BIOPRESERVATION AND BIOBANKING
Volume 00, Number 00, 2022

DOI: 10.1089/bio.2022.0097

Effect of Melatonin and Caffeine Supplementation to Freezing Medium on Cryosurvival of Peruvian Paso Horse Sperm Using a Two-Step Accelerating Cooling Rate

Erika Tamay,[1] Paula Palacios,[2] Gabriela Peláez,[2] Luis Rodrigo Saa,[1] Jesús Dorado,[3] Julián Santiago-Moreno,[4] and Diego A. Galarza[2]

This research examined the antioxidant and cryoprotective effects of melatonin (ME) and caffeine (CAF) supplementation in freezing medium on the cryosurvival of Peruvian Paso horse sperm using a two-step accelerating cooling rate. Twenty ejaculates from four adult and fertile stallions were recovered, initially diluted with INRA-96®, and finally frozen with INRA-Freeze® with either no supplementation (as control), 1 μM ME, or 2 mM CAF using a two-ramp freezing system content inside a cryogenic-box and liquid nitrogen vapors. The sperm kinematic parameters and integrity of the plasma and acrosomal membranes of fresh semen and cryopreserved samples were evaluated using the CASA system (SCA-Evolution® 2018) and PI/fluorescein isothiocyanate-conjugated peanut (Arachis hypogaea) agglutinin double fluorescent test, respectively. The oxidative stress of post-thaw sperm samples was also assessed using the CellRox Deep Red fluorescence test. The results showed that curvilinear velocity and average-path velocity were greater ($p < 0.05$) after freezing with CAF than the control group. In addition, there were significance differences ($p < 0.01$) between stallions (1–4) in post-thaw kinematic parameters regardless of ME or CAF addition. Both ME and CAF improved ($p < 0.05$) the proportion of sperm with intact plasma membranes and intact acrosomes. Nevertheless, neither CAF nor ME improved the oxidative stress after the cryopreservation process.

Keywords: melatonin, caffeine, Peruvian Paso horse, spermatozoa, oxidative stress

Introduction

The Peruvian Paso horse is a breed originating from Peru and is also bred in some other Andean countries (e.g., Ecuador and Colombia); it is a flagship breed of these countries. Therefore, it is very important to work on promoting and preserving their genetic material through semen. The use of cryopreserved Peruvian Paso stallion sperm is rare due to the low cryosurvival of sperm, with poor results after thawing.[1–3] In addition, to the best of our knowledge, there are no reports on the post-thaw sperm quality and fertility in this breed after artificial insemination (AI) of frozen-thawed semen.

The breed could influence pregnancy rates of cryopreserved semen. More recently, other studies determined that amides[3,4] and low-density lipoprotein[1] are suitable cryoprotectant agents for freezing spermatozoa. Thereby, the development of assisted reproductive techniques (ARTs) in this breed of horse could improve sperm cryosurvival for use in AI programs as in other breeds.[5]

[1]Laboratorio de Sanidad Animal y Zoonosis, Departamento de Ciencias Biológicas y Agropecuarias, Universidad Técnica Particular de Loja, Loja, Ecuador.
[2]Laboratorio de Biotecnología de la Reproducción Animal, Facultad de Ciencias Agropecuarias, Universidad de Cuenca, Cuenca, Ecuador.
[3]Veterinary Reproduction Group, Department of Animal Medicine and Surgery, University of Cordoba, Cordoba, Spain.
[4]Departamento de Reproducción Animal, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria-Consejo Superior de Investigaciones Científicas, Madrid, Spain.

There is considerable variability in the ability of spermatozoa from different stallions to survive the freezing process[6]; however, there are no reports that indicate individual effects of Peruvian Paso stallions and their relationship with sperm cryosurvival.

It is well known that the process of freezing can induce irreversible damage to equine sperm such as alterations in the plasma membrane due to oxidative stress, capacitation, and apoptosis-like changes.[7,8] The cooling rate affects the velocity at which ice forms, which influences sperm survival.[9] For instance, low cooling rates cause dehydration of the spermatozoa, and cell membranes are exposed to osmotic stress for longer periods with the generation of reactive oxygen species (ROS). Two-step acceleration cooling rates lead to the lowest cryoinjury of sperm in some species such as rams.[10]

The plasma membrane of stallion sperm contains elevated amounts of polyunsaturated fatty acids,[11] which makes this cell highly sensitive to oxidative stress and subsequent lipid peroxidation (LPO) during cryopreservation.[12,13] These events are associated with disrupted mitochondrial and plasma membranes; DNA fragmentation can affect the motility and fertilizing ability.[14]

Melatonin (ME) is a powerful free radical scavenger antioxidant that can repair damaged biomolecules in living organisms.[15] ME is amphiphilic and may cross cell membranes and easily reach intracellular compartments, including mitochondria. It can enhance the activities of the electron transport chain.[16–18] In this sense, the antioxidant effect of ME has previously been demonstrated to mitigate the increase in ROS produced by mitochondrial metabolism in equine sperm. This can reduce premature sperm aging.[19–21] In fact, some reports suggest that the supplementation of 1 μM ME to the extender can improve the mitochondrial function of equine spermatozoa subjected to cryopreservation.[19,22,23]

Caffeine (CAF) is a methylxanthine derivative phosphodiesterase inhibitor shown to enhance sperm motility.[24,25] The CAF binds to the adenosine receptors and stimulates adenylyl cyclase, an enzyme present in mitochondria, which, in turn, facilitates the conversion of adenosine triphosphate into cyclic adenosine monophosphate.[26] The addition of CAF to freezing medium produced different effects on sperm motility variables, improving the curvilinear velocity (VCL) and amplitude of lateral head displacement (ALH) of frozen-thawed equine spermatozoa.[25,27] However, to our knowledge, there is little information on the antioxidant effect of CAF in cryopreserved stallion semen.[28]

This work used an initially slower cooling rate, followed by a gradually faster cooling (accelerating cooling) along with the described antioxidant action of ME (1 μM) and the stimulating effect of CAF (2 mM) on sperm motility. The aim was to investigate the benefit of this freezing method along with ME and CAF supplementation to freezing medium. The hypothesis was that it could decrease the oxidative stress and improve the kinematic function and plasma and acrosome membrane integrity of Peruvian Paso stallion spermatozoa.

