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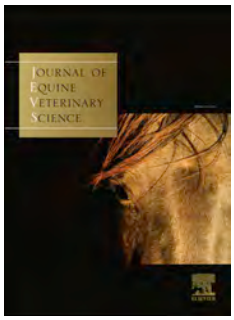

Effect of Mare Colostrum in Extenders for Freezing Stallion Semen

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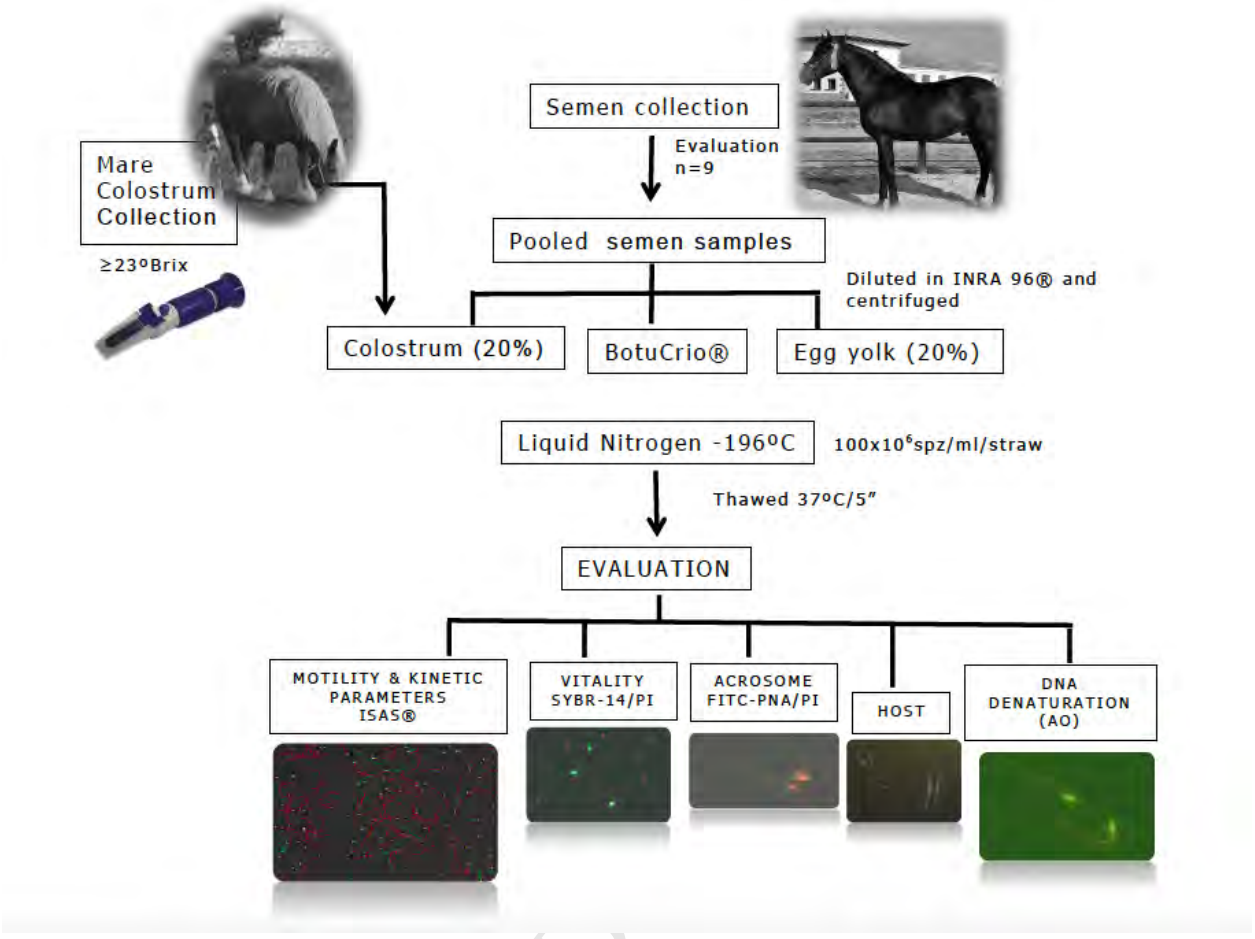
Mare
Colostrum
Collection
≥23ºBrix
Semen collection
Evaluation
n=9
Pooled semen samples
Diluted in INRA 96® and
centrifuged
Colostrum (20%)
BotuCrio®
Egg yolk (20%)
Liquid Nitrogen -196ºC
100x10⁶spz/ml/straw
Thawed 37ºC/5"
EVALUATION
MOTILITY & KINETIC
PARAMETERS
ISAS®
VITALITY
SYBR-14/PI
ACROSOME
FITC-PNA/PI
HOST
DNA
DENATURATION
(AO)

