



COMPARISON OF TWO SPERM FREEZING PROTOCOLS OF COLOMBIAN CRIOLLO HORSES: PRELIMINARY RESULTS

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Summary

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The equine industry is an essential source of job and income worldwide. The use of artificial insemination (AI) with frozen semen is an opportunity to continue disseminating the Colombian Criollo horse. Seminal cryopreservation studies in Colombian Criollo horses are limited, and there is no previous knowledge in deleterious changes induced by the cryopreservation process on the chromatin integrity of the spermatid cells. The present study aimed to verify the effect of the modified-INRA 82 and Botucurio® extenders on sperm kinematics, chromatin integrity, and morphology of cryopreserved Colombian Criollo sperm. Five ejaculates of five stallions were obtained, split into two samples, and frozen with two different extenders (modified-INRA 82 and Botucurio) and cooling curves. After freezing, samples were analysed with the CASA system. Sperm morphology, chromatin integrity, hypoosmotic, and thermoresistance tests performed. Data were analysed by MANOVA (parametrical variables) and Kruskal Wallis (non-parametrical variables) with SAS Studio ® software. Statistically significant differences ($P \leq 0.05$) were found for the progressive motility (14 ± 5 and 27 ± 13), and chromatin integrity (64 ± 3 and 69 ± 5) for modified-INRA 82 and Botucurio, respectively. Botucurio samples presented higher total motility at 30, 60, 90, 120, and 150 min and progressive motility at 60, 90, 120 min in thermoresistance test than modified-INRA 82. Botucurio and modified-INRA 82, are capable to conserve semen equine effectively from Colombian Criollo horses. However, Botucurio permitted an improved kinematics and higher chromatin integrity than modified-INRA 82.

Key words: cryopreservation, dimethylformamide, equine, freezing-thaw, glycerol, semen

INTRODUCTION

Colombian Criollo horses are a breed that stands out for its elasticity, rhythm, the

harmony of movements, temperament, verve and smoothness when walking

(Naranjo, 2012). Due to these characteristics the breed is in expansion, leading to the distribution of genetic material to different countries. Acceptance of frozen semen as a method to produce registered foals has stimulated interest in this technology (Loomis, 2001), being poorly used in this breed. Frozen semen adds a new dimension to the horse breeding industry by enabling long-term preservation of spermatozoa from stallions and permitting provision of this semen to breed farms worldwide (Miller, 2008; Nikitkina *et al.*, 2020). Currently, the agribusiness and the assisted reproductive technologies require high availability and constant use of seminal doses to produce more foals/stallion per year, demanding semen from genetically superior stallions (Hernández-Avilés *et al.*, 2018).

The use of frozen semen has several advantages: lower transportation costs, indefinite storage, better timing for AI, and reduced risk of transmission of venereal diseases (Miller, 2008). However, frozen-thawed semen also has several disadvantages: a decrease in pregnancy rate and the cost of semen processing (freezing and storage) versus breeding with cooled shipped semen. Thus, the freeze-thawing process can affect semen quality, influencing pregnancy rates (Sieme *et al.*, 2008).

The cryopreservation process reduces fertility compared with fresh semen caused by loss of sperm viability and impairment of function in the population of survivors (Watson, 2000). Membranes are initially destabilised during the freezing process, both by low-temperature effects and by exposure to high salt concentrations (Holt & North, 1994). Cryopreservation may compromise the acrosome, plasma membrane, and flagellum independently (McLaughlin *et al.*, 1993), and the decreased ability of spermatozoa to

attach to equine oviductal epithelial cells or zona pellucida, explaining in part the reduced fertility of cryopreserved compared to fresh spermatozoa in the horse (Dobrinski *et al.*, 1995).

Cryopreservation also induces oxidative damage with formation of reactive oxygen species (ROS) detrimental to subsequent sperm performance (Watson, 2000). Thus, the cryopreservation process and consequent ROS formation promote DNA fragmentation in equine spermatozoa (Baumber *et al.*, 2003), damaging the male germline causing a variety of adverse outcomes such as low fertilisation rates and decrease in embryo implantation (Valcarce *et al.*, 2013). Recent advances in extender composition have allowed increased quality and fertility of frozen stallion semen (Alvarenga *et al.*, 2016). Seminal cryopreservation and post-thaw chromatin integrity studies in Colombian Criollo horses are limited. However, equine agribusiness of the breed is growing and needs more information. Therefore, the present study aimed to verify the effect of modified INRA 82 and Botucurio® (Botucurio) extenders on the cinematic, chromatin integrity, and spermatozoa morphology from Colombian Criollo horses after cryopreservation.