Materials and Methods

Animal management, semen collection, and processing

All animals were handled in accordance with the chapter 7.8 of the Terrestrial Animal Health Code-2019© OIE (July 8, 2019) regarding the protection of animals used in scientific experiments. We used four fertile Peruvian Paso stallions aged between 5 and 12 years with a mean body score of 7.5 (range 1–9)[29] and assessed to be clinically healthy. The fertility of each stallion was confirmed, as all produced at least eight foals after mating with at least eight mares during the breeding season. They were housed in boxes ($4 \times 4 \times 3$ m of length, width and height, respectively) and fed with grass (70% green matter) and concentrate (grain, barley straw, and dry alfalfa supplements) with free access to water and mineralized salt.

Twenty semen ejaculates from all stallions (five ejaculates/stallion) were collected with an artificial vagina (46°C–48°C) during the December 2019 to January 2020 breeding season. Semen was collected with a 7-day interval collection; this range was used to conform to the weekly training of horses for Paso Fino competitions. Immediately after collection, the semen volume was measured, diluted 1:1 (v:v) with INRA-96® extender (016441; IMV, L'Aigle, France) at 37°C, and transported to the Animal Reproduction Biotechnology Research Laboratory (3°04′48.1″S 79°04′31.0″W) of the University of Cuenca at room temperature (22°C) <2 hours after initial evaluation.

The sperm concentration was determined using a Neubauer chamber (Marienfeld, Lauda-Königshofen, Germany). Ejaculates with a sperm motility of >70%, a score of >3 on a mass motility scale of 0–5, and a sperm concentration of $>100 \times 10^6$ sperm/mL were used for subsequent work. The fresh-extended samples were then centrifuged at 300 *g* for 10 minutes, and resuspended in 3 mL of INRA-Freeze® medium (021727; IMV) at 22°C; this resuspended sample was named the stock sample. The stock sample was divided into three groups: ME supplementation (Sigma M5250), CAF (Sigma CO750) supplementation, or none (control). The supplementation with additives was carried out in two steps.

The first step consisted of taking an aliquot from the stock sample for each group and adjusting to an initial volume and concentration of 1 mL and 200×10^6 sperm/mL using the freezing medium. The second step consisted of adding a further 1 mL of freezing medium (1:1, volume equal) supplemented with a double concentration of ME (2 μM), CAF (4 mM), or none, and mixing into the first 1 mL aliquots of each group. Thus, the final volume and final sperm concentration of the three samples before freezing were 2 mL and 100×10^6 sperm/mL, and the final concentration of ME and CAF was 1 μM and 2 mM, respectively. The stock solutions of antioxidants were previously prepared (ME: 1 mM in dimethyl sulfoxide [DMSO]; and CAF: 4 mM in freezing-medium) and stored at −20°C until use. The use of final concentrations of ME (1 μM) and CAF (2 mM) were based on previous studies performed in equine semen.[19,23,25]

Cryopreservation process

Samples from each group were stored for 75 minutes at 5°C (cooling rate: 0.3°C/min from +22°C to +5°C) and then packed manually into 0.25 mL French straws (IMV) carefully sealed with polyvinyl alcohol. After cooling, the subjective motility and score (1–5) of the sperm samples of all treatments were assessed to check that these values did not differ from the fresh samples.

The straws were frozen using a freezing protocol that included a styrofoam cryo-box ($30 \times 29 \times 31$ cm length, width, and height, respectively, with 3.4 L of liquid nitrogen [LN_2]) and two internal ramps at different distances. We used this freezing protocol according to preliminary studies that were performed in our laboratory to examine the efficacy of the protocol concerning the adjustment of the height of each ramp and time of exposure of straws to LN_2 vapors on sperm cryosurvival of the horse (data not shown).

The freezing protocol placed the straws in the first ramp 17 cm above the LN_2 surface for 4 minutes and then immediately placed them in a second lower ramp for 2 minutes at 7 cm above the LN_2. The descent of straws from the first to the second ramp was done quickly using a large precooled clamp while holding the end of the straws (filter section). Finally, the straws were plunged into LN_2 and kept there for 2 months. A total of 182 straws were frozen from 3 treatments: Control ($n = 64$), ME ($n = 55$), and CAF ($n = 63$).

The temperature inside (T_{in}) the straws was assessed to determine the cooling rates generated by this freezing protocol. The T_{in} assessment allowed us to register the occurrence of ice nucleation and estimate the duration of subsequent dissipation of the latent heat of fusion. For the latter purpose, a Digital Thermometer® (6802 II Bestdo, Shanghai, China) with two K-type thermocouple sensors from fine wire were used. The thermocouple sensor was introduced to a dummy 0.25-mL straw containing the freezing medium. The freezing procedure was started when the temperature inside the straws was near 5°C. The thermocouple inside the dummy straw registered the temperature drop (°C) over time (seconds) until the immersion into LN_2.

All frozen sperm samples were thawed by placing the straws in a water bath at 37°C for 30 seconds. The contents were poured into 1.5 mL dry Eppendorf tubes and incubated for 5 minutes at 37°C. After incubation, sperm motility, the status of sperm membranes, and oxidative stress were evaluated.

