stimulating hormone (ICSH), also known as luteinizing hormone, stimulates testosterone production by Leydig cells, whereas testosterone supports spermatogenesis through its actions within the seminiferous epithelium, including interactions with Sertoli cells [4]. The hypothalamic–pituitary–gonadal (HPG) axis regulates these processes through coordinated hormonal signaling. Physical stress and oxidative disturbances may alter HPG axis activity, potentially affecting gonadotropin secretion, circulating testosterone concentrations, and subsequent sperm production and maturation [5]. These relationships provide a physiological rationale for investigating reproductive endocrine responses alongside semen characteristics in stallions exposed to repeated exercise.

Equine semen quality is commonly assessed using sperm motility, viability, and plasma membrane integrity, which provide complementary information about sperm function [6]. Beyond the quality of freshly collected semen, spermatozoa's ability to retain functional characteristics during chilled storage is particularly important for artificial insemination (AI) programs. Chilled semen allows the transport of genetic material between breeding facilities without requiring the immediate presence of the stallion. However, sperm quality may deteriorate progressively during storage because of changes in cellular metabolism, membrane stability, and oxidative balance. This deterioration can reduce the proportion of motile, viable spermatozoa available for insemination. Consequently, interventions that maintain sperm function during chilled storage are of interest in equine reproductive management.

L-carnitine is a naturally occurring compound involved in cellular energy metabolism and has been investigated for its potential antioxidant and reproductive effects. It is present in the male reproductive tract, including the epididymis and seminal plasma [7]. Its principal metabolic function is to transport long-chain fatty acids into mitochondria for β-oxidation, supporting adenosine triphosphate (ATP) production [8]. Because sperm motility requires a continuous energy supply, L-carnitine-associated metabolic processes may be relevant to sperm function. Its reported antioxidant-related properties also provide a rationale for examining whether supplementation influences reproductive responses under conditions associated with physiological stress. Nevertheless, the presence of L-carnitine in reproductive tissues and its established metabolic functions do not, by themselves, demonstrate that systemic supplementation improves reproductive performance in exercised stallions.

Previous investigations have evaluated L-carnitine supplementation in different reproductive contexts. Studies involving L-carnitine and acetyl-L-carnitine combined with intracytoplasmic morphologically selected sperm injection (IMSI) have reported improvements in reproductive outcomes in cases of male infertility associated with abnormal sperm morphology [9, 10]. Experimental investigations in rats have also suggested that L-carnitine administration may alleviate reproductive impairment associated with hypobaric hypoxia and improve semen characteristics [11]. In roosters, supplementation of cryopreservation media with appropriate concentrations of L-carnitine has been associated with reduced ROS concentrations and fewer sperm abnormalities [12]. However, these findings have not been consistent across species or administration strategies. For example, dietary L-carnitine supplementation for 12 weeks in boars did not substantially improve sperm production or overall semen quality, although changes in certain sperm kinematic parameters were reported [13].

In stallions, previous studies have described favorable effects of nutraceutical preparations containing L-carnitine and other antioxidants on sperm motility, membrane integrity, and the quality of chilled or frozen–thawed semen [14]. Strenuous exercise has also been associated with changes in endocrine responses and semen characteristics in stallions [15]. Nevertheless, the available findings come from different experimental conditions, supplement formulations, and administration methods. In particular, evidence from combined nutraceutical preparations or the direct addition of L-carnitine to semen extenders cannot establish the independent effects of

intramuscular L-carnitine supplementation in exercised stallions.

Despite growing interest in L-carnitine as a metabolic cofactor and potential reproductive supplement, several questions remain regarding its effects in physically active Warmblood stallions. First, much of the available evidence comes from studies in other animal species, human reproductive medicine, or L-carnitine added directly to semen preservation media. These approaches differ substantially from systemic supplementation because the administered compound must be absorbed or distributed, taken up by tissues, and metabolically processed before it can affect reproductive tissues. Consequently, findings obtained from dietary supplementation in other species or direct supplementation of semen extenders cannot be assumed to apply to intramuscular administration in stallions.

Second, previous equine investigations involving multicomponent nutraceutical formulations have not necessarily distinguished the contribution of L-carnitine from that of other antioxidant or metabolic ingredients [14]. This limits the interpretation of L-carnitine-specific effects on sperm function. Furthermore, although researchers have reported exercise-related changes in reproductive hormones and semen characteristics [15], the extent to which systemic L-carnitine supplementation influences these responses during repeated exercise remains poorly characterized.

Third, researchers often treat reproductive hormone concentrations and semen quality as separate outcomes, although endocrine regulation and sperm function are physiologically interconnected. Simultaneously evaluating ICSH and testosterone concentrations alongside sperm motility, viability, and plasma membrane integrity may provide a broader picture of reproductive responses during supplementation. However, changes in circulating hormone concentrations do not necessarily reflect corresponding changes in testicular function or identify the mechanisms driving alterations in semen quality.

Finally, the effects of systemic L-carnitine administration on the preservation of semen quality during chilled storage require further investigation. An intervention associated with favorable characteristics in freshly collected semen may not necessarily reduce the subsequent deterioration of sperm function during refrigeration. Evaluating semen at successive storage intervals is therefore important for distinguishing differences in initial semen quality from differences in the pattern of deterioration during storage. This distinction matters for AI programs that rely on transporting and short-term preservation of stallion semen.

Together, these gaps indicate the need for preliminary investigations that assess endocrine responses, fresh semen characteristics, and chilled semen quality within the same experimental framework. Such investigations may help identify measurable responses to L-carnitine supplementation and inform the design of larger studies, while recognizing that findings from a small number of stallions cannot establish generalizable reproductive benefits.

This preliminary study aimed to evaluate the effects of intramuscular L-carnitine supplementation on reproductive hormone concentrations and fresh and chilled semen quality in Warmblood stallions subjected to a standardized repeated exercise protocol.

Specifically, the study compared circulating ICSH and testosterone concentrations across conditions without exercise or supplementation, repeated exercise with placebo, and repeated exercise with L-carnitine supplementation. It also aimed to assess differences in fresh semen characteristics across these conditions, particularly progressive sperm motility, viability, and plasma membrane integrity.

The study also aimed to evaluate chilled semen quality during storage at 0, 24, 48, and 72 h to determine whether the responses observed during L-carnitine supplementation were reflected in semen characteristics at successive preservation intervals. By integrating endocrine measurements with fresh and chilled semen assessments, the study sought to provide exploratory evidence regarding the reproductive responses associated with intramuscular L-carnitine administration in exercised Warmblood stallions.

The investigation aimed to characterize these responses under the specified experimental conditions rather than establish a definitive mechanism of action, improved fertility, or clinical effectiveness for routine breeding management.

MATERIALS AND METHODS

Ethical approval

The study was conducted using horses owned by Nirwana Stable, Semarang City, Central Java, Indonesia. Written informed consent was obtained from the owners before the animals were enrolled. All experimental procedures, including the exercise protocol, intramuscular administration of L-carnitine or placebo, and collection

of blood and semen samples, were reviewed and approved by the Research Ethics Committee of the Faculty of Veterinary Medicine, Universitas Airlangga, Indonesia, on August 13, 2025 (approval number: 1.KEH.127.08.2025)

Before enrollment, each stallion underwent a clinical examination to assess its general health and reproductive status. Only clinically healthy stallions with a history of proven fertility were included. Throughout the experiment, the animals were maintained under consistent management and feeding conditions, with unrestricted access to drinking water. The study incorporated washout intervals between experimental conditions to minimize residual effects from preceding treatments.

