Cryobiology 115 (2024) 104884

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/cryo

L-*carnitine* enhances the kinematics and protects the sperm membranes of chilled and frozen-thawed Peruvian Paso horse spermatozoa

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ARTICLE INFO

Keywords:
L-*carnitine*
Cryopreservation
Spermatozoa
Peruvian Paso horse

ABSTRACT

L-*carnitine* (LC) transports fatty acids to the mitochondria for energy production, reducing lipid availability for peroxidation through β-oxidation. This research examines the effect of LC supplementation to two skimmed milk-based extenders on the cryosurvival of chilled (5°C) and frozen-thawed Peruvian Paso horse spermatozoa .An initial experiment determined the optimal LC concentration (0, 1, 5, 10, 25, and 50 mM) when added to INRA-96® and UHT (skimmed milk + 6% egg yolk) extenders, using nine ejaculates from three stallions chilled for up to 96 h. Subsequently, the effect of 25 mM LC supplementation (the optimal concentration) on chilling (INRA-96) and freezing (INRA-Freeze®) extenders was evaluated using eight pooled samples from sixteen ejaculates (2 ejaculates/pool) from four stallions. Results indicated that all LC concentrations produced significantly higher values ($P<0.05$) for kinematic variables (total [TM] and progressive motilities, curvilinear [VCL] and straight-line [VSL] velocity, and beat-cross frequency [BCF]), and the integrity of plasma/acrosome membranes (IPIA) compared to non-supplemented chilled sperm samples for up to 96 h with both extenders. Moreover, the use of 25 mM LC was more efficient ($P<0.05$) in preserving the post-chilled values of velocity, BCF, and IPIA for the long term than lower LC concentrations (1–10 mM). Post-thaw values of total motility, the amplitude of lateral head displacement (ALH), and IPIA were significantly improved ($P<0.05$) when INRA-Freeze extender was supplemented with 25 mM LC. In conclusion, supplementation of L-*carnitine* to skimmed milk-based extenders enhanced kinematic variables and protected the membrane integrity in chilled and frozen-thawed Peruvian Paso horse spermatozoa.

1. Introduction

The cryopreservation of Peruvian Paso horse sperm has enabled the long-term preservation of their genetic material [6,37]. However, the utilization of cryopreserved semen from this breed has faced challenges due to low sperm cryosurvival rates following chilling-warming [16] or freezing-thawing processes [10,50]. Indeed, fertility outcomes following artificial insemination (AI) using chilled or frozen-thawed semen remain uncertain. In this context, the cryopreservation of the Peruvian Paso horse sperm for use in AI programs is of paramount importance for their promotion. In a preliminary effort to enhance the cryosurvival of Peruvian Paso horse sperm, the freezing medium was supplemented with melatonin and caffeine, yielding promising results [53] although optimization is still pending.

During the cooling or freezing process, various subpopulations of equine spermatozoa experience irreversible damage. The primary injuries are associated with alterations in the plasmatic membrane due to oxidative stress, capacitation, and apoptosis-like changes [47,54]. These impairments lead to reduced sperm motility and compromised structural integrity of equine spermatozoa [9]. Additionally, cryopreservation induces an elevation in oxidative stress, primarily due to the increased production of reactive oxygen species (ROS), rendering sperm cells susceptible to attack by free radicals such as H_2O_2, O_2- and OH-. This heightened vulnerability is attributed to the abundance of polyunsaturated fatty acids (PUFAs) within the sperm cells [39,41]. The high PUFA content in the plasma membrane of stallion sperm intensifies its susceptibility to oxidative stress and subsequent lipid peroxidation (LPO), a process exacerbated by ROS [4,44]. These cumulative events

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https://doi.org/10.1016/j.cryobiol.2024.104884
Received 23 November 2023; Received in revised form 3 March 2024; Accepted 6 March 2024
Available online 7 March 2024

disrupt both the mitochondrial and plasma membranes, leading to DNA fragmentation that adversely affects motility and fertilization capability [51].

The application of antioxidants has shown potential in mitigating the deleterious impacts of ROS, thereby enhancing the quality of frozen-thawed sperm [7]. Furthermore, antioxidants have been identified as effective agents for safeguarding both plasmatic and mitochondrial membranes, while also minimizing DNA damage in cryopreserved equine sperm [15,55].

L-*carnitine* (LC), a compound derived from the synthesis of lysine and methionine, plays a pivotal role in facilitating the transport of short-, medium- and long-chain fatty acids into the mitochondria for *β-oxidation,* leading to the generation of readily available energy (ATP) that positively influences sperm motility [30]. The antioxidant capacity of LC has been attributed to its ability to augment glutathione peroxidase activity [24]. Previous studies have demonstrated the antioxidant, cold shock protective, and locomotion-stimulating effects of LC in the spermatozoa of humans [2,9], bulls [12], rams [22], mice [5], and pigs [32]. Previous studies on stallion sperm have reported the positive impact of LC on sperm motility [29]. Notably, it has been established that the supplementation of LC and pyruvate to skimmed milk extenders effectively maintains sperm motility parameters after 24 h of semen chilling at temperatures of 5 °C or 15 °C [8] as well as for 72 h at 22 °C [26]. The supplementation of 1 or 2 mM of LC into skimmed milk-based extenders has been shown to preserve the motility of equine sperm when chilled at 5 °C for 48 h [45]. Additionally, other studies have revealed that LC supplementation in post-thawed semen acts as an antioxidant and stimulates equine sperm metabolism [33]. To the best of our knowledge, there are few reports concerning the cryoprotective effects of LC in frozen-thawed stallion semen. Thus, the present study aims to evaluate the impact of LC supplementation in two skimmed milk-based extenders on kinematic variables and membrane integrity of both chilled and frozen-thawed Peruvian Paso horse spermatozoa.