Sperm quality assessment

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 Ci-E, negative phase contrast [Ph1] with green filter; Nikon Instruments, Inc., New York). Fresh or frozen-thawed sperm samples were adjusted to a concentration of 20×10^6 sperm/mL with the INRA medium. Briefly, the 5-μm aliquot of sperm samples was loaded into a warmed (37°C) slide and covered with a coverslip. At least 3 fields and at least 200 sperm tracks per field (average: 600 spermatozoa per sample evaluated) were evaluated at 100× on each sample slide (image acquisition rate 25 frames/s).[9]

The percentage of total sperm motility (TM), percentage of progressive sperm motility (PSM), VCL (μm/s), average-path velocity (VAP, μm/s), straight line velocity (VSL, μm/s), straightness index (STR, %), linearity index (LIN, %), wobble index (WOB, %), ALH (μm), and beat-cross frequency (Hz) were assessed.

The plasma and acrosome membrane status were assessed using a double association of fluorescent probes—propidium iodide (PI; Sigma P4170) and fluorescein isothiocyanate-conjugated peanut (Arachis hypogaea) agglutinin (PNA-FITC, Sigma L 7381).[30] A total of 200 sperms per slide were examined, and 4 subpopulations of cells were quantified into 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. In addition, the total percentages of cells presenting an intact plasma membrane (IPM) equivalent to live sperm (IPIA+IPDA) and intact acrosomal membrane (IPIA+DPIA) were calculated.

The oxidative stress of frozen-thawed sperm samples was assessed using CellROX Deep Red Reagent® fluorescent probe (2.5 mM; CAT 10422 Life Technologies) diluted in DMSO for a final concentration of 2 mM (working solution) and stored at −20°C in the dark. An aliquot of 100 μL (25×10^6 sperm/mL) from each semen sample was added to 2 μL of CellROX® (2 mM) and 2 μL of Hoescht 33342 (in phosphate-buffered saline; 1 mg/mL) and incubated at 37°C for 30 minutes.[31]

After incubation, each sample was centrifuged for 5 minutes at 2000 *g*, and the supernatant was then removed; the pellets were resuspended in 200 μL of TALP-sperm medium (113.94 mM NaCl, 3.08 mM KCl, 0.30 mM NaH_2PO_4 H_2O, 1 mM Na-lactate, 1.97 mM CaCl $2H_2O$, 0.50 mM MgCl $6H_2O$, 10 mM HEPES sodium, and 25 mM $NaHCO_3$, 6 mg/mL bovine serum albumin, 0.11 mg/mL Na pyruvate, and 5 μL/mL gentamycin; 326 mOsm/kg, pH 7.6). Two hundred cells were counted and classified into three categories: (1) sperm under mild or no oxidative stress (non-stained midpiece) (No-OE), (2) sperm under moderate oxidative stress (midpiece stained pale red, M-OE), and (3) sperm under intense oxidative stress (midpiece stained strong red, I-OE).

Statistical analysis

Data were analyzed using Statistica software for Windows, version 12.0 (StatSoft, Tulsa, OK). Sperm variables that showed non-normal distributions, as determined by the Shapiro–Wilk test, were transformed to *arcsine* (percentages values) or *log10* (numeric values) before analysis. To evaluate the effect of ME or CAF on sperm quality before and after cryopreservation, one-way ANOVA and the Tukey multiple comparison test were used, using the general linear model procedure. In addition, the male (four stallions) factor was included as a fixed effect to evaluate differences between stallions.

Results

Typical examples of the temperature changes during the freezing protocol are shown in Figure 1. Ice nucleation occurred in the first ramp at −8.5°C. The apparent duration of the dissipation of heat of fusion, here defined as the time between the onset of ice nucleation (15 seconds) and return to the same T_{in} (41 seconds), was 26 seconds with an average cooling rate of 9.2°C/min. This freezing protocol produced two accelerating cooling rates. The first ramp from +5°C to −74°C registered an average cooling rate of 19°C/min, and the second ramp from −74°C to −168°C registered an average of 47°C/min (Fig. 1).

Fresh and post-thaw values for the sperm kinematic parameters are shown in Table 1. Differences in sperm kinematic parameters between stallions and between groups

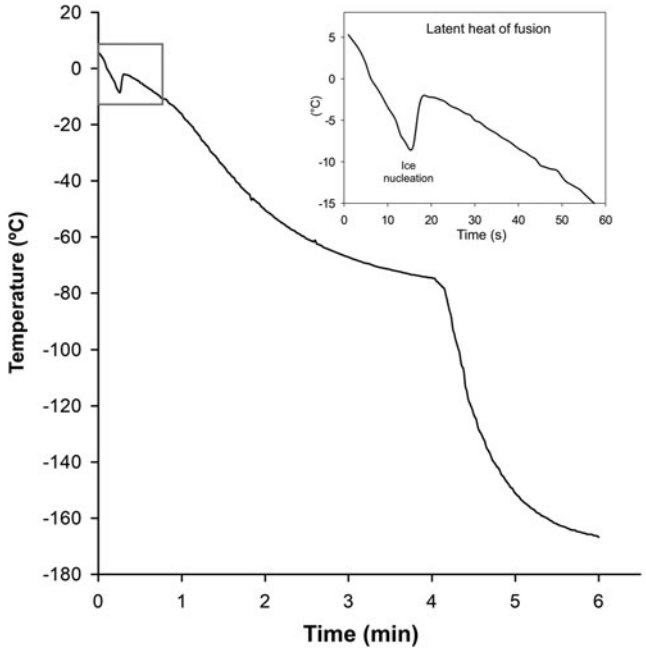

FIG. 1. Time courses of measured temperature inside the straws (T_{in}) in the freezing procedure. The duration time (seconds) and temperature at which latent heat of fusion (ice nucleation) occurs inside straws are shown in the small *box*.

are shown in Figure 2. Data regarding the status of sperm membranes and oxidative stress are included in Table 2.