Study period and location

The study was conducted at Nirwana Stable from August 2025 to May 2026, where the stallions were housed and underwent the experimental exercise, supplementation, and sampling procedures. The experimental protocol comprised three treatment periods of 8 weeks each, separated by two washout intervals of 8 weeks each.

Study design

A preliminary randomized-sequence crossover design was used to investigate the effects of repeated exercise and intramuscular L-carnitine supplementation on reproductive hormone concentrations and semen quality. Two clinically healthy, proven fertile Warmblood stallions, aged 5–7 years and weighing approximately 500–550 kg, were enrolled. Each stallion served as its own control across three experimental conditions, with the order of the conditions independently randomized for each animal.

The experimental conditions were as follows: negative control (C−), without the experimental exercise protocol or supplementation; positive control (C+), repeated high-intensity exercise with placebo administration; and treatment (T), repeated high-intensity exercise with intramuscular L-carnitine supplementation.

Each experimental condition lasted 8 weeks. An 8-week washout interval was implemented between successive treatment periods to minimize potential carryover effects. This duration was selected with reference to the approximately 57-day period required for spermatogenesis in horses [16]. No experimental exercise or supplementation was administered during the washout intervals.

The stallion was considered the biological experimental unit (n = 2). Repeated sampling occasions, duplicate assays, and storage interval measurements were treated as repeated observations nested within stallion and were not considered independent replicates. The crossover design aimed to reduce between-animal variability by allowing comparisons within the same animals.

Both stallions remained at Nirwana Stable throughout the experiment and were maintained under consistent husbandry conditions. They received the same feeding regimen during each experimental phase and had free access to drinking water. The experimental exercise workload was standardized within the two exercised conditions. Body weight was monitored throughout the study to assess changes associated with the experimental conditions.

Exercise protocol

In the C+ and T conditions, stallions underwent a standardized high-intensity exercise protocol 6 times per week for 8 weeks, designed to elicit exercise-associated physiological responses. Each session lasted about 60 min and included a 10-min warm-up, 40 min of continuous trotting and cantering at 70%–80% of maximum heart rate, and a 10-min cool-down. The protocol was selected with reference to previous investigations describing physiological and hormonal responses to high-intensity exercise in horses [1, 17].

The negative control condition did not include the experimental exercise protocol. During washout intervals, neither the experimental exercise protocol nor supplementation was administered.

L-carnitine supplementation

During the T condition, stallions received L-Carnitine Injection for Horse (Ceva Animal Health Pty Ltd., Australia; 200 mg/mL) by intramuscular injection at a dose of 6 mg/kg body weight twice weekly. Injections were administered on days 1 and 4 of each week throughout the 8-week treatment period.

The administered dose was based on the manufacturer's recommended dose of 3 mL/100 kg body weight, equivalent to 600 mg/100 kg (6 mg/kg body weight). Injections were administered into the cervical portion of the trapezius muscle.

During the C+ condition, stallions received an equivalent volume of sterile saline using the same injection schedule and administration procedure. The placebo procedure was intended to maintain blinding between the

exercised treatment conditions.

Blood collection

Blood samples were collected aseptically from the jugular vein of each stallion using sterile disposable needles and syringes. Samples were transferred into 5-mL blood collection tubes without anticoagulant.

Sampling was performed in the morning at three standardized time points during each experimental condition: baseline (week 0, before the treatment period), midpoint (week 4), and endpoint (week 8).

After collection, blood samples were allowed to clot at room temperature for 30–45 min and were subsequently centrifuged at 1,500 rpm for 15 min at 4°C [18]. The separated serum was transferred into labeled 2-mL polypropylene microtubes and stored at −80°C until hormonal analysis.

Serum samples were handled under aseptic conditions to minimize contamination and preanalytical variation.

Semen collection

Semen was collected using a Hannover-type artificial vagina by the standing four-feet method. Collections occurred in the morning under each experimental condition, with assessments at baseline (week 0), midpoint (week 4), and endpoint (week 8). No semen was collected at other times.

A standardized sexual rest interval of 7 days was maintained before semen collection to reduce variation associated with ejaculation frequency. Before collection, the artificial vagina was prepared with warm water and maintained at approximately 45°C. A sterile collection liner and filter were used.

Immediately after collection, the gel fraction was removed by filtration, and the sperm-rich fraction was retained for evaluation. Two consecutive ejaculates were collected during each sampling session; only the second ejaculate was used for analysis. Consequently, the two ejaculates were not considered separate experimental units.

Fresh semen evaluation

Fresh semen was evaluated immediately after collection. Macroscopic characteristics included ejaculate volume (mL), consistency, odor, pH, and color. Semen consistency was assessed descriptively, ranging from watery to thick, while odor and color were evaluated based on observed characteristics.

Semen pH was measured using indicator strips (MQuant®, Merck KGaA, Darmstadt, Germany) with a measurement range of 6.5–10.0 and a color-scale resolution of approximately 0.2–0.3 pH units.

Microscopic examination was performed using a phase-contrast microscope (E100, Nikon, Tokyo, Japan). Progressive sperm motility was assessed subjectively at 200× magnification by evaluating 100 spermatozoa across five microscopic fields.

Sperm viability was assessed using Eosin–Nigrosin staining at 400× magnification. A total of 200 spermatozoa across five microscopic fields were evaluated to determine the percentage of viable spermatozoa.

Assessment of sperm plasma membrane integrity

Functional sperm plasma membrane integrity was evaluated using the hypo-osmotic swelling test (HOST). The HOST solution was prepared by dissolving 0.735 g sodium citrate and 1 g fructose in 100 mL distilled water, yielding an osmolarity of 150 mOsm/kg. For each assessment, 0.1 mL of semen was mixed with 0.9 mL of HOST solution at a ratio of 1:9 and incubated at 23°C for 60 min.

Following incubation, samples were examined under light microscopy at 400× magnification. Spermatozoa exhibiting tail swelling, coiling, or bending were classified as HOST-positive, indicating a functional response of the plasma membrane to hypo-osmotic conditions. Spermatozoa with straight tails were classified as HOST-negative.

The percentage of spermatozoa exhibiting functional plasma membrane integrity was calculated from the proportion of HOST-positive spermatozoa among 200 spermatozoa examined across five microscopic fields.

Chilled semen preparation and evaluation

Semen was initially diluted with the commercial extender INRA 96 at a ratio of 1:1. The diluted samples were centrifuged at 4,000 rpm for 17 min, after which the supernatant was discarded to remove seminal plasma. The relative centrifugal force could not be calculated because the rotor radius and centrifuge specifications were not recorded. The same centrifugation protocol was applied to all samples across all experimental conditions. The cooling rate and sperm concentration after resuspension were not recorded.