2. Materials and methods

L-*carnitine* (Ref: 8400920025) was obtained from the Sigma-Aldrich Chemical Co. Both INRA-96® (Cod: 016441) and INRA-Freeze® (Cod: 021727) extenders were acquired from IMV-Technology (IMV, L 'Aigle, France). The extenders were meticulously prepared at the Animal Reproduction Biotechnology Research Laboratory located at 'Irquis Farm,' affiliated with the University of Cuenca (3°04′48.1″S 79°04′31.0″W), utilizing reagent-grade chemicals sourced from Sigma Chemical Co.

2.1. Animals and semen collection

Four sexually mature and clinically healthy Peruvian Paso horses, aged between 5 and 12 years and possessing an average body score of 7,5 (ranging from 1 to 9) as assessed by the Henneke scale [28], were enlisted for participation in this experimental study. These males were housed in 15 m^2 stables located at "*Las Marías*" farm (Paute, Azuay, Ecuador, 2°46′26.0″S 78°44′44.9″W). Food and water were provided *ad libitum*. The horses' diet primarily comprised 70% grass (green matter) and concentrated supplements (including grains, barley straw, and dried alfalfa) in accordance with the recommendations outlined by the National Research Council (NRC) guidelines of 2007. The daily management of the horses encompassed daytime grazing in open fields followed by confinement within the stables during the night.

Prior to the commencement of the experimental phase, two semen ejaculates were collected from each horse, separated by a 7-day interval, to deplete the extragonadal sperm reserves. All animal handling procedures adhered to protocols sanctioned by the Honorable Board of Directors of the Faculty of Agricultural Sciences at the University of Cuenca. Furthermore, this research was conducted in strict accordance with the principles delineated in chapter 7.8 of the Terrestrial Animal Health Code-2019© of the World Organization for Animal Health (OIE), dated 07/8/2019, pertaining to the ethical treatment of animals utilized in scientific investigations.

Prior to each semen collection, the penis was meticulously cleaned, and subsequent ejaculates were collected using an artificial vagina (Hannover model, Cod: 11220/0145, Minitube®, Germany) that had been pre-warmed to temperatures ranging between 45 °C and 48 °C. Immediately after semen collection, the ejaculate volume was determined, and two 10-mL aliquots were diluted in a 1:1 (v:v) ratio with both INRA-96 and UHT extenders at a temperature of 37 °C. These diluted samples were then expeditiously transported to the Laboratory of the University of Cuenca at 22 °C, all within a timeframe of less than 2 h from the initial collection.

Subsequently, the sperm concentration of each ejaculate was determined utilizing a Neubauer chamber (Marienfeld, Lauda-Königshofen, Germany). Sperm motility and quality scores (rated on a mass motility scale of 0 [lowest] to 5 [highest]) were initially assessed using a phase contrast microscope (Nikon Eclipse model 50i; employing negative contrast) at a magnification of 10X. Only ejaculates with more than 200 $\times$ 10^6 sperm/mL,70% of sperm motility, and motility quality score above were included in the two subsequent experiments.

Freshly-extended samples, pre-diluted in a 1:1 (v:v) ratio with INRA-96 and UHT extenders, underwent centrifugation at 400×*g* for 10 min. The resultant pellets were resuspended with 3 mL of the respective extenders, in preparation for either the chilling or freezing procedures. These rediluted samples were collectively referred to as *'stock samples'*.

2.2. Experimental design

This study was conducted in two consecutive experiments to assess the effect of L-*carnitine* (LC) on the viability and functionality of Peruvian Paso horse spermatozoa, encompassing fresh-extended, chilled-warmed, and frozen-thawed semen.

2.2.1. Experiment 1: Assessment of suitable L-carnitine concentration

The first experiment aimed to ascertain the optimal LC concentration for supplementation in both INRA-96 and UHT extenders, considering their impact on sperm kinematic parameters and membrane integrity. The UHT extender was made by blending *ultra-high temperature-treated* commercial skimmed milk, supplemented with antibiotics (100,000 IU penicillin sodium and 100 mg dihydrostreptomycin per 100 ml), and 6% (v:v) egg yolk. This extender was characterized by an osmolarity range of 298–310 mOsm/kg and a pH level of 6.9–7.2 [22].

In total, nine ejaculates were collected from three Peruvian Paso horses (three ejaculates/male). The *stock samples* from each ejaculate, utilizing either the INRA or UHT extender, were split into six aliquots. These aliquots were subsequently allocated to create six distinct treatment groups, each varying in L-*carnitine* concentration: 0 (as control), 1 mM (LC-1), 5 mM (LC-5), 10 mM (LC-10), 25 mM (LC-25), or 50 mM (LC-50). In total, twelve treatment combinations were established for both extender types, with all treatments standardized to attain a final sperm concentration of 50 $\times$ 10^6 sperm/mL.

The kinematic parameters and the integrity of both plasma and acrosome membranes were evaluated in samples from all treatments at three time points: immediately after collection (referred to as 'fresh'), as well as after 48 and 96 h of cold storage at 5 °C.

2.2.2. Experiment 2: Effect of L-carnitine on chilled and frozen-thawed equine semen

The second experiment assessed the impact of the optimal LC concentration (i.e., 25 mM) along with the extender exhibiting the superior kinetic motion (INRA, based on VCL and VSL values) determined in the first experiment. Subsequently, 25 mM LC was incorporated into both the chilling extender (INRA-96) and the freezing extender (INRA-Freeze), aiming to enhance the cryosurvival of equine sperm.

Eight pooled samples from sixteen ejaculates (two ejaculates

randomly selected) obtained from four Peruvian Paso horses, were employed in accordance with the methodology established by Dorado et al. [17].

The INRA-96 *stock samples* were employed to create two distinct chilling treatments, each characterized by LC supplementation levels: 0 (control) and 25 mM (LC-25). All treatments were meticulously standardized to achieve a final sperm concentration of 50×10^6 sperm/mL. Kinematic parameters and the integrity of both plasma and acrosome membranes were methodically assessed at three-time intervals: immediately following collection (0 h, designated as 'fresh'), as well as after 24 and 72 h of cold storage at 5 °C. The selection of the 24 h and 72 h time points at 5 °C was based on the observations from the preceding experiment, wherein a substantial decline in motility was identified at 48 h. Furthermore, no data were available for the 24 h time point. It is noteworthy that chilled equine semen is commonly employed for *in vivo* AI after 24 h of cold-storage, while cold-storage for up to 72 h is utilized to discern differences between treatments [49].