MATERIALS AND METHODS

Animals

Five clinically healthy Colombian Criollo horses, with proven fertility, aged between 5 to 12 years old, weighing 350–430 kg were used as semen donors in Chiquinquirá, Boyacá, Colombia (5°37'N 73°48'W). The stallions were housed in individual boxes with free access to water and mineral salt, receiving daily oat and alfalfa hay.

The Animal Ethical Use Committee of the La Salle University approved the use of animals and the procedures included in the study. Besides, the study involved the use of client-owned animals, where high standard veterinary care ensured animal welfare and, with informed consent in writing.

Semen collection, handling, and initial evaluation

Sperm samples were collected with a Missouri artificial vagina with a semen nylon filter (Minitüb, Tiefenbach, Germany) inserted in the collection bottle to remove the gel fraction. Stallions were initially collected to eliminate the extragonadal sperm reserves from the cauda epididymis until stabilising the sperm parameters. Sperm analysis was performed with one aliquot of 500 μ L of raw semen to assess total motility, sperm concentration (haemocytometer), and morphology (eosin-nigrosin staining) with phase-contrast microscopy (Olympus CBA Microscopy; 1000 \times). Only ejaculates that met the following characteristics were used in the experiment: sperm concentration $\geq 60 \times 10^6$ sperm/mL, progressive motility $> 60\%$, and $> 70\%$ morphologically normal sperm (Perez-Osorio *et al.*, 2008).

Sperm cryopreservation

Five ejaculates were obtained from each stallion, the semen samples were diluted 1+1 (vol/vol) in Kenney extender (Kenney, 1975) and split into two samples:

1) Modified-INRA 82. The sample was centrifuged at 600 \times g for 10 min. Pellet was resuspended to 150×10^6 sperm/mL with modified-INRA 82 extender (HEPES, skim milk-egg yolk, raffinose, trehalose, sucrose, fructose, and glucose) using 5% dimethylformamide (Sigma-

Aldrich), packaged into 0.5 mL straws and maintained at 5 $^{\circ}$ C for 80 min (Perez-Osorio *et al.*, 2008).

2) Botucio. The sample was centrifuged at 600 \times g for 10 min. Pellet was resuspended to 150×10^6 sperm/mL with Botucio® (Botupharma, Botucatú, Brazil), packaged into 0.5 mL straws and maintained at 5 $^{\circ}$ C for 30 min (Papa *et al.*, 2002).

Straws were kept 4 cm above liquid nitrogen for 10 min and then plunged in liquid nitrogen. Samples were stored for 2 weeks, until their evaluation.

Sperm post-thaw evaluation

From each cryopreserved ejaculate and experimental sample (modified-INRA 82 and Botucio), five straws were analysed. Straws were thawed in a water bath at 37 $^{\circ}$ C for 30 sec.

Sperm kinematic evaluation was performed using a computer-assisted sperm analysis system (Hamilton Thorne IVOS II, v 14.0), analysing 25 fields. The variables considered in this study were total motility (TM, %), progressive motility (PM, %), average path velocity (VAP, m/s), straight-line velocity (VSL, m/s) and curvilinear velocity (VCL, m/s).

Sperm morphology assessment was performed on smears on slides. The samples were stained with aqueous solutions of malachite green, eosin, and gentian violet. Two hundred spermatozoa per slide were analysed by a phase-contrast microscope (Olympus CBA Microscopy; 1000 \times) to determine changes or injuries in head, acrosome (swelling, breakage, or absence of acrosomes) and flagella (neck, mid-piece, and tail) (Brito, 2007).

A live sperm assessment was performed with eosin-nigrosin supravital stain. Two hundred spermatozoa were analysed per slide with a light microscope

(Olympus CBA Microscopy; 1000×). Unstained sperm, intact membranes were considered live, and those that stained red (even partially), disrupted membranes were considered dead (Brito, 2007).

Chromatin integrity was determined with acridine orange epifluorescence. Smears were made with 200 µL of thawed sperm mixed in 200 µL stock solution (1 g of acridine orange). After staining, the slides were gently rinsed in a stream of distilled water and air-dried. The smears stained were assessed under an epifluorescence microscope (Leica Microsystems, DMLB, Wetzlar, Germany) using a 490-nm excitation filter and a 530-nm barrier filter. The observation time per field was not longer than 40 sec. Two hundred spermatozoa were counted per slide. Sperm heads with intact chromatin showed green fluorescence, and the sperm heads with diminished chromatin integrity exhibited orange-red staining (Tejada *et al.*, 1984).