The sperm pellet was resuspended in INRA 96 extender, and additional extender was added to restore the

initial volume. The prepared samples were subsequently cooled to 5°C using a controlled cooling procedure and maintained at this temperature throughout storage.

Chilled semen quality was evaluated after 0, 24, 48, and 72 h of storage. Progressive sperm motility was assessed using a computer-assisted sperm analysis (CASA) system (TOX IVOS® II, Hamilton-Thorne, Beverly, MA, USA). Subjective motility assessments were also performed for comparison. Both CASA-based and subjective evaluations were conducted by operators blinded to treatment allocation. The CASA system was calibrated before each analytical session according to the manufacturer's recommendations.

Sperm viability and functional plasma membrane integrity were evaluated at the designated storage intervals using the procedures described for fresh semen.

Hormonal assays

Serum concentrations of ICSH and testosterone were measured using enzyme-linked immunosorbent assays (ELISAs).

ICSH was quantified using a sandwich ELISA validated for horses (Horse LH ELISA Kit, catalog number BZ-22118257-EB, BIOENZY, East Jakarta, Indonesia). Testosterone was quantified using a general testosterone ELISA kit (catalog number MDBEA0009Ge, MEDIKBIO, Malang, Indonesia).

According to the manufacturers, the analytical sensitivities were 0.05 ng/mL for ICSH and 0.1 ng/mL for testosterone. The corresponding detection ranges were 0.1–50 ng/mL and 0.2–25 ng/mL, respectively.

All samples were analyzed in duplicate, with duplicate measurements representing analytical replicates of the same serum sample. The reported intra-assay and interassay coefficients of variation were below 10% and 12%, respectively.

Serum samples were thawed once and diluted 1:2 with assay buffer according to the manufacturers' protocols. Absorbance was measured at 450 nm using a Multiscan Reader (Thermo Scientific, Vantaa, Finland) with Genesis V 3.00 software (VWR International Ltd., Lutterworth, UK).

Statistical analysis

Data were analyzed using repeated-measures analysis of variance (ANOVA) to evaluate the effects of experimental condition (C−, C+, and T), sampling time (baseline, week 4, and week 8), and their interaction on reproductive hormone concentrations and semen quality parameters.

The evaluated outcomes included sperm motility, viability, plasma membrane integrity, chilled semen characteristics, and circulating ICSH and testosterone concentrations.

When a main effect or interaction was statistically significant, Duncan's post hoc test was used to compare group means. Statistical significance was defined as $p < 0.05$.

Quantitative results were presented as mean ± standard deviation (SD). Different superscript letters were used to identify statistically significant differences among experimental conditions, where applicable.

Statistical analyses were performed using SPSS version 27.0 for Windows (IBM Corp., Armonk, NY, USA).

RESULTS

Body weight

Body weight changes during the three experimental conditions are presented in Figure 1. During the negative control condition (C−), body weight remained relatively stable, increasing from 510.0 ± 2.1 kg at baseline to 512.0 ± 2.5 kg at week 8, representing a 0.39% increase. In contrast, body weight during the exercise-plus-placebo condition (C+) decreased from 509.0 ± 1.6 kg to 491.0 ± 1.9 kg, corresponding to a 3.54% reduction.

During the exercise-plus-L-carnitine condition (T), body weight decreased from 512.0 ± 2.1 kg at baseline to 502.0 ± 1.5 kg at week 8, representing a 1.95% reduction. The absolute reduction was 18 kg during C+ and 10 kg during T. Thus, the reduction observed during T was 8 kg smaller, equivalent to approximately 44.4% less body weight loss than during C+. These descriptive findings indicate a smaller reduction in body weight during L-carnitine supplementation than during exercise with placebo.

Serum ICSH concentrations

Serum ICSH concentrations differed among the experimental conditions (Figure 2). The highest concentration was observed during C− (2.13 ± 0.19 ng/mL), whereas the lowest was recorded during C+ (1.12 ± 0.14 ng/mL). During T, the concentration was 1.79 ± 0.28 ng/mL, which was reported to be significantly higher than during C+ ($p < 0.05$).

The ICSH concentration during T was approximately 59.8% higher than during C+ and represented

approximately 84.0% of the concentration observed during C−. These comparisons indicate that the reduction in ICSH concentration during exercise with placebo was less pronounced with L-carnitine supplementation.

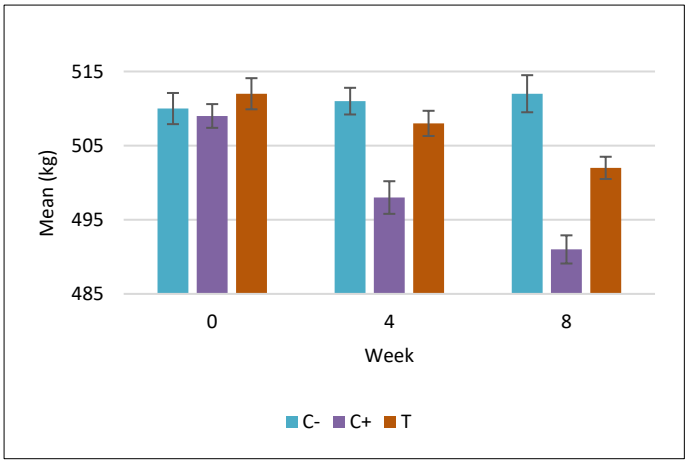


Figure 1: Body weight of Warmblood stallions during the 8-week experimental periods. C− = Negative control (no experimental exercise or supplementation); C+ = Positive control (repeated exercise with saline placebo); T = Repeated exercise with intramuscular L-carnitine supplementation. Values are expressed in kilograms (kg).

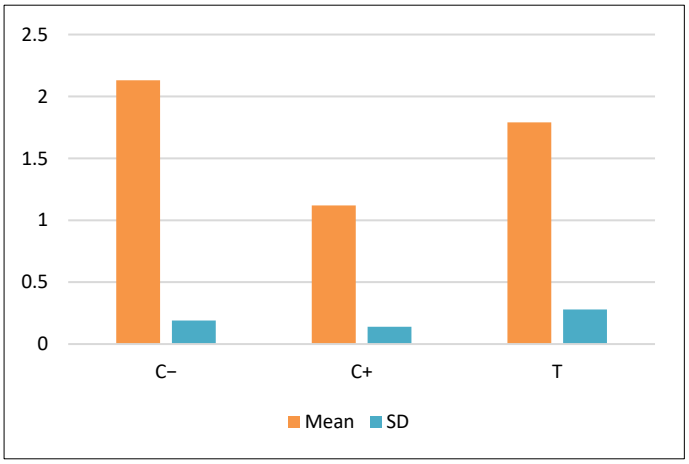


Figure 2: Serum interstitial cell-stimulating hormone concentrations in Warmblood stallions under the three experimental conditions. ICSH = Interstitial cell-stimulating hormone; C− = Negative control (no experimental exercise or supplementation); C+ = Positive control (repeated exercise with saline placebo); T = Repeated exercise with intramuscular L-carnitine supplementation. Hormone concentrations are expressed in ng/mL.

Serum testosterone concentrations

Serum testosterone concentrations across the experimental conditions are shown in Figure 3. The highest concentration was observed during C− (2.12 ± 0.18 ng/mL), followed by T (1.91 ± 0.26 ng/mL), whereas C+ showed the lowest concentration (1.43 ± 0.12 ng/mL).