Similarly, the INRA-Freeze *stock samples* were employed to create two freezing treatments, each characterized by LC supplementation: 0 (as control) or 25 mM (LC-25), both meticulously adjusted to achieve a final sperm concentration of 50×10^6 sperm/mL. Subsequently, both the control and LC-25 treatments were subjected to a 75 min of cold storage at 5 °C. Thereafter, the samples were manually loaded into 0.25 mL straws (IMV, L'Aigle, France), with each straw meticulously sealed using polyvinyl alcohol. The freezing procedure, as outlined by Tamay et al. [53], involved a well-defined protocol encompassing the use of a Styrofoam cryo-box ($30 \times 29 \times 31$ cm dimensions for length, width, and height, respectively) with a capacity of 3.4 L of liquid nitrogen (LN_2). The freezing process incorporated two internal ramps placed at distinct distances. Initially, the straws were placed on the first ramp, situated 17 cm above the LN_2 surface for 4 min. Subsequently, the straws were promptly transferred to the second, lower ramp and maintained at a height of 7 cm above the LN_2 for an additional 2 min. Ultimately, the straws were submerged into LN_2 and maintained for 2 months. A total of 112 straws were frozen, equally divided between the two treatments: control (n = 56) and LC-25 (n = 56). All frozen sperm samples underwent thawing subsequent to a two-month cryopreservation period.

The thawing process by placing the straws in a water bath set to 37 °C for 30 s. The thawed contents were subsequently transferred into dry 1.5 mL Eppendorf tubes and subjected to a 5-min incubation at 37 °C.

2.3. Sperm analysis

Prior to semen analysis, all samples were incubated at 37 °C for 5 min. Sperm kinematic parameters were objectively assessed using a CASA system (*Sperm Class Analyzer, SCA-Evolution® 2018, v.6.4.0.99 software. Microptic S.L., Barcelona, Spain*) coupled to a phase contrast microscope (Nikon Eclipse model 50i; negative contrast) with the following settings: 25 frames/s, head area 20–70 μm^2, velocity limit for slow sperm 10 mm/s, velocity limit for medium sperm 45 μm/s, velocity limit for fast sperm 90 μm/s, and minimal straightness for progressive spermatozoa 75%. Sperm samples (5 μL) were placed on slides warmed at 37 °C and covered with a coverslip. A minimum of three fields and at least 200 sperm tracks per field (average: 600 spermatozoa per sample evaluated) were evaluated at 10 × magnification on each sample slide. The percentage of total (TM) and progressive (PSM) sperm motility, curvilinear velocity (VCL, μm/s), average-path velocity (VAP, μm/s), straight-line velocity (VSL, μm/s), straightness (STR, %), linearity (LIN, %), wobble (WOB, %), the amplitude of lateral head displacement (ALH, μm) and beat-cross frequency (BCF, Hz) were assessed as described by Tamay et al. [53].

The status of plasma and acrosome membranes was assessed using a double association of fluorescent probes – propidium iodide (PI, Sigma P-4170) and fluorescein isothiocyanate conjugated peanut (*Arachis hypogaea*) agglutinin (PNA-FITC, Sigma L7381), according to Tamay et al. [6]. A total of 200 sperm per slide were examined using a Nikon Eclipse E200 epifluorescence light microscope (Nikon Instruments Inc. New York, NY, USA) with a triple-band pass filter (40 × magnification with an excitation: 450–490 nm, and emission: 520 nm) and four subpopulations of cells were quantified, i.e., those showing in percentages: (1) intact plasma membrane/intact acrosome (IPIA); (2) intact plasma membrane/damaged acrosome (IPDA); (3) damaged plasma membrane/intact acrosome (DPIA); and (4) damaged plasma membrane/damaged acrosome (DPDA). In addition, the total percentage of cells presenting an intact plasma membrane equivalent to live sperm (Total IP: IPIA + IPDA) and total intact acrosomal membrane (Total IA: IPIA + DPIA) were calculated.

2.4. Statistical analysis

Results are presented as mean ± SEM. Values for sperm variables that showed non-normal distributions, as determined by the Shapiro–Wilk test, were *arcsine*- or *log10*-transformed (percentages and numeric values, respectively) before analysis. In the first experiment, the effects of the *L-carnitine concentrations*, *extender type*, *chilling time,* and its interaction on the kinematic variables and integrity of sperm membranes were compared using repeated-measures ANOVA and Bonferroni's multiple range test, employing the General Linear Model procedure. In addition, the *male* factor as covariable was included in this analysis due to variability between stallions.

In the second experiment, the effect of 25 mM LC supplementation on INRA-96 or INRA-freeze extenders was analyzed by a one-way ANOVA, and Tukey's post hoc multiple comparison tests were used. Significance was set at $P < 0.05$. All calculations were made using Statistica software for Windows v.12.0 (StatSoft Inc, Tusla, OK, USA).

3. Results

3.1. Experiment 1: effect of extenders and storage time on chilled semen

The interaction *LC concentrations* × *extender type* × *chilling time* have a significant effect on IPIA ($P < 0.05$), IPDA ($P < 0.05$), and total IP percentages ($P < 0.001$). However, the interaction *LC concentrations* × *chilling time* ($P < 0.05$) had an effect on VSL, STR, and LIN. Additionally, the interaction *extender type* × *chilling time* significantly influenced TM ($P < 0.01$), VCL ($P < 0.001$), VSL ($P < 0.01$), ALH ($P < 0.001$), and BCF ($P < 0.001$) (See Figs. 1 and 2). In fact, VCL and VSL values remained above 60 μm/s and 40 μm/s, respectively, at 96 h of cold storage using INRA-96 extender, unlike the UHT extender (Fig. 1).