After freezing and thawing, significant reductions ($p < 0.01$) were recorded for all sperm quality variables in frozen sperm versus fresh sperm for all groups. Both TM and PSM percentages were drastically reduced ($p < 0.001$) after freeze-thawing regardless of ME or CAF addition to freezing medium. Both VCL and VAP values were greater ($p < 0.05$) after freezing with CAF compared with either ME or the non-supplemented group (control). There were no significant differences ($p > 0.05$) between control, ME, or CAF groups for the remaining kinetic variables (Table 1).

Regarding male effects, there were significant differences ($p < 0.05$) between ejaculates from the four stallions regarding kinematic parameters. The VSL and STR values were greater ($p < 0.05$) for ejaculates from stallions 1, 2, and 3 than those obtained from stallion 4 (Fig. 2). After cryopreservation, the kinematic parameters decreased drastically ($p < 0.01$) compared with fresh samples in all stallions regardless of ME or CAF supplementation. There were no significant differences ($p > 0.05$) between groups in terms of the kinematic parameters in any stallions. However, stallion 1 returned higher ($p < 0.05$) PSM and VSL values than all other stallions. Also, stallion 1 returned greater ($p < 0.05$) TM, VCL, and ALH values than stallion 4 (Fig. 2).

The cryopreservation process affected the integrity of sperm plasma membranes and acrosome membranes, leading to a significant reduction ($p < 0.001$) of their percentages in all groups versus fresh samples (Table 2). The percentage of frozen-thawed sperm with intact plasma and acrosome membranes (IPIA) was greater ($p < 0.05$) after the ME and CAF treatments than the control. Lower ($p < 0.05$) percentages of sperm with damaged plasma membranes were observed after freezing with either ME or CAF groups versus the control group in the category of sperm with a DPIA.

Moreover, total numbers of sperm with IPM (or viability) were greater ($p < 0.05$) with both ME and CAF treatments than the control group. Finally, after cryopreservation, there were no significant differences ($p > 0.05$) between treatments (control, ME and CAF) in any of the oxidative stress categories (Total-EO, M-OE, and I-OE) (Table 2).

Discussion

These results suggest that CAF or ME supplementation could protect the plasma and acrosome membranes of stallion spermatozoa during cryopreservation using a two-step accelerating cooling rate. The supplementation of CAF to the freezing medium improved the post-thaw VSL and VAP values. However, ME and CAF had no beneficial effects on the remaining sperm motility variables, nor did they reduce

TABLE 1. SPERM MOTILITY VARIABLES (MEAN ± STANDARD ERROR OF THE MEAN) ASSESSED BY CASA FOR PERUVIAN PASO HORSE SEMEN BEFORE (FRESH) AND AFTER FREEZING-THAWING WITH MELATONIN AND CAFFEINE SUPPLEMENTED TO THE FREEZING MEDIUM

Kinematic parameters	*Fresh samples* (n = *20*)	*Frozen-thawed samples*		
		Control (n = *63*)	*ME* (n = *53*)	*CAF* (n = *64*)
TM, %*	87.9 ± 4.95^{a}	37.7 ± 2.79^{c}	40.5 ± 2.54^{c}	43.3 ± 2.79^{c}
PSM, %*	61.6 ± 6.40^{a}	8.3 ± 1.31^{c}	8.8 ± 1.24^{c}	11.9 ± 1.46^{c}
VCL, μm/s*	139.0 ± 5.15^{a}	34.0 ± 1.45^{c}	36.0 ± 1.67^{bc}	40.0 ± 1.85^{b}
VAP, μm/s*	95.4 ± 2.27^{a}	19.6 ± 1.04^{c}	21.0 ± 1.08^{bc}	23.7 ± 1.28^{b}
VSL, μm/s*	70.2 ± 5.51^{a}	13.9 ± 0.83^{c}	15.1 ± 0.92^{c}	16.8 ± 0.98^{c}
STR, %*	75.1 ± 4.99^{a}	61.6 ± 1.25^{c}	63.6 ± 1.47^{c}	64.5 ± 1.28^{c}
LIN, %*	54.8 ± 5.04^{a}	36.0 ± 1.24^{c}	38.2 ± 1.52^{c}	38.9 ± 1.37^{c}
WOB, %*	70.5 ± 3.04^{a}	53.8 ± 1.04^{c}	55.8 ± 1.11^{c}	56.3 ± 1.07^{c}
ALH, μm*	13.4 ± 8.53^{a}	1.7 ± 0.06^{c}	1.8 ± 0.07^{c}	1.9 ± 0.07^{c}
BCF, Hz	7.9 ± 0.37^{a}	4.7 ± 0.21^{c}	5.1 ± 0.24^{c}	5.4 ± 0.25^{c}

Different superscripts within the same row differ significantly ($^{abc}p < 0.05$ and $^{abc}p < 0.001$). The asterisk indicates differences between stallions (males) ($^{*}p < 0.05$).

ALH, amplitude of lateral head displacement; BCF, beat-cross frequency; CAF, caffeine; LIN, linearity index; ME, melatonin; PSM, progressive sperm motility; STR, straightness index; TM, total sperm motility; VAP, average-path velocity; VCL, curvilinear velocity; VSL, straight line velocity; WOB, wobble index.