The testosterone concentration during T was reported to be significantly higher than during C+ ($p < 0.05$). It was approximately 33.6% higher than the C+ value and about 90.1% of the C− value. Conversely, the concentration during C+ was approximately 32.5% lower than during C−. These observations indicate that testosterone concentrations were higher during L-carnitine supplementation than during exercise with placebo.

Fresh semen quality

Macroscopic semen characteristics: Macroscopic characteristics of fresh semen under the three experimental conditions are presented in Table 1. Ejaculate volume was highest during C− (60.0 ± 2.5 mL), followed by T (54.0 ± 2.1 mL), and lowest during C+ (42.0 ± 1.8 mL). The volume during C+ was 30.0% lower than during C−. In contrast, ejaculate volume during T was 28.6% higher than during C+ and represented 90.0% of the C− value. Differences in ejaculate volume among the conditions were reported as statistically significant ($p < 0.05$).

Semen consistency was thick during C−, watery during C+, and moderate during T. Normal odor was recorded during C− and T, whereas a slightly abnormal odor was reported during C+. Semen color was creamy white during C− and T but pale yellowish during C+.

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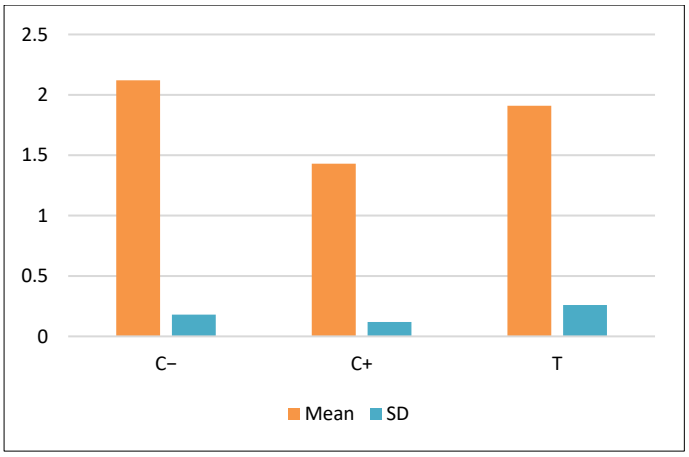


Figure 3: Serum testosterone concentrations in Warmblood stallions under the three experimental conditions. C− = Negative control (no experimental exercise or supplementation); C+ = Positive control (repeated exercise with saline placebo); T = Repeated exercise with intramuscular L-carnitine supplementation. Hormone concentrations are expressed in ng/mL.

Semen pH was highest during C− (7.20 ± 0.05), followed by T (7.10 ± 0.06), and lowest during C+ (6.80 ± 0.04). pH differences among the experimental conditions were statistically significant ($p < 0.05$). The qualitative observations of consistency, odor, and color are presented descriptively.

Table 1: Macroscopic characteristics of fresh semen from Warmblood stallions under the three experimental conditions.

Experimental condition	Ejaculate volume (mL)	Consistency	Odor	pH	Color
C−	60.0 ± 2.5	Thick	Normal	7.20 ± 0.05	Creamy white
C+	42.0 ± 1.8	Watery	Slightly abnormal	6.80 ± 0.04	Pale yellowish
T	54.0 ± 2.1	Moderate	Normal	7.10 ± 0.06	Creamy white

C− = Negative control (no experimental exercise or supplementation); C+ = Positive control (repeated exercise with saline placebo); T = Repeated exercise with intramuscular L-carnitine supplementation; SD = Standard deviation; ANOVA = Analysis of variance. Quantitative values are presented as mean ± SD. Consistency, odor, and color are descriptive qualitative observations.

Progressive sperm motility in fresh semen: Progressive sperm motility differed among the experimental conditions (Figure 4). The highest motility was recorded during C− (77.00 ± 1.77%), followed by T (73.40 ± 4.68%), whereas the lowest value was observed during C+ (60.40 ± 1.47%).

Progressive motility during T was reported to be significantly higher than during C+ ($p < 0.05$). The T value was approximately 21.5% higher than the C+ value and corresponded to approximately 95.3% of the C− value. Conversely, motility during C+ was approximately 21.6% lower than during C−. These results show that progressive motility was higher during L-carnitine supplementation than during exercise with placebo.

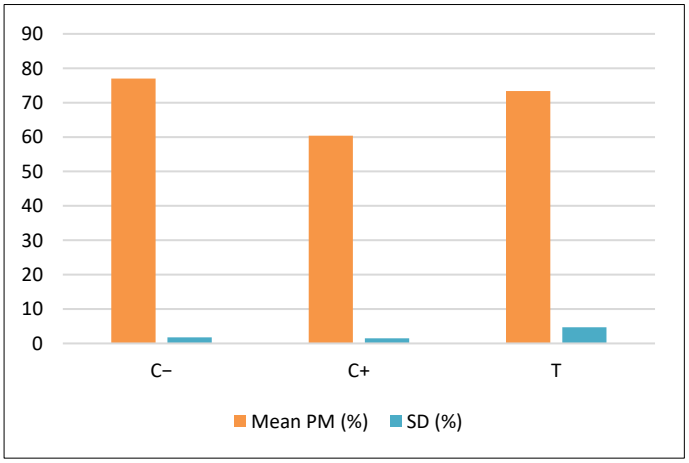


Figure 4: Progressive sperm motility in fresh semen from Warmblood stallions under the three experimental conditions. C− = Negative control (no experimental exercise or supplementation); C+ = Positive control (repeated exercise with saline placebo); T = Repeated exercise with intramuscular L-carnitine supplementation. Progressive motility is expressed as the percentage of spermatozoa exhibiting progressive movement.

Sperm viability in fresh semen: Fresh sperm viability was highest during C− (82.70 ± 2.28%), followed by T (80.00 ± 3.74%), and lowest during C+ (68.50 ± 1.77%) (Figure 5).

Viability during T was significantly higher than during C+ ($p < 0.05$) and approximately 16.8% higher than C+. The T value represented approximately 96.7% of the viability recorded during C−. In comparison, viability during C+ was approximately 17.2% lower than during C−. These findings indicate higher fresh sperm viability with L-carnitine supplementation than with placebo exercise.

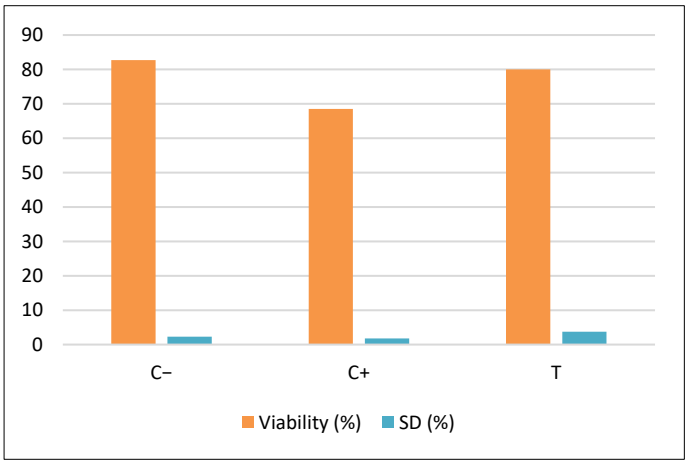


Figure 5: Sperm viability in fresh semen from Warmblood stallions under the three experimental conditions. C− = Negative control (no experimental exercise or supplementation); C+ = Positive control (repeated exercise with saline placebo); T = Repeated exercise with intramuscular L-carnitine supplementation. Viability is expressed as the percentage of viable spermatozoa determined using eosin–nigrosin staining.