In fresh semen samples, LC supplementation to the UHT extender improved ($P < 0.05$) the values of VSL (i.e., LC-1, LC-10 and LC-25 treatments) and BCF (LC-50) compared to their respective controls. Furthermore, in chilled semen samples, all LC concentrations (1–50 mM) added to both the INRA-96 and UHT extenders resulted in significantly higher values ($P < 0.01$) for motilities (i.e., TM and PSM), velocities (i.e., VCL and VSL), progression ratio values (i.e., STR and LIN), ALH, and BCF at 48 and 96 h of cold storage compared to their respective control groups (Fig. 1).

All kinematic parameters in non-supplemented sperm samples (controls) significantly reduced their values ($P < 0.01$) after 48 h and/or 96 h of cold storage, regardless of extender type. At 48 h of cold-storage, the LC-10 and LC-25 treatments yielded higher ($P < 0.05$) values for TM, VSL, LIN, and BCF compared to the LC-50 treatment with UHT extender. Meanwhile, with INRA-96, the LC-10 treatment showed a greater ($P < 0.05$) value of VSL than the LC-50 treatment. At 96 h of cold storage with the INRA-96 extender, the values of VCL, VSL, LIN, and ALH were higher ($P < 0.05$) following the LC-25 and LC-50 treatments than after LC-1 and LC-5 treatments. In fact, with the LC-25 treatment, the BCF was higher ($P < 0.05$) than with all other LC treatments (including the control) (Fig. 1).

In terms of sperm membranes status, the LC-25 and LC-50 treatments with the INRA-96 extender exhibited higher percentages ($P < 0.05$) of