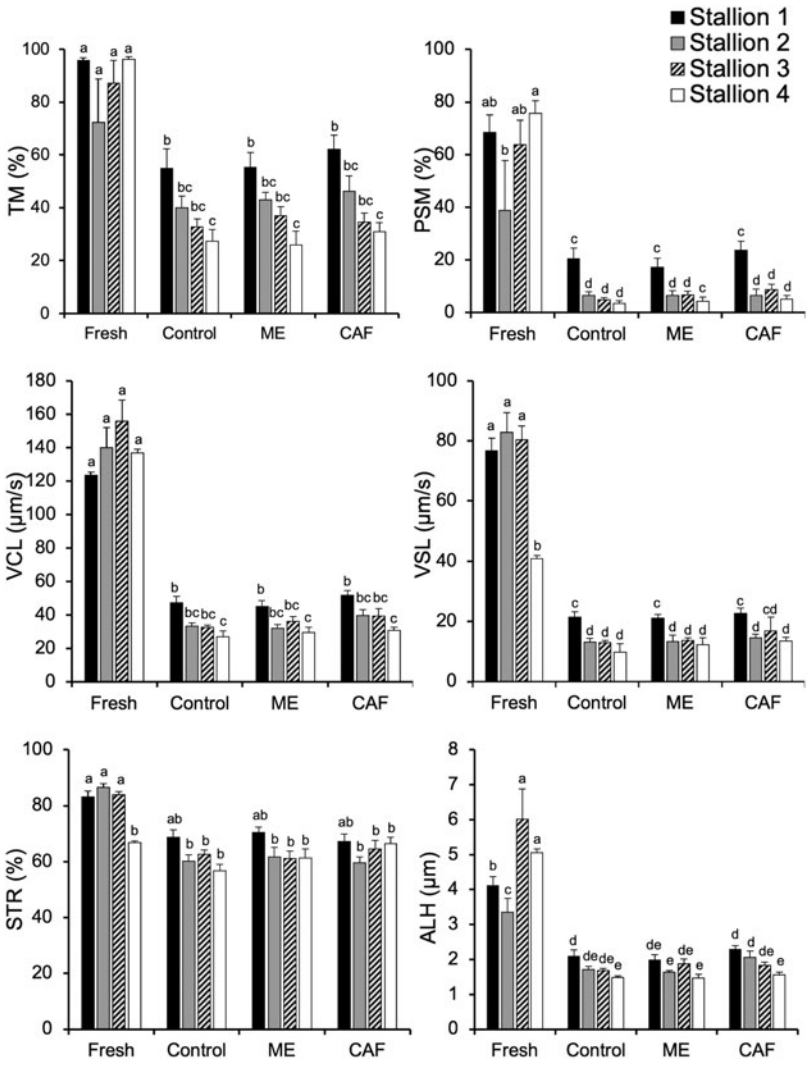

FIG. 2. Post-thaw kinetic parameters of sperm from Peruvian Paso stallions frozen with ME and CAF. Different *letters* (a–e) in each treatment and in each kinematic parameter indicate significant differences between stallions ($p < 0.05$ for a, b, c, d and e). CAF, caffeine; ME, melatonin.

TABLE 2. PERCENTAGES OF SPERM IN THE VARIOUS CATEGORIES OF MEMBRANE INTEGRITY (ASSESSED WITH FLUORESCENT MARKERS PROPIDIUM IODIDE/FLUORESCEIN ISOTHIOCYANATE-CONJUGATED PEANUT [ARACHIS HYPOGAEA] AGGLUTININ OR OXIDATIVE STRESS (ASSESSED WITH FLUORESCENT MARKERS CELLROX DEEP RED/HOESCHT 33342) IN PERUVIAN PASO HORSE SEMEN BEFORE (FRESH) AND AFTER FREEZING-THAWING WITH MELATONIN AND CAFFEINE SUPPLEMENTED TO FREEZING MEDIUM

Fluorescence parameters	*Fresh samples* (n = *20*)	*Frozen-thawed samples*		
		Control (n = *64*)	*ME* (n = *55*)	*CAF* (n = *63*)
IPIA, %	82.7 ± 1.45^{a}	24.3 ± 1.62^{c}	46.4 ± 2.45^{b}	41.8 ± 2.16^{b}
IPDA, %	0.33 ± 0.17^{a}	8.41 ± 1.47^{c}	5.75 ± 0.62^{ac}	5.9 ± 0.55^{ac}
DPIA, %	12.7 ± 2.35^{c}	61.2 ± 2.00^{a}	41.2 ± 2.10^{b}	46.5 ± 2.07^{b}
DPDA, %	4.33 ± 1.30	6.13 ± 0.92	6.67 ± 0.73	5.8 ± 0.70
IPM (viability), %	83.0 ± 1.61^{a}	32.7 ± 1.99^{c}	52.1 ± 2.19^{b}	47.7 ± 2.17^{b}
IAM, %	95.3 ± 1.30^{a}	85.5 ± 1.58^{c}	87.6 ± 0.92^{c}	88.2 ± 0.89^{c}
No-OE, %*	—	69.1 ± 5.17	65.8 ± 5.10	70.1 ± 4.72
Total-OE, %*	—	30.9 ± 5.17	34.2 ± 5.10	29.9 ± 4.72
M-OE, %	—	14.8 ± 3.90	16.8 ± 3.28	14.8 ± 3.90
I-OE, %	—	16.1 ± 2.58	17.4 ± 2.68	15.8 ± 2.33

Different superscripts within the same row differ significantly ($^{abc}p < 0.05$ and $^{abc}p < 0.001$). Asterisks indicate differences between stallions (males) ($^{*}p < 0.05$).

DPDA, damaged plasma membrane/damaged acrosome; DPIA, damaged plasma membrane/intact acrosome; IAM, intact acrosome membrane; I-OE, sperm under intense oxidative stress; IPDA, intact plasma membrane/damaged acrosome; IPIA, intact plasma membrane/intact acrosome; IPM, intact plasma membrane or viability; M-OE, sperm under moderate oxidative stress; No-OE, sperm under mild or no oxidative stress; Total-OE, sperm with total oxidative stress.

oxidative stress after freezing-thawing. In addition, one stallion produced better post-thaw sperm motility variables than the others; however, the freezing process, nevertheless, drastically affected the sperm motility variables of the four stallions. These results suggest that sperm from stallions of this breed respond differently to the cryopreservation process due to the differences between males.