Sperm plasma membrane integrity in fresh semen: Functional plasma membrane integrity, assessed using the HOST, differed among the experimental conditions (Figure 6). The highest percentage of spermatozoa with intact plasma membranes was recorded during C− (67.20 ± 2.28%), followed by T (64.10 ± 3.97%) and C+ (55.30 ± 1.10%).

Plasma membrane integrity during T was reported to be significantly higher than during C+ ($p < 0.05$). The T value was approximately 15.9% higher than the C+ value and represented approximately 95.4% of the C− value. Conversely, plasma membrane integrity during C+ was approximately 17.7% lower than during C−. These results indicate that a greater proportion of spermatozoa maintained functional plasma membrane integrity during L-carnitine supplementation than during placebo exercise.

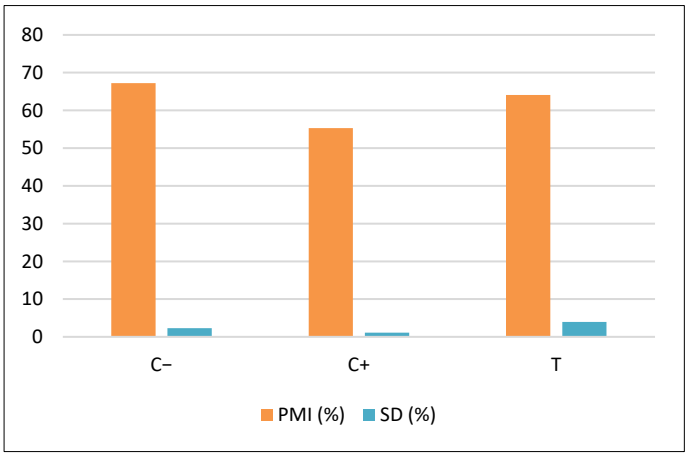


Figure 6: Functional plasma membrane integrity of spermatozoa in fresh semen from Warmblood stallions under the three experimental conditions. HOST = Hypo-osmotic swelling test; C− = Negative control (no experimental exercise or supplementation); C+ = Positive control (repeated exercise with saline placebo); T = Repeated exercise with intramuscular L-carnitine supplementation. Plasma membrane integrity is expressed as the percentage of HOST-positive spermatozoa.

Chilled semen quality

Progressive sperm motility during chilled storage: Progressive sperm motility decreased during the 72-h chilled storage period under all three experimental conditions (Figure 7). At 0 h, motility was highest during C− (62.20 ± 1.70%), followed by T (58.79 ± 1.61%) and C+ (55.40 ± 1.51%).

At 24 h, progressive motility decreased to 54.87 ± 1.50%, 51.85 ± 1.42%, and 48.86 ± 1.34% during C−, T, and C+, respectively. The corresponding values at 48 h were 47.53 ± 1.30%, 44.92 ± 1.23%, and 42.33 ± 1.16%. After 72 h, progressive motility decreased further to 39.06 ± 1.07% during C−, 36.92 ± 1.01% during T, and 34.79 ± 0.95%

during C+.

At every storage interval, motility was highest during C− and lowest during C+, with intermediate values during T. Progressive motility during T was approximately 6.1% higher than during C+ at each interval. The manuscript reports statistically significant differences between T and C+ at the evaluated storage times ($p < 0.05$).

From 0 to 72 h, progressive motility decreased by approximately 37.2% under each experimental condition. Thus, although motility remained higher during T than during C+ throughout storage, the proportional decline was similar across conditions. The observed differences therefore primarily reflect differences in motility levels rather than a clearly reduced rate of deterioration during chilled storage.

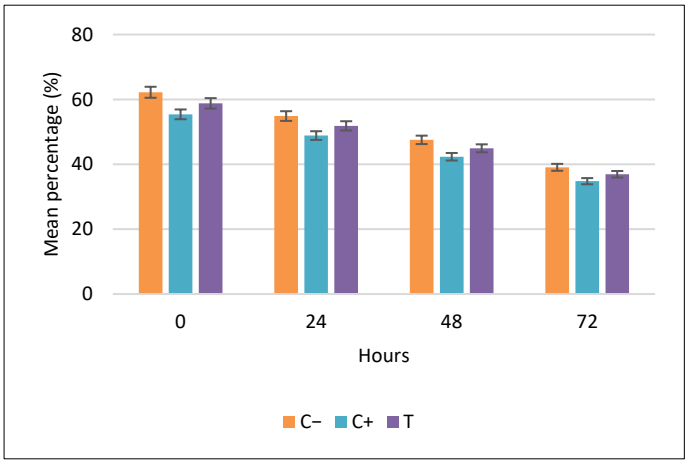


Figure 7: Progressive sperm motility in chilled semen from Warmblood stallions during 72 h of storage at 5°C. C− = Negative control (no experimental exercise or supplementation); C+ = Positive control (repeated exercise with saline placebo); T = Repeated exercise with intramuscular L-carnitine supplementation; CASA = Computer-assisted sperm analysis. Progressive motility is expressed as a percentage and was assessed at 0, 24, 48, and 72 h of storage.

Sperm viability during chilled storage: Sperm viability declined progressively during chilled storage under all experimental conditions (Figure 8). At 0 h, viability was highest during C− (73.33 ± 2.05%), followed by T (71.13 ± 1.99%) and C+ (68.13 ± 1.90%).

After 24 h, viability decreased to 67.00 ± 1.88%, 65.00 ± 1.82%, and 62.25 ± 1.74% during C−, T, and C+, respectively. At 48 h, the corresponding values were 57.16 ± 1.60%, 55.45 ± 1.55%, and 53.10 ± 1.49%. At 72 h, viability declined further to 49.00 ± 1.37% during C−, 47.53 ± 1.33% during T, and 45.45 ± 1.27% during C+.

Viability remained highest during C− and lowest during C+ throughout storage. Values during T were consistently intermediate and approximately 4.4%–4.6% higher than those during C+.

Between 0 and 72 h, viability decreased by approximately 33.2% during C− and T and by 33.3% during C+. These findings show a similar proportional decline in viability across conditions, despite consistently higher values during L-carnitine supplementation than during exercise with placebo.