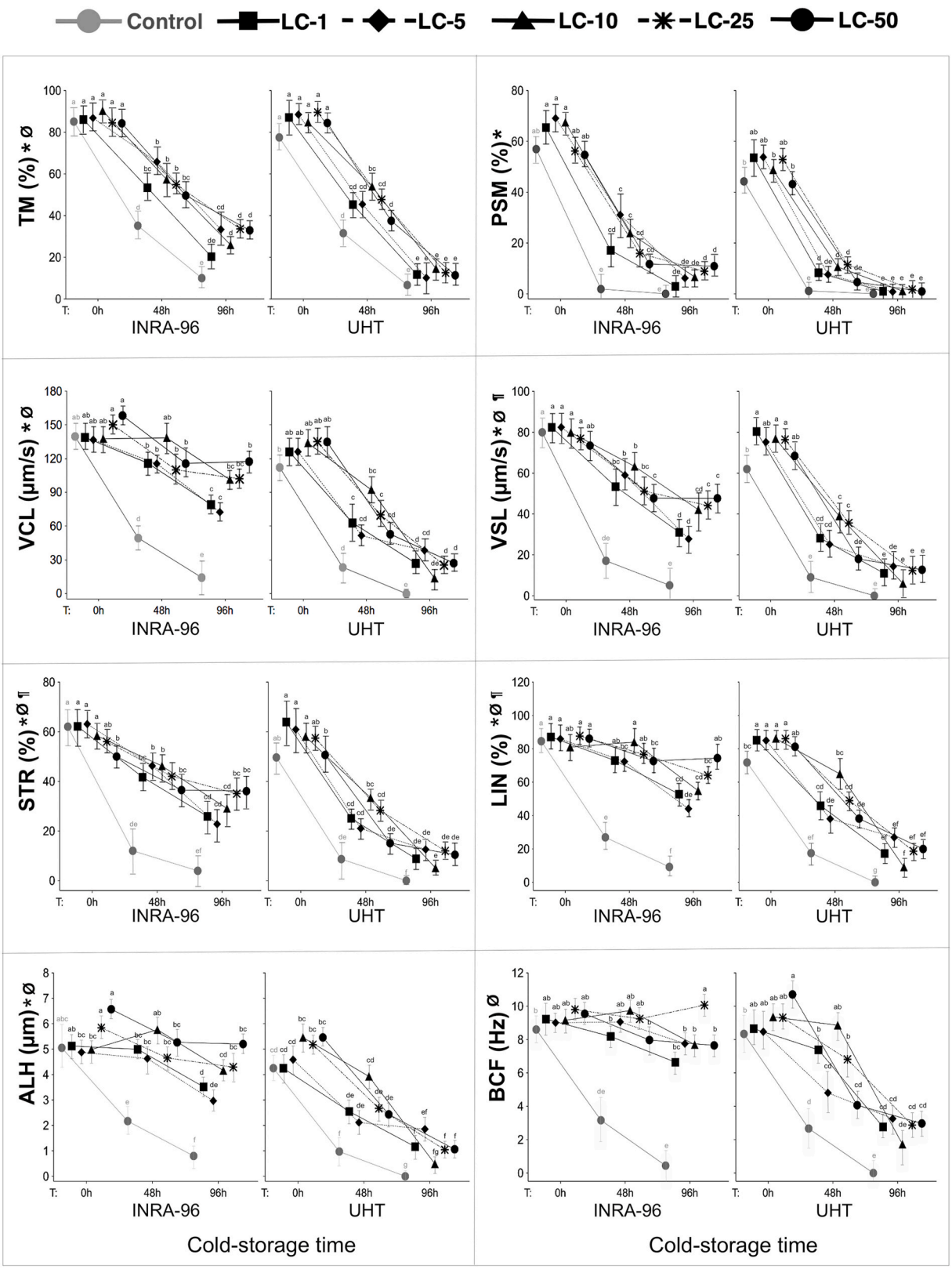

Fig. 1. Kinematic variables for Peruvian Paso horse sperm diluted with both INRA-96 and UHT (skimmed milk + egg yolk) extenders supplemented with either 0 (control), 1 (LC-1), 5 (LC-5), 10 (LC-10), 25 (LC-25), and 50 mM (LC-50) L-*carnitine*, and chilled (5 °C) for up to 96 h. TM (%), total motility; PSM (%), progressive sperm motility; VCL (µm/s), curvilinear velocity; VSL (µm/s), straight line velocity; STR (%) straightness; LIN (%), linearity, ALH (µm), amplitude of lateral head displacement; and BCF (Hz) beat–cross frequency. * Differences between stallions (males) ($P < 0.05$). ø Interaction between *extender type* × *chilling time* ($P < 0.01$). ¶ Interaction between *LC concentrations* × *chilling time* ($P < 0.05$). Different letters in superscript (a–e) at each evaluation time indicate significant differences between values for extenders and cold storage times in each kinematic variables ($P < 0.05$ for a – b – c – d – e – f – g; $P < 0.01$ for a – c, b – d, c – e, d – f, and e – g; and $P < 0.001$ for a – d, a – e, b – e, and c - f).

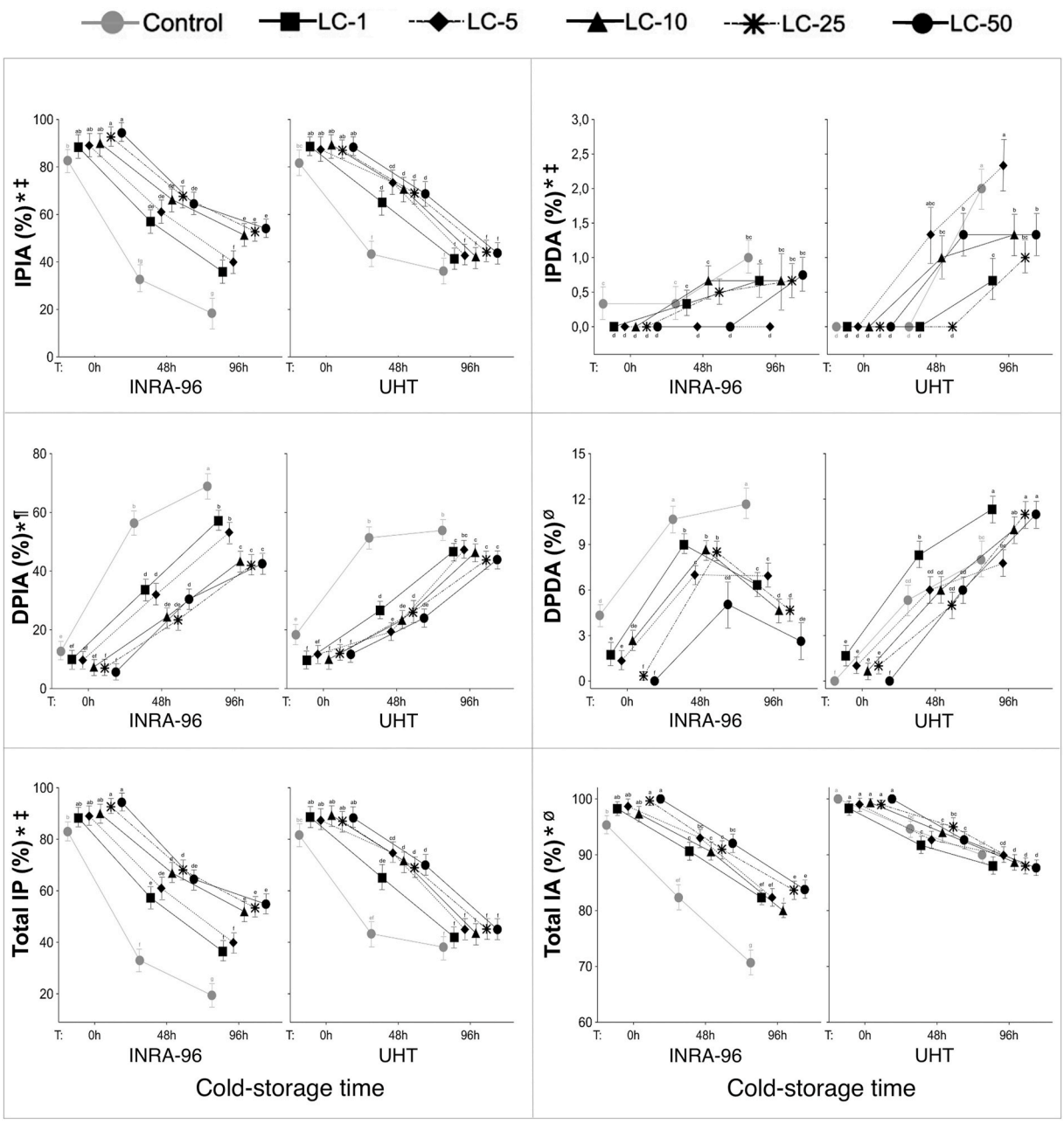

Fig. 2. Simultaneous integrity of plasma and acrosome membranes (IPIA) for Peruvian Paso horse sperm diluted with both INRA-96 and UHT (skimmed milk + egg yolk) extenders supplemented with either 0 (control), 1 (LC-1), 5 (LC-5), 10 (LC-10), 25 (LC-25), and 50 mM (LC-50) L-*carnitine*, and chilled (5 °C) for up to 96 h * Differences between stallions (males) ($P < 0.05$). ‡ Interaction between *LC concentrations* × *extender type* × *chilling time* ($P < 0.05$). ¶ Interaction between *LC concentrations* × *chilling time* ($P < 0.05$). ø Interaction between *extender type* × *chilling time* ($P < 0.01$). Different letters in superscript (a–e) at each evaluation time indicate significant differences between values for extenders and cold storage times in each kinematic variables ($P < 0.05$ for a – b – c – d – e – f – g; $P < 0.01$ for a – c, b – d, c – e, d – f, and e – g; and $P < 0.001$ for a – d, a – e, a – g, b – e, b – f, b – f, and c – g).

simultaneous integrity (IPIA) and total integrity of plasma (total IP) and acrosome (total IA) membranes in fresh samples. Similarly, all LC treatments showed higher percentages of IPIA and total IP ($P < 0.05$) than their respective controls during 48 and 96 h of cold storage with the INRA-96 extender. However, with the UHT extender, all LC treatments showed higher percentages ($P < 0.01$) of IPIA and total IP only after 48 h of cold storage. In this same way, all LC treatments resulted in lower undesirable percentages ($P < 0.01$) of DPIA and DPDA during the 48 and 96 h of chilling (Fig. 2).