Slow cooling rates at the onset of freezing followed by accelerating cooling rates are known to improve sperm survival[9,10,32,33] because cells dehydrate efficiently and minimize exposure to the deleterious effects of unfrozen, extracellular, and hypertonic solutions during this critical temperature range (−5°C to −35°C) when ice growth occurs.[34] The freezing protocol of this study produced a slow initial cooling rate when ice nucleation and dissipation of latent heat of fusion occurred during the first ramp.

Previous research has examined the effect of ME on cryopreserved stallion sperm with variable results. Some studies have shown improvements in sperm kinematic parameters and viability values.[35] The mechanism by which ME improves these characteristics is due to the fact that ME is a highly lipophilic molecule that crosses cell membranes, thus easily reaching intracellular compartments[36,37] and leading to an improvement in the activity of the electron transport chain.[17] Supplementation of 50 pM to 1 μM of ME to the INRA-96 extender inhibited the LPO of stallion sperm, but it did not improve sperm motility or kinetics.[19] Unlike previous work that supplemented chilling[22] or freezing[23] extenders with ME, the mitochondrial function of equine spermatozoa was improved.

The improvement of the sperm motility in the aforementioned work may have been because ME, together with hormones (e.g., triiodothyronine), directly influenced the physiological regulation of mitochondrial homeostasis[38] even after freezing.

Here, the ME effect was not seen with relatively low motility in all groups. Cryopreserved equine sperm must reach a minimum PSM of 25%–35% to achieve successful pregnancy rates after AI.[39] Our post-thaw PSM results are low, but they can be used efficiently in ART such as intracytoplasmic sperm injection.[40] This negative effect could probably be due to the high sensitivity to cellular stress of the mitochondria during the cryopreservation process of the semen.[41] These contrary findings lead us to speculate that factors inherent to the stallion semen directly influence sperm cryosurvival (e.g., factors such as individual, breed, seminal plasma, or reproductive season).

Only 30%–40% of stallions produce semen that can be satisfactorily cryopreserved and retain their fertilizing capacity.[42,43] In fact, horses have been categorized as good and bad freezers; the male factor has been described as the main conditioning factor for the response to cryopreservation.[44] Our results demonstrate significant differences between stallions on post-thaw sperm kinetic parameters irrespective of ME or CAF supplementation. Our sperm freezing results obtained in the Peruvian Paso stallion may also vary from other studies due to different protocols[4] and the type of extender[1,3] freezing used. This makes it difficult to compare results. Our freezing protocol included a commercial freezing medium (defined) supplemented with ME or CAF, and freezing used a two-ramp cryogenic-box system. However, this work did demonstrate the male effect on cryopreserving semen from this breed.

Our findings also demonstrated that the ME or CAF improved the integrity of the plasma and acrosomal membranes, but the maintenance of the integrity of the plasma membrane is not necessarily associated with increased sperm motility.[45] These results make this research relevant and illustrate the beneficial effect of both ME and CAF, which agree well with previous reports.[22,23] Since CAF improved some kinematic variables, and both CAF and ME improved plasma and acrosome sperm membranes, a synergistic effect has been reported between ME and CAF when they are used together in the same extender. Indeed, synergistic effects using different additives in stallion semen extenders have been reported.[46]

Our results do not show any differences between groups perhaps because of the production of ROS during the processing of the samples, together with the higher ROS production from non-viable or poor quality spermatozoa of this breed, which may saturate the scavenger effect of ME and CAF; previous studies have shown this positive effect in other horses.[19,23,47] Unfortunately, the fertilization capacity of sperm was not evaluated by AI, and thus our findings should be considered as a preliminary approach for conservation of this breed.

Conclusion

The supplementation of ME or CAF to the freezing medium protected the integrity of the plasma and acrosomal membranes of the cryopreserved Peruvian Paso horse spermatozoa when a two-step accelerating cooling rate was used. The CAF improved the relative efficacy of some sperm motility parameters, but neither additive could reduce the oxidative stress after cryopreservation. More research, such as the use of these additives and amides, is required to improve the sperm cryosurvival of this breed of horse; standardization of protocols for individual stallions could lead to better cryosurvival rates.

Acknowledgments

The authors thank the ''Universidad de Cuenca'' and the ''Universidad Técnica Particular de Loja (UTPL)'' from the Ecuador through agreement number 254-20202. Also, the authors are grateful for the support granted by Universidad Técnica Particular de Loja to Erika Tamay via the ''Vicerrectorado Académico de Investigación.''

Author Disclosure Statement

No conflicting financial interests exist.

Funding Information

No funding was received for this article.

References

1. Barriga X, Rodríguez J, Herrera S, et al. Evaluation of low-density lipoprotein and egg yolk as cryoprotectants non-penetrating of Peruvian Paso horse semen. Spermova 2019; 9(2):88–91; doi: 10.18548/aspe/0007.11
2. Dellepiane H, Chilge S, Vexelman D, et al. Evaluation of the viability, acrosomal integrity and mitochondrial activity in chilled semen of Peruvian Paso horse. Preliminary report. Spermova 2015;5(1):38–41; doi: 10.18548/aspe/0002.8