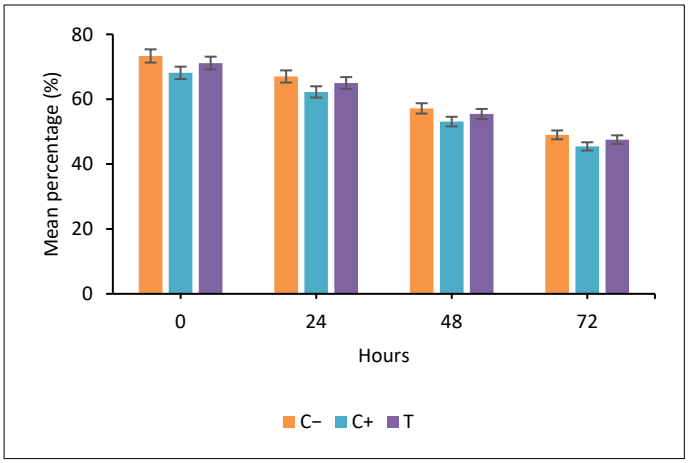


Figure 8: Sperm viability in chilled semen from Warmblood stallions during 72 h of storage at 5°C. C− = Negative control (no experimental exercise or supplementation); C+ = Positive control (repeated exercise with saline placebo); T = Repeated exercise with intramuscular L-carnitine supplementation. Viability is expressed as the percentage of viable spermatozoa assessed at 0, 24, 48, and 72 h of storage.

Sperm plasma membrane integrity during chilled storage: Functional plasma membrane integrity decreased progressively during the 72-h storage period under all experimental conditions (Figure 9).

At 0 h, plasma membrane integrity was highest during C− (60.66 ± 1.80%), followed by T (58.80 ± 1.70%) and C+ (55.40 ± 1.65%). At 24 h, the corresponding values were 53.50 ± 1.60%, 51.90 ± 1.55%, and 49.20 ± 1.47%, respectively.

After 48 h, plasma membrane integrity decreased to 44.00 ± 1.32% during C−, 42.70 ± 1.28% during T, and 40.50 ± 1.22% during C+. At 72 h, the corresponding values were 36.16 ± 1.08%, 34.90 ± 1.04%, and 33.00 ± 0.99%.

Throughout chilled storage, plasma membrane integrity remained highest during C− and lowest during C+, with intermediate values during T. Plasma membrane integrity during T was approximately 5.4%–6.1% higher than during C+ across the evaluated storage intervals.

From 0 to 72 h, plasma membrane integrity decreased by approximately 40.4% during C−, 40.7% during T, and 40.4% during C+. Therefore, the proportional decline was broadly similar across the experimental conditions, although the absolute percentages of spermatozoa exhibiting functional plasma membrane integrity remained higher during T than during C+.

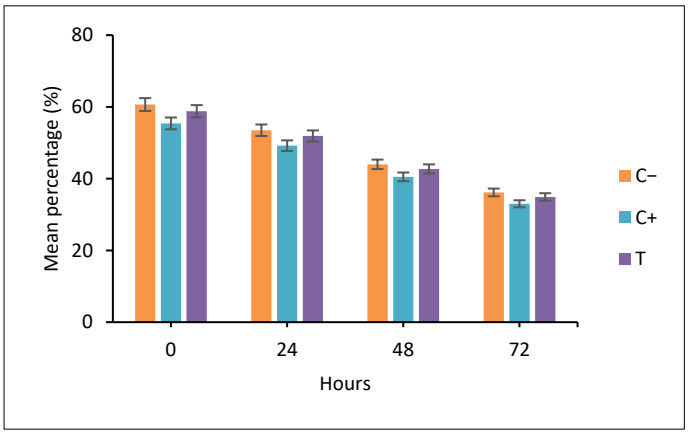


Figure 9: Functional plasma membrane integrity of spermatozoa in chilled semen from Warmblood stallions during 72 h of storage at 5°C. HOST = Hypo-osmotic swelling test; C− = Negative control (no experimental exercise or supplementation); C+ = Positive control (repeated exercise with saline placebo); T = Repeated exercise with intramuscular L-carnitine supplementation. Plasma membrane integrity is expressed as the percentage of HOST-positive spermatozoa assessed at 0, 24, 48, and 72 h of storage.

DISCUSSION

Exercise-associated changes in body weight and reproductive hormones

This study provides preliminary observations on relationships among repeated exercise, intramuscular L-carnitine supplementation, reproductive hormone concentrations, and semen quality in Warmblood stallions. Compared with the exercise-plus-placebo condition (C+), the exercise-plus-L-carnitine condition (T) was associated with a smaller reduction in body weight, higher circulating ICSH and testosterone concentrations, and more favorable fresh semen characteristics. During chilled storage, sperm motility, viability, and plasma membrane integrity were consistently higher in T than in C+. However, these findings should be interpreted cautiously because only two stallions were enrolled, and the repeated measurements do not constitute independent biological replicates.

Body weight remained relatively stable during the negative control condition (C−), whereas reductions of 3.54% and 1.95% were observed during C+ and T, respectively. The absolute reduction in body weight during T was 8 kg smaller than during C+, corresponding to approximately 44.4% less body weight loss. These observations suggest that the physiological responses associated with the experimental exercise protocol differed between the supplemented and placebo conditions. Nevertheless, body weight is influenced by multiple factors, including energy intake, expenditure, hydration, and changes in body composition. Because these components were not measured independently, the smaller reduction in body weight during T cannot be attributed to a specific metabolic action of L-carnitine.

The lower ICSH and testosterone concentrations observed during C+ compared with C− are consistent with the possibility that repeated exercise was associated with altered reproductive endocrine responses. Previous investigations have suggested that physical stress may influence HPG axis activity and reproductive behavior [19].

In the present study, ICSH concentrations were 1.12 ± 0.14 ng/mL during C+ and 1.79 ± 0.28 ng/mL during T, whereas the corresponding testosterone concentrations were 1.43 ± 0.12 and 1.91 ± 0.26 ng/mL. Thus, ICSH and testosterone concentrations during T were approximately 59.8% and 33.6% higher, respectively, than during C+.

These observations suggest a possible association between L-carnitine supplementation and higher circulating reproductive hormone concentrations under the experimental exercise condition. However, the measurements do not establish whether the differences originated at the hypothalamic, pituitary, or testicular level. In addition, circulating hormone concentrations are subject to temporal and physiological variation. Consequently, the observed differences do not definitively show that supplementation restored HPG axis function.

Regarding chilled semen, sperm motility, viability, and plasma membrane integrity were consistently higher during T than during C+. However, the proportional declines in these parameters during storage were similar between conditions. Therefore, the findings suggest that L-carnitine supplementation was associated with higher semen quality values during chilled storage, but do not demonstrate a slower rate of semen deterioration. This distinction is important when interpreting the potential benefits of supplementation for chilled semen preservation.

Potential metabolic and oxidative mechanisms

One possible explanation for the observed endocrine differences involves the relationship between energy availability and reproductive hormonal regulation. Gonadotropin-releasing hormone (GnRH) secretion is sensitive to physiological and metabolic conditions, and previous research has discussed the potential relevance of carnitine-dependent energy metabolism to reproductive endocrine function [20]. Because L-carnitine participates in the mitochondrial transport of long-chain fatty acids for β-oxidation, supplementation could theoretically influence cellular energy metabolism under conditions of increased physiological demand.

Nevertheless, neither GnRH secretion nor hypothalamic energy metabolism was directly assessed in this study. The higher ICSH and testosterone concentrations observed during T therefore cannot be attributed specifically to changes in GnRH synthesis or release. These pathways remain mechanistic hypotheses requiring direct investigation.