3.2. *Effect of L-carnitine on chilled and frozen-thawed semen*

Data for the kinematic variables and the status of sperm membranes are included in Tables 1 and 2, respectively. Overall, except for the progression ratio values (i.e., STR, LIN, and WOB), the kinematic variables decreased ($P < 0.05$) after chilling (24 or 72 h) or freezing-thawing processes compared to their fresh values. In the kinematic analysis, the chilled samples decreased ($P < 0.05$) at 24 h (TM, PSM, VCL, VAP, and VSL) and 72 h (BCF), regardless of LC supplementation. However, only after LC-25 treatment, the BCF was greater ($P < 0.05$) than in the control. After the freezing-thawing process, the TM and ALH improved ($P < 0.05$) with LC-25 treatment when compared to the control (Table 1).

Table 1
Kinematics variables (mean ± SEM) for samples either fresh, chilled or frozen-thawed sperm of Peruvian Paso horse supplemented or not with 25 mM L-*carnitine* (LC-25).

Kinematic variables	Fresh samples		Chilled samples				Frozen-thawed samples	
			24 h		72 h			
	Control	LC-25	Control	LC-25	Control	LC-25	Control	LC-25
TM (%)	87.3 ± 2.75^{a}	93.3 ± 1.88^{a}	80.0 ± 4.14^{ab}	86.4 ± 3.22^{a}	65.3 ± 7.12^{b}	69.7 ± 6.50^{b}	38.0 ± 3.48^{c}	52.8 ± 2.40^{b}
PSM (%)	65.1 ± 4.50^{ab}	74.7 ± 1.68^{a}	47.4 ± 3.16^{b}	52.4 ± 3.67^{ab}	32.6 ± 5.75^{cd}	32.6 ± 6.67^{cd}	17.4 ± 2.26^{d}	20.3 ± 1.96^{d}
VCL (μm/s)	136.5 ± 2.38^{a}	137.0 ± 3.94^{a}	74.2 ± 1.51^{b}	75.6 ± 2.55^{b}	63.6 ± 5.80^{b}	66.6 ± 8.69^{b}	66.0 ± 2.74^{b}	65.0 ± 1.85^{b}
VAP (μm/s)	91.3 ± 1.91^{a}	91.6 ± 2.65^{a}	48.8 ± 0.97^{b}	49.9 ± 1.72^{b}	35.9 ± 4.46^{b}	43.7 ± 6.54^{b}	36.9 ± 4.73^{b}	43.8 ± 2.80^{b}
VSL (μm/s)	76.6 ± 1.90^{a}	78.6 ± 1.52^{a}	37.8 ± 0.90^{b}	37.2 ± 1.36^{b}	27.3 ± 3.40^{c}	31.3 ± 4.38^{bc}	32.1 ± 2.46^{bc}	34.6 ± 2.67^{bc}
STR (%)	83.8 ± 0.57	84.1 ± 0.42	71.2 ± 1.70	71.5 ± 1.93	66.2 ± 3.00	78.5 ± 3.60	87.6 ± 0.79	86.8 ± 0.87
LIN (%)	58.1 ± 1.61^{ab}	58.9 ± 2.83^{ab}	48.5 ± 1.02^{b}	47.4 ± 2.43^{b}	35.1 ± 2.89^{b}	41.6 ± 3.07^{b}	63.3 ± 2.56^{a}	57.4 ± 2.62^{ab}
WOB (%)	70.2 ± 2.19	69.2 ± 3.21	67.7 ± 2.28	66.6 ± 2.81	51.1 ± 2.84	58.6 ± 3.45	70.5 ± 2.54	65.7 ± 2.66
ALH (μm)	5.1 ± 0.19^{a}	5.1 ± 0.33^{a}	4.1 ± 1.18^{ab}	3.3 ± 0.45^{b}	2.7 ± 0.19^{c}	2.7 ± 0.27^{c}	3.9 ± 0.21^{b}	4.3 ± 0.18^{a}
BCF (Hz)	9.4 ± 0.36^{b}	9.6 ± 0.23^{b}	8.0 ± 0.23^{c}	8.1 ± 0.29^{c}	6.6 ± 0.67^{d}	7.5 ± 0.82^{c}	11.9 ± 0.53^{a}	11.1 ± 0.36^{a}

TM: total motility; PSM: progressive sperm motility; VCL: curvilinear velocity; VAP: average path velocity; VSL: straight line velocity; STR: straightness; LIN: linearity; WOB: wobble; ALH: amplitude of lateral head displacement; BCF: beat-cross frequency. $^{a–e}$ Different superscripts within a same row differ significantly between treatments ($^{a-b-c}$ $P < 0.05$; $^{a-c}$ $P < 0.01$).

Table 2
Categories of membranes integrity (mean ± SEM) in fresh, chilled and frozen-thawed sperm of Peruvian Paso horse supplemented or not with 25 mM L-carnitine (LC-25).