3. Santiani A, Evangelista-Vargas S, Vargas S, et al. Cryopreservation of Peruvian Paso horse spermatozoa: Dimethylacetamide preserved an optimal sperm function compared to dimethyl sulfoxide, ethylene glycol and glycerol. Andrologia 2017;49(6):e12672; doi: 10.1111/and.12672
4. Mellisho E, Olmos R, Ancco E, et al. 15 Thermoresistance sperm test for epididymal stallion sperm frozen with two cryoprotectants. Reprod Fertil Dev 2014;26(1):121–122; doi: 10.1071/rdv26n1ab15
5. Gomes RG, da Silva CB, González SM, et al. Equine Reproduction: Physiology and Advances in Technology. In: Marcondes M, Silva-Santos K, Rafagnin L, eds. *Biotechnology of Animal Reproduction*. NY, USA: Nova Science Publishers Inc.; 2016:237–270.
6. Al-Essawe E, Johannisson A, Wulf M, et al. Improved cryosurvival of stallion spermatozoa after colloid centrifugation is independent of the addition of seminal plasma. Cryobiology 2018;81:145–152; doi: 10.1016/j.cryobiol.2018.01.009
7. Peña FJ, Macías García B, Samper JC, et al. Dissecting the molecular damage to stallion spermatozoa: The way to improve current cryopreservation protocols? Theriogenology 2011;76(7):1177–1186; doi: 10.1016/j.theriogenology.2011.06.023
8. Thomas AD, Meyers SA, Ball BA. Capacitation-like changes in equine spermatozoa following cryopreservation. Theriogenology 2006;65(8):1531–1550; doi: 10.1016/j.theriogenology.2005.08.022
9. Galarza DA, López-Sebastián A, Woelders H, et al. Two-step accelerating freezing protocol yields a better motility, membranes and DNA integrities of thawed ram sperm than three-steps freezing protocols. Cryobiology 2019;91:84–89; doi: 10.1016/j.cryobiol.2019.10.007
10. Galarza D, López-Sebastián A, Santiago-Moreno J. Effectiveness of two-step accelerating cooling rate on post-thaw characteristics of ram sperm. Reprod Domest Anim 2019; 54:113.
11. Aitken RJ, De Iuliis GN, Drevet JR. Role of oxidative stress in the etiology of male infertility and the potential therapeutic value of antioxidants. In: Henkel R, Samanta L, Agarwal A, eds. *Oxidants, Antioxidants, and Impact of the Oxidative Status in Male Reproduction*. London, UK: Academic Press; 2018:91–100; doi: 10.1016/B978-0-12-812501-4.00010-9
12. Agarwal A, Said TM. Oxidative stress, dna damage and apoptosis in male infertility: A clinical approach. BJU Int 2005;95(4):503–507; doi: 10.1111/J.1464-410X.2005.05328.X
13. Aitken RJ, Wingate JK, De Iuliis GN, et al. Cis-unsaturated fatty acids stimulate reactive oxygen species generation and lipid peroxidation in human spermatozoa. J Clin Endocrinol Metab 2006;91(10):4154–4163; doi: 10.1210/jc.2006-1309
14. Sieme H, Oldenhof H, Wolkers WF. Sperm membrane behaviour during cooling and cryopreservation. Reprod Domest Anim 2015;50:20–26; doi: 10.1111/rda.12594
15. Tan D, Reiter R, Manchester L, et al. Chemical and physical properties and potential mechanisms: Melatonin as a broad spectrum antioxidant and free radical scavenger. Curr Top Med Chem 2005;2(2):181–197; doi: 10.2174/1568026023394443
16. Ahmadi S, Bashirip R, Ghadiri-Anarip A, et al. Antioxidant supplements and semen parameters: An evidence based review. Int J Reprod Biomed 2016;14(12):729; doi: 10.29252/ijrm.14.12.729
17. Carpentieri A, Díaz De Barboza G, Areco V, et al. New perspectives in melatonin uses. Pharmacol Res 2012;65(4): 437–444; doi: 10.1016/j.phrs.2012.01.003
18. Kocyigit A, Selek S. Exogenous antioxidants are double-edged swords. Bezmialem Sci 2016;4(2):70–75; doi: 10.14235/bs.2016.704
19. Balao Da Silva CM, MacÍas-García B, Miró-Morán A, et al. Melatonin reduces lipid peroxidation and apoptotic-like changes in stallion spermatozoa. J Pineal Res 2011; 51(2):172–179; doi: 10.1111/J.1600-079X.2011.00873.X
20. Gibb Z, Lambourne SR, Aitken RJ. The paradoxical relationship between stallion fertility and oxidative stress. Biol Reprod 2014;91(3):77–78; doi: 10.1095/biolreprod.114.118539/2434147
21. Mayo JC, Sainz RM, Antolín I, et al. Melatonin regulation of antioxidant enzyme gene expression. Cell Mol Life Sci 2002 5910 2002;59(10):1706–1713; doi: 10.1007/pl00012498
22. Affonso FJ, Carvalho HF, Lançoni R, et al. Addition of antioxidants myoinositol, ferulic acid, and melatonin and their effects on sperm motility, membrane integrity, and reactive oxygen species production in cooled equine semen. J Equine Vet Sci 2017;59:57–63; doi: 10.1016/j.jevs.2017.09.006
23. Lançoni R, Celeghini ECC, Alves MBR, et al. Melatonin added to cryopreservation extenders improves the mitochondrial membrane potential of postthawed equine sperm. J Equine Vet Sci 2018;69:78–83; doi: 10.1016/j.jevs.2018.06.006
24. Rota A, Sabatini C, Przybył A, et al. Post-thaw addition of caffeine and/or pentoxifylline affect differently motility characteristics of horse and donkey cryopreserved spermatozoa. J Equine Vet Sci 2019;66:85–86; doi: 10.1016/j.jevs.2018.05.052
25. Stephens TD, Brooks RM, Carrington JL, et al. Effects of pentoxifylline, caffeine, and taurine on post-thaw motility and longevity of equine frozen semen. J Equine Vet Sci 2013;33(8):615–621; doi: 10.1016/j.jevs.2012.10.004
26. Pariz JR, Raneá C, Monteiro RAC, et al. Melatonin and caffeine supplementation used, respectively, as protective and stimulating agents in the cryopreservation of human sperm improves survival, viability, and motility after thawing compared to traditional TEST-Yolk buffer. Oxid Med Cell Longev 2019;2019:6472945; doi: 10.1155/2019/6472945
27. Rota A, Sabatini C, Przybył A, et al. Post-thaw addition of caffeine and/or pentoxifylline affect differently motility of horse and donkey-cryopreserved spermatozoa. J Equine Vet Sci 2019;75:41–47; doi: 10.1016/J.JEVS.2019.01.003
28. Alves N de C, Diniz S de A, Viegas RN, et al. Addition of caffeine to equine thawed sperm increases motility and decreases nitrite concentration. Andrologia 2021;53(2): e13918; doi: 10.1111/and.13918
29. Henneke DR, Potter GD, Kreider JL, et al. Relationship between condition score, physical measurements and body fat percentage in Mares. Equine Vet J 1983;15(4):371–372; doi: 10.1111/J.2042-3306.1983.tb01826.x
30. Galarza DA, López-Sebastián A, Woelders H, et al. Sephadex filtration as successful alternative to density-gradient centrifugation procedures for Ram sperm selection with improved kinetics. Anim Reprod Sci 2018;192:261–270; doi: 10.1016/j.anireprosci.2018.03.022
31. Rodrigues Alves MB, Andrade AF, Arruda RP, et al. An efficient technique to detect sperm reactive oxygen species: The CellRox Deep Red® fluorescent probe. Biochem Physiol Open Access 2015 42 2015;4(2):e13918; doi: 10.4172/2168-9652.1000157
32. Moore AI, Squires EL, Bruemmer JE, et al. Effect of cooling rate and cryoprotectant on the cryosurvival of