Oxidative stress represents another potential pathway linking strenuous exercise with reproductive changes. Exogenous antioxidants have been investigated for their ability to reduce oxidative damage associated with excessive free radical production [21]. L-carnitine has also been studied for its potential contribution to the balance between ROS production and antioxidant defenses [22]. Previous investigations have reported changes in antioxidant enzyme activity following carnitine supplementation, although the magnitude of these responses may depend on supplementation duration and experimental conditions [23].

These findings provide a biological rationale for investigating L-carnitine in exercised stallions. However, the present study did not measure ROS concentrations, lipid peroxidation products, antioxidant enzyme activities, or other direct indicators of oxidative stress. Therefore, it cannot establish that the observed endocrine or semen quality differences resulted from reduced oxidative damage. The proposed antioxidant contribution of L-carnitine should be regarded as a plausible mechanism rather than a demonstrated finding.

Macroscopic characteristics of fresh semen

The exercise-plus-placebo condition was associated with less favorable macroscopic semen characteristics than the negative control condition. Ejaculate volume decreased from 60.0 ± 2.5 mL during C− to 42.0 ± 1.8 mL during C+, while semen pH decreased from 7.20 ± 0.05 to 6.80 ± 0.04. During T, ejaculate volume was 54.0 ± 2.1 mL and semen pH was 7.10 ± 0.06. Thus, ejaculate volume during T was approximately 28.6% higher than during C+ and represented approximately 90.0% of the C− value.

Previous research has suggested that exercise-associated physiological stress may influence accessory reproductive gland function and seminal plasma composition [24]. Such changes could contribute to variation in ejaculate volume and other macroscopic characteristics. However, accessory gland secretory activity and seminal plasma composition were not directly measured in the present study. Accordingly, the observed differences in ejaculate volume and pH cannot be attributed to a specific alteration in accessory gland function.

The study also observed differences in consistency, odor, and color among the experimental conditions. Semen was described as thick and creamy white during C−, watery and pale yellowish during C+, and moderately consistent and creamy white during T. These findings provide descriptive information about the ejaculates but should not be interpreted as independent evidence of altered fertility or accessory gland pathology. The

qualitative nature of these assessments, along with the small number of stallions, limits the conclusions that can be drawn.

Progressive motility, viability, and plasma membrane integrity of fresh semen

The microscopic evaluation of fresh semen revealed lower progressive motility, viability, and functional plasma membrane integrity during C+ than during C−. During T, all three parameters were higher than during C+, suggesting that L-carnitine supplementation was associated with less pronounced changes in fresh semen characteristics under the experimental exercise condition.

Progressive motility was 60.40 ± 1.47% during C+ and 73.40 ± 4.68% during T, representing an approximately 21.5% higher value during supplementation. The T value corresponded to approximately 95.3% of the motility observed during C−. Similarly, sperm viability was approximately 16.8% higher during T than during C+, reaching approximately 96.7% of the C− value. Functional plasma membrane integrity was approximately 15.9% higher during T than during C+ and represented approximately 95.4% of the C− value.

Sperm motility depends on adequate energy availability and the maintenance of cellular structures involved in movement. Oxidative disturbances may affect mitochondrial activity, adenosine triphosphate production, and membrane stability, potentially compromising sperm function [24, 25]. L-carnitine participates in mitochondrial fatty acid metabolism and has been investigated for its potential contribution to cellular energy production and protection against lipid peroxidation [26, 27]. These properties provide possible explanations for the differences observed in the present study.

However, mitochondrial function, intracellular energy availability, and sperm lipid peroxidation were not measured. It is therefore not possible to determine whether the higher motility, viability, and plasma membrane integrity observed during T were mediated by improved energy metabolism, reduced oxidative damage, or another mechanism. Furthermore, the reported percentage differences represent descriptive comparisons between experimental conditions rather than independently established treatment effects.

Previous human reproductive research has reported improvements in semen characteristics following L-carnitine supplementation, including findings discussed by Khaw *et al.* [28]. Although these reports support continued investigation of L-carnitine in reproductive medicine, differences in species, reproductive physiology, supplementation protocols, and semen evaluation methods limit their applicability to stallions. The present findings therefore support a potential association between L-carnitine supplementation and favorable sperm characteristics, but they do not establish a generalizable reproductive benefit.

Chilled semen quality and the pattern of deterioration during storage

Chilled semen quality decreased progressively during the 72-h storage period under all experimental conditions. Progressive sperm motility, viability, and functional plasma membrane integrity were highest during C−, lowest during C+, and intermediate during T at each evaluated storage interval. These observations indicate that the differences among experimental conditions persisted during chilled storage.

At 0 h, progressive motility was 55.40 ± 1.51% during C+ and 58.79 ± 1.61% during T. After 72 h, the corresponding values were 34.79 ± 0.95% and 36.92 ± 1.01%. Thus, progressive motility remained approximately 6.1% higher during T than during C+ throughout storage. Similarly, viability and functional plasma membrane integrity remained higher during T than during C+ at each assessment.

Importantly, higher semen quality values during storage do not necessarily indicate a slower rate of deterioration. From 0 to 72 h, progressive motility decreased by approximately 37.2% under each experimental condition. Viability decreased by approximately 33.2% during C− and T and 33.3% during C+. Functional plasma membrane integrity decreased by approximately 40.4% during C−, 40.7% during T, and 40.4% during C+.

These nearly identical proportional reductions indicate that the supplemented condition was associated with higher semen quality values at the evaluated time points, but not with a clearly slower proportional decline during chilled storage. Therefore, the present data do not support the conclusion that intramuscular L-carnitine supplementation extended the functional lifespan of spermatozoa or reduced the rate of deterioration during refrigeration.

Previous investigations have explored the potential roles of antioxidants and metabolic cofactors in maintaining sperm function during semen storage [29, 30]. Such interventions may act through mechanisms involving membrane stabilization, metabolic support, or protection against oxidative injury. However, this study did not directly evaluate these mechanisms, and deterioration patterns were similar across the three conditions.

The higher chilled semen-quality values observed during T may, at least partly, reflect differences already

present at the beginning of chilled storage. This interpretation is consistent with the higher fresh semen quality values observed during supplementation. Nevertheless, fresh progressive motility was assessed subjectively, whereas chilled progressive motility was assessed using CASA. Therefore, differences in assessment methods limit direct numerical comparisons between fresh and chilled motility measurements.

Taken together, these findings suggest that the supplemented condition was associated with more favorable sperm function during chilled storage, without demonstrating a specific protective effect against the subsequent proportional loss of motility, viability, or plasma membrane integrity.

Relevance of previous reproductive studies and unmeasured mechanisms

This investigation extends earlier studies by evaluating systemic L-carnitine supplementation in exercised Warmblood stallions while assessing circulating reproductive hormones, fresh semen characteristics, and chilled semen quality.

Previous equine investigations have reported favorable sperm responses following supplementation with nutraceutical preparations containing L-carnitine and other antioxidant compounds [14]. However, the use of multicomponent preparations limits the extent to which those findings can be attributed specifically to L-carnitine. Moreover, results from semen extenders or cryopreservation media do not necessarily reflect the effects of systemic supplementation.