The functional integrity of the spermatid plasma membrane was analysed by the hypo-osmotic swelling test (HOST). One hundred µL of thawed sperm was mixed in 1.0 mL hypoosmotic solutions (sucrose to 100 mOsm/L) and incubated by 30 min in a water bath at 37 °C. After incubation, samples were fixed in saline formaldehyde buffer 10%, and 20 µL of the solutions containing semen were placed on a slide, covered with a cover glass. With a phase-contrast microscope (Olympus CBA Microscopy; 1000×), 200 cells were analysed per sample. Spermatozoa were classified as swollen (coiled) or not swollen (Turner, 2005).

Thermoresistance test was performed, incubating the thawed sperm at 37 °C. Total and progressive motility were evaluated at 30-minute intervals, until 210 min (Perez-Osorio *et al.*, 2008).

Statistical analysis

Data were evaluated using the Statistical Analysis System (SAS, Cary, NC, USA). TM, PM, VAP, VSL, VCL, sperm morphology, life sperm, sperm chromatin integrity, HOST and thermoresistance test were considered as dependent variables. Treatment (modified-INRA 82 or Botucurio), repetition, and stallion were considered as independent variables. Variables were evaluated for normality using the PROC UNIVARIATE procedure; those not meeting normal distribution were transformed using natural logarithm, and those still not meeting normality by this method were evaluated by non-parametric statistics.

Variables with normal distribution were analysed using the PROC GLM procedure (MANOVA), evaluating non-balanced variables and tests interactions between treatments (modified-INRA 82 or Botucurio). Means were evaluated using Tukey's test using the LSMEANS procedure. Variables not meeting normal distribution were analysed by the PROC NPAR1WAY procedure, to evaluate the means by Kruskal-Wallis test. Data are presented as means ± standard deviation. Differences at $P \leq 0.05$ were considered significant.

RESULTS

Stallions presented a mean age of 6.9 ± 2 years old. Raw semen values were volume 52 ± 3 mL, TM $72 \pm 9\%$, PM $64 \pm 9\%$ and vigour 3.0 ± 0 . The percentages of morphologically normal sperm and live sperm for fresh semen were $77.4 \pm 7.3\%$ and $71.4 \pm 10\%$ respectively.

Thawed sperm characteristics are described in Table 1. Semen frozen with Botucurio showed better PM ($P=0.024$) and chromatin integrity ($P=0.034$), in

Table 1. Mean values with standard deviations (n=15) and probabilities (P) of post-thawing parameters of Colombian Criollo horse sperm cryopreserved with either modified-INRA 82 or Botucurio

Variable	Modified-INRA 82	Botucurio	P
Total motility (%)	50 ± 14	61 ± 9	0.121
Progressive motility (%)	14 ± 5	27 ± 13	0.024
Average path velocity (VAP) (µ/S)	64 ± 13	63 ± 11	0.597
Straight line velocity (VSL) (µ/S)	45 ± 13	49 ± 8	0.928
Curvilinear velocity (VCL) (µ/S)	132 ± 11	127 ± 18	0.573
Normal morphology	63 ± 11	67 ± 13	0.672
Live sperm (%)	64 ± 3	63 ± 5	0.186
Chromatin integrity (%)	64 ± 3	69 ± 5	0.034
HOST (%)	64 ± 5	72 ± 5	0.095

Table 2. Mean values with standard deviations (n=15) and probabilities (P) of total motility and progressive motility in the thermoresistance test of Colombian Criollo horse sperm cryopreserved with either modified-INRA 82 or Botucurio

Time (min)	Total motility (%)			Progressive motility (%)		
	Modified-INRA 82	Botucurio	P	Modified-INRA 82	Botucurio	P
0	39 ± 8	47 ± 6	0.574	27 ± 13	35 ± 8	0.681
30	34 ± 8	44 ± 4	0.048	27 ± 13	35 ± 8	0.095
60	22 ± 3	36 ± 4	0.045	12 ± 3	30 ± 8	0.045
90	12 ± 6	27 ± 7	0.045	5 ± 3	16 ± 7	0.043
120	8 ± 7	17 ± 8	0.045	3 ± 4	11 ± 8	0.050
150	2 ± 4	11 ± 9	0.039	1 ± 2	4 ± 5	0.143
180	1 ± 2	5 ± 9	0.585	1 ± 1	4 ± 7	0.343
210	0 ± 0	2 ± 4	0.648	0 ± 0	0 ± 0	0.655

comparison to samples frozen with modified-INRA 82. No differences ($P > 0.05$) were observed in samples cryopreserved with modified-INRA 82 and Botucurio regarding TM, VAP, VSL, VCL, sperm morphology, live/dead sperm, and HOST.