equine spermatozoa. J Equine Vet Sci 2006;26(5):215–218; doi: 10.1016/j.jevs.2006.03.003
33. Oldenhof H, Bigalk J, Hettel C, et al. Stallion sperm cryopreservation using various permeating agents: Interplay between concentration and cooling rate. Biorpeserv Biobank 2017;15(5):422–431; doi: 10.1089/BIO.2017.0061
34. Woelders H, Chaveiro A. Theoretical prediction of 'optimal' freezing programmes. Cryobiology 2004;49(3):258–271; doi: 10.1016/j.cryobiol.2004.09.001
35. Izadpanah G, Zare-Shahneh A, Zhandi M, et al. Melatonin has a beneficial effect on stallion sperm quality in cool condition. J Equine Vet Sci 2015;35(7):555–559; doi: 10.1016/j.jevs.2015.02.007
36. Bucak MN, Ateşşahin A, Yüce A. Effect of anti-oxidants and oxidative stress parameters on Ram semen after the freeze–thawing process. Small Rumin Res 2008;75(2–3):128–134; doi: 10.1016/j.smallrumres.2007.09.002
37. Itagaki S, Kurokawa T, Nakata C, et al. In vitro and in vivo antioxidant properties of ferulic acid: A comparative study with other natural oxidation inhibitors. Food Chem 2009;114(2):466–471; doi: 10.1016/j.foodchem.2008.09.073
38. López A, García JA, Escames G, et al. Melatonin protects the mitochondria from oxidative damage reducing oxygen consumption, membrane potential, and superoxide anion production. J Pineal Res 2009;46(2):188–198; doi: 10.1111/J.1600-079X.2008.00647.X
39. Ng CM, Blackman MR, Wang C, et al. The role of carnitine in the male reproductive system. Ann N Y Acad Sci 2004;1033(1):177–188; doi: 10.1196/annals.1320.017
40. Choi YH, Love CC, Varner DD, et al. Equine blastocyst development after intracytoplasmic injection of sperm subjected to two freeze-thaw cycles. Theriogenology 2006;65(4):808–819; doi: 10.1016/j.theriogenology.2005.04.035
41. Leon J, Acuña-Castroviejo D, Sainz RM, et al. Melatonin and mitochondrial function. Life Sci 2004;75(7):765–790; doi: 10.1016/j.lfs.2004.03.003
42. Ángel D, Bran JA. Assisted reproduction in horses: Contributions from theory. Rev CES Med Vet y Zootec 2010;5(1):56–69.
43. Aurich C. Reprint of: Seasonal influences on cooled-shipped and frozen-thawed stallion semen. J Equine Vet Sci 2016;43:S1–S5; doi: 10.1016/j.jevs.2016.07.007
44. Tischner M. Evaluation of deep-frozen semen in stallions. J Reprod Fertil Suppl 1979;27:53–59.
45. Ball BA. Oxidative stress, osmotic stress and apoptosis: Impacts on sperm function and preservation in the horse. Anim Reprod Sci 2008;107(3–4):257–267; doi: 10.1016/j.anireprosci.2008.04.014
46. Freitas ML, Bouéres CS, Pignataro TA, et al. Quality of fresh, cooled, and frozen semen from stallions supplemented with antioxidants and fatty acids. J Equine Vet Sci 2016;46:1–6; doi: 10.1016/j.jevs.2016.07.003
47. Ortega-Ferrusola C, Sotillo-Galán Y, Varela-Fernández E, et al. Detection of ''Apoptosis-Like'' changes during the cryopreservation process in equine sperm. J Androl 2008;29(2):213–221; doi: 10.2164/jandrol.107.003640

Address correspondence to:
Diego A. Galarza, PhD
Laboratorio de Biotecnología de la Reproducción Animal
Facultad de Ciencias Agropecuarias
Universidad de Cuenca
Cuenca EC010205
Ecuador

E-mail: andres.galarza@ucuenca.edu.ec