Fluorescence parameters	Fresh samples		Chilled samples				Frozen-thawed samples	
			24 h		72 h			
	Control	LC-25	Control	LC-25	Control	LC-25	Control	LC-25
IPIA (%)	74.2 ± 2.07^{b}	83.0 ± 1.77^{a}	69.9 ± 1.98^{c}	81.9 ± 1.22^{ab}	57.8 ± 5.02^{d}	66.9 ± 3.22^{cd}	37.0 ± 1.47^{e}	49.3 ± 1.63^{cd}
IPDA (%)	0.88 ± 0.47^{c}	0.00^{c}	2.13 ± 0.30^{ab}	0.75 ± 0.16^{c}	3.0 ± 0.38^{a}	2.25 ± 0.37^{a}	2.0 ± 0.37^{b}	2.13 ± 0.36^{b}
DPIA (%)	22.2 ± 2.37^{dc}	15.1 ± 1.70^{d}	22.9 ± 2.17^{cd}	14.3 ± 1.36^{d}	31.1 ± 4.49^{b}	22.6 ± 2.46^{c}	43.9 ± 1.93^{a}	34.1 ± 2.27^{b}
DPDA (%)	2.7 ± 1.03^{b}	1.7 ± 0.88^{b}	5.1 ± 1.01^{b}	3.1 ± 0.72^{b}	8.1 ± 0.69^{ab}	8.3 ± 1.19^{ab}	17.1 ± 1.75^{a}	14.5 ± 1.66^{a}
Total IP (%)	75.1 ± 2.06^{b}	85.3 ± 1.27^{a}	72.0 ± 2.01^{b}	82.6 ± 1.31^{a}	60.8 ± 4.74^{bc}	69.1 ± 3.06^{b}	39.0 ± 1.53^{d}	51.4 ± 1.71^{c}
Total IA (%)	96.4 ± 1.01^{a}	98.1 ± 0.88^{a}	92.8 ± 0.92^{a}	96.1 ± 0.64^{a}	88.9 ± 0.85^{ab}	89.5 ± 1.39^{ab}	80.9 ± 1.73^{b}	83.4 ± 1.82^{b}

IPIA: intact plasma membrane/intact acrosome; IPDA: intact plasma membrane/damaged acrosome; DPIA: damaged plasma membrane/intact acrosome; DPDA: damaged plasma membrane/damaged acrosome; IP: total sperm with plasmalemma integrity (or viability); and IA: total sperm with intact acrosome. $^{a–e}$ Different superscripts within a same row differ significantly between treatments ($^{a-b-c-d}$ $P < 0.05$; $^{a-c,\ b-d}$ $P < 0.01$; $^{a-d}$ $P < 0.001$).

After the LC-25 treatment, the IPIA and total IP percentages improved ($P < 0.05$) in fresh and 24-h chilled samples compared to their controls. In fact, with the LC-25 treatment, the IPIA percentage decreased ($P < 0.05$) after 72 h of cold storage. In this regard, lower ($P < 0.05$) undesirable percentages of IPDA (at 24 h) and DPIA (at 72 h) were observed after the LC-25 treatment compared to their controls. Furthermore, the freezing-thawing process affected the IPIA, total IP, and total IA percentages, decreasing ($P < 0.05$) their values, regardless of LC supplementation. However, the post-thaw IPIA and total IP percentages were greater ($P < 0.05$) after the LC-25 treatment than after the controls. Additionally, a lower ($P < 0.05$) undesirable DPIA percentage was obtained after the LC-25 treatment compared to its control (Table 2).

4. Discussion

The results indicated that LC supplementation (1–50 mM) in skimmed-milk-based extenders enhanced kinematic variables and protected sperm membranes of chilled Peruvian Paso horse sperm for medium-term (e.g., 48 h) or long-term (e.g., 96 h) preservation. Moreover, the concentration of 25 mM LC was more efficient than lower concentrations (i.e., 1–10 mM LC) due to a better response in locomotion stimulation and the preservation of the integrity of the plasmatic and acrosomal membranes under chilled conditions. Consequently, supplementing with 25 mM LC in both chilling and freezing extenders showed an improvement in the integrity of sperm membranes in fresh, chilled (24 h), and frozen-thawed pooled sperm samples, respectively. Based on the aforementioned findings, L-*carnitine* may be an useful additive to extenders for chilling or freezing Peruvian Paso horse spermatozoa since it improves cryosurvival.

L-*carnitine* is well-known for its antioxidant and free radical scavenging properties, which facilitate a reduction in mitochondrial and cytosolic ROS and, consequently, result in a decrease in LPO. These effects of LC have been previously demonstrated in cold-stored equine semen [14]. LC has a powerful antioxidant effect by reducing the availability of lipids for peroxidation and increasing the activity of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase [27]. Furthermore, LC acts as a carrier for the translocation of long-chain fatty acids across the inner membrane of mitochondria for β-oxidation. Consequently, ATP production provides readily available energy for use by spermatozoa [1]. It has been reported that LC can protect cell membranes against ROS damage and maintain membrane structure by regulating carbohydrate metabolism [27,34]. Our results showed that LC supplementation to skimmed milk-based extenders significantly improved motilities (TM and PSM), motion kinetics (VCL and VSL), and the integrity of membranes (plasma and acrosome) in fresh and cryopreserved sperm samples (chilled and frozen-thawed). Previous studies have reported a direct correlation between sperm motility and velocity parameters (VCL and VSL) with fertility, emphasizing their pivotal role in sperm fertilizing oocytes within the oviductal tract [3,31] In fact, it has been shown that male fertility is determined mainly by sperm swimming velocity and sperm morphology [40].

A noteworthy finding in the present study was the significant increase in ALH and BCF values of equine sperm following supplementation with L-carnitine (LC) in both chilling and freezing media. It is well-established that LC enhances ATP production by facilitating mitochondrial *β*-oxidation. Existing evidence supports the notion that ATP activates purinergic P2 receptors, potentially leading to elevated

intracellular Ca_2+ levels, resulting in hyperactivation and acrosome exocytosis [18,38]. These processes, in turn, contribute to improved flagellar movement. We propose that through this mechanism, LC-induced sperm hyperactivation and enhanced flagellar movement culminated in the observed increase in ALH and BCF values.