The sperm frozen with Botucurio showed higher TM ($P \leq 0.05$) at 30, 60, 90, 120 and 150 min and PM ($P \leq 0.05$) at 60, 90 and 120 min after thawing in the thermoresistance test, regarding sperm frozen

with modified-INRA 82 (Table 2). No differences ($P > 0.05$) were observed in the remaining times of the test.

Total motility, progressive motility, normal morphology, percentage of live sperm, and vigour decreased ($P < 0.05$) after freezing compared to fresh semen. No differences were observed between stallions ($P = 0.093$) and repetitions ($P = 0.156$).

DISCUSSION

The present results correspond to the evaluation of two equine sperm cell cryopreservation protocols with two different refrigeration curves. The parameters in raw semen were similar to those reported in other studies about the breed (Restrepo *et al.*, 2013; Hernández-Avilés *et al.*, 2018), who concluded that raw seminal characteristics of the Colombian Criollo horse are similar to other horse breeds and presents parameters compatible with technical requirements used in biotechnologies such as insemination and cryopreservation.

As reported in other breeds, better results were observed in progressive motility, chromatin integrity, and thermoresistance in sperm cryopreserved with Botucrío than with modified-INRA 82 (Candeias *et al.*, 2012; Macedo *et al.*, 2018; Moreno *et al.*, 2013; Terraciano *et al.*, 2008). The observed results suggested better protection of Botucrío against the cryoinjury. Modified INRA 82 has been used in the cryopreservation of equine seminal material, showing a beneficial effect on sperm survival post-thawing in the stepwise or immediate addition of cryoprotectants (Perez-Osorio *et al.*, 2008; Pérez *et al.*, 2017). The chemical composition of freezing extenders plays a significant role in sperm cell survival during cryopreservation (Pillet *et al.*, 2008). Thus, Botucrío results may be associated with chemical composition, since this extender combines cryoprotection (methyl formamide at 4% and glycerol at 1%) enriched with buffer system (substrate of cellular cultivation as sources of macromolecules), sugars, egg yolk and skimmed milk (Papa *et al.*, 2002; Samper & Garcia, 2008).

Several studies have evaluated methyl formamide (MF) and dimethylformamide

(DMF) alone or combined with glycerol for freezing equine sperm, showing higher post-thaw motility than glycerol alone. Amides cryoprotection (MF and DMF) have lower molecular weight and higher membrane permeability than glycerol, resulting in lower osmotic damage to the cell (Squires *et al.*, 2004). Thus, amides and glycerol association at low concentrations provide superior results in post-thaw semen viability (Medeiros *et al.*, 2002; Vidament *et al.*, 2002; Terraciano *et al.*, 2008), comparable to the results in the present work.

The buffer system is another essential component of semen extender. The variation of buffer capacity may affect the sperm biological and enzymatic system (Yániz *et al.*, 2011). In the present study, modified-INRA 82 extender contained HEPES as a buffer, whereas Botucrío – a substrate of cellular cultivation with sources of macromolecules as amino acids (Papa *et al.*, 2002). Previously, sperm cryopreserved using an extender with an amino acid as glutamine and proline showed better sperm motility after thawing than sperm cryopreserved without amino acids (Trimeche *et al.*, 1999). Glutamine has a synergistic cryoprotective effect with glycerol, and an action mechanism independent from that of glycerol, playing an extra-cellular role towards stallion spermatozoa (Trimeche *et al.*, 1999; Khlifiaoui *et al.*, 2005).

In other species, amino acids have been associated with osmoregulation, antioxidant (Wright, 1986), maintenance of motility (Kundu *et al.*, 2001), and affinity for nucleic acids (Sokól *et al.*, 2009), to neutralise the generation of reactive oxygen species and reduce DNA damage (Cabrita *et al.*, 2011; Zhang *et al.*, 2004). The present study results showed a probable relationship between amino acid

content with a better performance of Botucurio in the thermoresistance test and a lower DNA fragmentation, suggesting better protection of Botucurio in comparison with modified-INRA 82. DNA integrity is an essential factor in the evaluation of gamete's function and fertility, as it is reflected in the ability for the correct transmission of paternal genetic information and the maintenance of embryonic development (Hamamah *et al.*, 1990; Küçük, 2018). The present study is the first which evaluated post-thawing changes of the seminal DNA integrity in Colombian Criollo horses.