L-carnitine has also been investigated as a potential strategy for supporting sperm membrane integrity and limiting oxidative injury [31]. Seminal plasma proteins, including HSP-1/2, may contribute to sperm membrane interactions and function. However, these proteins were not measured in the present study, and their involvement in the observed differences cannot be determined. Future investigations incorporating seminal plasma protein profiling could help clarify whether such pathways are associated with the reproductive responses observed during supplementation.

Evidence from other animal species provides additional context. For example, L-carnitine supplementation during the breeding season has been associated with changes in metabolic status and reproductive performance in sheep [32]. Although these observations are relevant to the broader investigation of carnitine in animal reproduction, direct extrapolation to exercised Warmblood stallions is not warranted.

The descriptive differences observed in the present study are broadly consistent with previous reports of improved semen characteristics following L-carnitine supplementation in equine and non-equine settings [7, 14, 28, 32]. Nevertheless, quantitative comparisons across studies are constrained by differences in species, study populations, administration routes, supplementation duration, exercise exposure, and semen evaluation procedures.

Study limitations and implications for future research

The principal limitation of this study was the inclusion of only two stallions. Although the crossover design allowed each animal to serve as its own control, it did not overcome the limited number of independent biological experimental units. Repeated measurements collected from the same stallions across experimental conditions and sampling time points are correlated observations and cannot be treated as independent biological replicates.

Consequently, the reported statistical significance and the apparent magnitude of the differences among conditions require cautious interpretation. The findings should be considered exploratory and hypothesis-generating rather than confirmatory evidence of supplementation efficacy. In addition, independently randomized treatment sequences in only two animals cannot reliably exclude potential period, order, or carryover effects.

Several mechanistic limitations should also be acknowledged. The study did not measure oxidative stress biomarkers, antioxidant enzyme activities, mitochondrial function, intracellular energy metabolism, GnRH secretion, or seminal plasma proteins. Therefore, the proposed endocrine, antioxidant, and metabolic explanations remain unverified.

The study also did not directly assess fertility outcomes, such as pregnancy rates following AI. Accordingly, differences in semen quality measurements cannot be assumed to translate into improved fertility or breeding efficiency. Furthermore, although the study evaluated chilled semen for 72 h, it did not investigate frozen–thawed semen; its findings should not be extended to cryopreservation outcomes.

Several procedural details of semen processing, including the relative centrifugal force, the cooling rate, and the sperm concentration after resuspension, were not recorded, which limits exact reproducibility of the processing protocol. The same procedure was applied to all samples.

Another methodological consideration is the use of different approaches to assess progressive motility in fresh and chilled semen. Fresh motility was evaluated subjectively, whereas chilled motility was assessed using CASA. This difference limits direct comparisons between the two sets of measurements.

Future research should include more stallions, appropriately balanced treatment sequences, and statistical models that account for the data's repeated-measures structure. Studies that directly measure oxidative stress, mitochondrial function, reproductive endocrine activity, and seminal plasma composition would help clarify the mechanisms potentially underlying the observed responses.

Further investigations should also distinguish effects on semen quality at the beginning of storage from effects on the rate of deterioration during preservation. Direct assessment of fertility outcomes would be necessary before conclusions could be drawn regarding the practical value of systemic L-carnitine supplementation in equine breeding programs.

Practical relevance

The present findings suggest that intramuscular L-carnitine supplementation warrants further investigation as a potential intervention for physically active breeding stallions. Under the conditions examined, supplementation was associated with smaller reductions in body weight, higher circulating ICSH and testosterone concentrations, and more favorable fresh semen characteristics than exercise with placebo.

Although chilled semen quality remained higher during supplementation, the similar proportional decline across conditions indicates that supplementation did not specifically slow deterioration during storage. This distinction is important when considering the potential relevance of the findings to AI programs.

The observations do not establish that L-carnitine supplementation improves fertility, extends the usable storage period of chilled semen, or enhances breeding efficiency. Given the small sample size and the lack of direct mechanistic and fertility assessments, the present study alone does not support routine supplementation.

Overall, this preliminary investigation provides an initial description of reproductive responses associated with repeated exercise and intramuscular L-carnitine supplementation in Warmblood stallions. The findings support further controlled investigation but require confirmation in larger, adequately powered studies before their practical significance can be established.

CONCLUSION

This preliminary randomized-sequence crossover study evaluated the effects of intramuscular L-carnitine supplementation on reproductive hormone concentrations and fresh and chilled semen quality in two Warmblood stallions subjected to repeated exercise. Compared with exercise plus placebo, exercise plus L-carnitine supplementation was associated with a smaller reduction in body weight, higher circulating ICSH and testosterone concentrations, and higher progressive sperm motility, viability, and functional plasma membrane integrity in fresh semen.

During chilled storage at 5°C, progressive motility, viability, and plasma membrane integrity remained higher under the supplemented condition than under the exercise-plus-placebo condition at the evaluated time points. However, the proportional declines in these parameters over 72 h were similar across experimental conditions. Thus, the findings indicate differences in semen quality levels but do not demonstrate that L-carnitine supplementation slowed the rate of deterioration during chilled storage.

The crossover design allowed each stallion to undergo all three experimental conditions, facilitating within-animal comparisons while reducing the influence of differences between individual stallions. The exercise plus placebo condition provided a baseline for evaluating observations associated with L-carnitine administration during repeated exercise.

The study also assessed several complementary reproductive outcomes within the same experimental framework. These included circulating reproductive hormone concentrations, macroscopic and microscopic characteristics of fresh semen, and sperm function at successive intervals during chilled storage. Evaluating semen quality at 0, 24, 48, and 72 h allowed us to distinguish differences in semen quality levels from differences in their proportional decline during storage.

Intramuscular L-carnitine supplementation was associated with higher reproductive hormone concentrations and more favorable fresh and chilled semen-quality measurements than placebo administration during repeated exercise in the two Warmblood stallions studied. However, supplementation was not associated with a clearly slower proportional decline in sperm motility, viability, or plasma membrane integrity during chilled storage.

These findings should be regarded as hypothesis-generating observations rather than evidence of a confirmed reproductive benefit. Larger, adequately powered studies that include direct mechanistic measurements and fertility outcomes are needed before the practical value of intramuscular L-carnitine supplementation in breeding stallions can be established.

DATA AVAILABILITY

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

GENERATIVE AI DECLARATION

Generative artificial intelligence (AI) tools were utilized exclusively for language editing, grammatical refinement, and improving textual clarity. No AI tools were employed in data analysis, result interpretation, or the development of scientific conclusions.

AUTHORS' CONTRIBUTIONS

ASH: Study conceptualization and design, research methodology, experimental procedures, statistical analysis, and manuscript revision. ES: Study conceptualization and design, research methodology, interpretation of results, and manuscript writing and revision. TDL, SU, HAH, ISH, and SPM: Study design, experimental procedures, sample collection and processing, data acquisition, and statistical analysis. KKR, SRT, YBB, and PA: Experimental procedures, sample processing, data collection, and preparation of the initial manuscript draft. GJ and KSA: Interpretation of results and critical revision of the manuscript. All authors contributed to the study, reviewed the manuscript, and approved the final version for publication.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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