The appropriate LC concentration for stallion sperm has varied between extenders, breeds, and storage temperatures. Stradaioli et al. [52] determined that oral LC administration to stallions (light-horse breeds) with oligoasthenospermic ejaculates improved sperm kinematic variables and morphological characteristics. Gibb et al. [26] reported that LC supplementation at 50 or 100 mM to a synthetic based-extender (modified Biggers, Whitten, and Whittingham, BWW) yielded higher percentages of TM and PSM than their controls in Shetland and Miniature crossbred pony stallions. Furthermore, the authors combined 50 mM LC with 10 mM *pyruvate,* obtaining better motilities when storing equine semen for 72 h at room temperature. Lisboa et al. [35,36] improved some sperm kinematic variables (e.g., TM, PSM, and VSL) of chilled stallion semen for 48 h when the skimmed milk extender was supplemented with LC (0.1 mM/mL), *acetyl-L-carnitine* (0.1 mM/mL), or their combination. Likewise, Nery et al. (2020) determined that the addition of 0.5, 1, and 2 mM of LC a skimmed milk-based extender preserved the motility of Quarter Horse stallions sperm stored at 5 °C for up to 48 h. Consistent with the results reported by Gibb et al. [27], and in contrast to the previously mentioned works, the present study showed that a concentration of 25 mM LC was the most suitable for improving kinematics variables and preserving the integrity of membranes in chilled sperm for up to 96 h from Peruvian Paso horse. Storing equine semen at 5 °C was successful in preserving kinematics and the integrity of membranes in the long term. A temperature of 5 °C was sufficient to restrict metabolism and maintain sperm functionality for more than 72 h [14,25].

The use of chilled-warmed or frozen-thawed semen from Peruvian Paso horses is rarely employed in AI programs due to the low survival of sperm after thawing or warming processes, respectively. Indeed, pregnancy rates in this breed following AI with fresh, chilled, or frozen-thawed sperm are unknown. There are few reports on chilled Peruvian Paso horse sperm, indicating that semen quality progressively decreases as chilling time increases (24 or 48 h). Dellepiane et al. [16] demonstrated that the percentage of live sperm of Peruvian Paso horse with an intact acrosome decreased after chilling at 5 °C (0 h: 55% compared to 48 h: 31%). Ávila et al. [8] showed that the addition of 6 mM LC along with 6 mM *pyruvate* to the Kenney extender maintained the quality of Peruvian Paso horse sperm for only during 24 h of cold storage (5 or 15 °C). In contrast to previous studies, the present work, in the first instance, demonstrated that 25 mM LC maintains the kinematic variables and effectively protects sperm membranes during chilling storage for short, medium, and long durations.

Efforts to improve the cryopreservation response of Peruvian Paso horse sperm have been made. The use of cryoprotectant agents (e.g., amides and glycerol) [50] and supplementation to freezing medium with low-density lipoproteins + egg-yolk [10] or melatonin and caffeine [53] has been explored. However, the achievements in post-thaw motility and the integrity of sperm membranes have been low, ranging from 18% to 46%. Cryopreservation and thawing processes reduce acrosomal integrity, viability, and motility of stallion sperm [44,53]. Deleterious effects induced by the cryopreservation process is mainly attributed to the osmotic stress. The result of this osmotic stress includes membrane damage, DNA damage, and ROS production, which causes premature capacitation-like changes [13,43].

The supplementation with 25 mM LC improved the motility, ALH, viability (total IP), and simultaneous integrity of plasmatic and acrosome membranes (IPIA) after the freezing-thawing process. It has been suggested that the mechanism of action of LC during the freezing-thawing process involves its antioxidant properties, which reduce ROS levels and play a role in equine sperm metabolism [33]. L-*carnitine* has been shown to increase glutathione levels and mitochondrial activity in frozen-thawed sperm [48]. Consequently, these beneficial effects of LC help decrease LPO and protein carbonylation, ultimately improving sperm motility, viability, as well as acrosome and DNA integrity [48].

The cryoprotective effect of LC has been demonstrated in humans [9], goats [11], roosters [19], and mice [48] sperm, improving motility and protecting plasma (viability) and acrosome membranes after cryopreservation while reducing ROS levels. In stallion sperm, however, only one previous report assessed the effect of oral supplementation with nutraceuticals (antioxidants such as LC and fatty acids) for 60 days and demonstrated an improvement in the quality of fresh, cooled, and frozen-thawed sperm from Mangalarga Marchador stallions [20]. Nonetheless, the administration pathway and the synergy between primary antioxidants (L-*carnitine* and *selenium*) and fatty acids (omega-3, omega-6) did not allow us to define the direct antioxidant or cryoprotective effect of LC.

The supplementation with 25 mM LC improved the motility, ALH, viability (total IP), and simultaneous integrity of plasmatic and acrosome membranes (IPIA) after the freezing-thawing process. It is likely that the two-ramp freezing system used in this study, which yielded accelerated cooling rates, as shown by Refs. [21,23,53], resulted in higher sperm cryosurvival rates. This effect has been previously demonstrated by Moore et al. [42] and Oldenhof et al. [46] in other horse breeds, such as Hanoverian warmblood and light-horse stallions. The other works mentioned earlier froze Peruvian Paso horse sperm using LN_2 vapors under a conventional system [10] or with a controlled-rate freezer [50]. Our findings regarding the cryoprotective effect of LC on equine spermatozoa are consistent with the results of previous research. This study is likely the first report to evaluate the cryoprotective effect of LC in stallion semen.

In conclusion, L-carnitine supplementation to skimmed milk-based extenders enhanced kinematic variables and protected the integrity of the plasmatic and acrosome membranes of chilled Peruvian Paso horse spermatozoa for the long-term. Moreover, the concentration of 25 mM L-*carnitine* was the most suitable, as it protected both plasmalemma and acrosome of Peruvian Paso horse sperm in fresh, chilled (24 h), or frozen-thawed pooled samples.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Declaration of competing interest

None of the authors have any conflict of interest to declare.

Acknowledgements

The authors thank the Peruvian Paso horse farm "Las Marías", especially its owner, Dr. Xavier Palacios, for their collaboration with the stallions.

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