Milk and egg yolk are components of both semen extenders used in the present study. They contain sugars, lipids, and proteins that are sources of energy and protection against cold shock in the sperm cell, although these substances are complex and contain components that benefit and impair sperm viability in the cryopreservation process. β -lactoglobulin and native phosphocaseinate appear to be two primary components of milk with a beneficial effect on sperm cells (Pillet *et al.*, 2008). Egg yolk protects sperm from thermal shock during freezing (Wall & Foote, 1999; Amirat *et al.*, 2004); however, a few of its components exert a noxious effect on spermatozoa (Pace & Graham, 1974), as decrease of the respiration, altered spermatozoa motility (Kampschmidt *et al.*, 1953) and source of bacterial contamination (Van Wagendonk-de Leeuw *et al.*, 2000). Low-density lipoproteins (LDL) have been identified as the principal component in egg yolk (Pace & Graham, 1974; Snoeck *et al.*, 2019) and the cryoprotective fraction, being isolated and purified in several experiments (Pace & Graham, 1974; Bergeron & Manjunath, 2006; Plante *et al.*, 2016). Egg yolk and egg yolk plasma demonstrated beneficial

effects using frozen stallion sperm (Pillet *et al.*, 2011). Extenders containing 2% and 3% LDL showed higher motile spermatozoa percentages during freezing-thaw process in comparison with INRA 96 (Moreno *et al.*, 2013). Botucurio has a higher percentage of egg yolk LDL than modified-INRA 82, resulting, probably, in better cryopreservation. The lipoproteins seemingly adhere to cell membranes during freezing, restoring the lost phospholipids, inducing a transient change in the composition of phospholipids to prevent the rupture of cell membrane protecting the sperm cells (Alvarenga *et al.*, 2016; Snoeck *et al.*, 2019).

It is difficult to determine chemically if the components of egg yolk and milk are associated with the sperm after dilution, incorporating or attaching to its surface or remaining in the spaces between cells. The family of phospholipid-binding proteins (BSP proteins) identified in seminal plasma play a vital role in the protection of egg yolk LDL (BSP protein: lipoprotein interaction) and milk proteins (BSP protein: casein micelle interaction) during the cryopreservation, promoting modifications in sperm membrane (Bergeron & Manjunath, 2006; Plante *et al.*, 2016).

The carbohydrates are another component of Botucurio and modified-INRA 82, that provide a nonpenetrating cryoprotection, acting on the osmotic pressure, dehydrating the cells, and decreasing the amount of intracellular water available for potential ice formation (Alvarenga *et al.*, 2016). Monosaccharides such as glucose and fructose are readily metabolised by spermatozoa (Hammerstedt, 1975), contributing to the spermatogenic metabolism by the glycolytic pathway yielding pyruvate (Darr *et al.*, 2016). In the present study, the monosaccharides may

aid the spermatid metabolism to guarantee motility and the more complex carbohydrate, not metabolised, protected the sperm membrane integrity, resulting in similar total motility, average path velocity, straight-line velocity, curvilinear velocity, elongation and functional integrity of the spermatid plasma membrane for both extenders. These results are similar to those reported in other studies (Medeiros *et al.*, 2002; Moreno *et al.*, 2013; Moura *et al.*, 2022).

The dilution effect, low temperature, freezing, and thawing produced a reduction in total sperm motility, progressive motility, normal morphology, percentage of live sperm, and vigour to fresh semen. The biophysical and metabolic alterations associate this decrease in sperm viability with events the plasma membrane undergoes in the cryopreservation process, going from a phase of a liquid state to a colloid; generating thermal shock, osmotic stress, ion exchange disorders and membrane destabilisation characterised by reduced motility, fertility and closed circular movements (Melo *et al.*, 2007; Snoeck *et al.*, 2007; Perez-Osorio *et al.*, 2008; Sieme *et al.*, 2008).

In conclusion, both Botucurio® and modified-INRA 82 extenders guarantee viable seminal cryopreservation of equine semen of Colombian Criollo horses. However, the use of Botucurio® presented a better kinematics and chromatin integrity compared to the modified-INRA 82, suggesting a more significant cryoprotective effect.

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