Effect of Mare Colostrum in Extenders for Freezing Stallion Semen

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Abstract

This study aimed to evaluate the addition of mare colostrum in stallion freezing extenders to improve sperm quality. First, colostrum samples were collected from four mares after the foal´s birth and their composition was determined. Ejaculates were collected from nine fertile stallions. Sperm samples were pooled, diluted and cryopreserved into three experimental extender groups: Lactose-based extender supplemented with mare colostrum (20%), Lactose-based extender supplemented with egg yolk (20%), and BotuCrio®. The quality of the post-thaw semen samples were evaluated assessing sperm motility by means of computer-assisted analysis, viability bySYBR-14 and propidium iodine (PI) stain, acrosome integrity by FITC-PNA and PI stain, plasma membrane functionality by HOS-test and DNA denaturation by Acridine Orange (AO) test. **There were no significant differences in the percentages of total motility, acrosome integrity and DNA fragmentation among the extenders after thawing. Kinematics parameters showed significantly higher values in BotuCrio® than in Lactose extenders (*P*< .05). BotuCrio® and Lactose colostrum extender yielded significantly better rates for HOS-test, LIN, STR and WOB, than**

egg-yolk extender (*P*< .05). However, in relation to sperm viability, Lactose egg yolk extender showed significantly better results in comparison to the others seminal experimental media (*P*< .05). In conclusion, the incorporation of mare colostrum into cryopreservation media protected the sperm against cold-shock; therefore, it may be a good cryoprotectant agent alternative in extenders for freezing stallion semen.

Key words: Mare colostrum; Stallion; Semen; Cryopreservation.

1. Introduction:

In equine species the fertilizing capacity of frozen semen is lower than in other species [1]. There is also a strong individual factor, thus, stallions are qualified as "poor or good freezers" [2,3]. In addition, pregnancy rates in mares inseminated with frozen semen doses are lower in comparison to chilled semen or natural conditions (30-40%) [4–6]. The cryopreservation medium should ensure the sperm quality after freezing-thawing process due to specific compounds, as cryoprotectants, which decrease cold-shock damage [7]. Egg yolk is currently used as an **external** cryoprotective agent to freeze equine sperm and other species sperm [8]. It is considered as a good non-penetrating cryoprotectant due to cholesterol, phospholipids and **Low-density lipoprotein** (LDL), proteins that have been identified as protective agents against temperature related injury [9].

Previous research has demonstrated that milk contains some factors and components, such as caseinates, that provide protection to semen during the storage [1,10]. In addition, milk has been shown to have an antioxidant effect responsible for strong plasma membrane protection [1,11]. Colostrum, as the first form of milk produced by the mammary gland, has similar compounds as milk. Furthermore, it has been recognized to protect the foal during its first days of life by providing Immunoglobulins

(Ig) [12,13]. Notwithstanding, the composition of colostrum differs from milk in the Ig concentrations (IgG>80%),fat and lactose levels [13–15]. Likewise, colostrum shows anti-inflammatory and antimicrobial effects because of some active compounds such as lactoferrine, lactoperoxidase and lysozyme [16].

Mares develop an endometritis in the uterus during 24-36h after insemination [6], which is in some cases persistent and incompatible with pregnancy. Some extender components such us glycerol or egg yolk proteins may induce a greater migration of polymorphonuclear neutrophils (PMNN) in the endometrium [17,18]. A recent study, investigated the anti-inflammatory effect of exogenous lactoferrine on breeding- induced endometritis [19].

Colostrum may be a viable alternative instead of milk or egg yolk in cryopreservation stallion extender because it could provide protection to spermatozoa against cold-injury, and protection in the mare´s uterus after insemination [20,21]**.**

Based on the research presented above and taking into account the strong goal to find alternative cryoprotectants, the aim of this study was to test the protective action of mare colostrum in stallion freezing extender.

2. Materials and methods

2.1. Reagents and media

All chemicals were obtained from Sigma-Aldrich Química S.A. (Madrid, Spain), unless otherwise indicated. Dimethylformaide (DMF) was from Panreac Química S.L.U. (Barcelona, Spain) and Equex Paste from Minitube Ibérica S.L. (la Selva del Camp, Spain). The medium used for washing semen and centrifugation was INRA 96® (IMV Technologies, L'Aigle, France).

The care and the use of animals were in accordance with the Spanish Policy for Animal Protection RD 1201/05 and the directive 2010/63/EU for animal experiments.

2.2. Mare colostrum collection, evaluation and preservation

Colostrum was collected from four mares of different breeds and ages, from the Military Horse Breeding Centre (MHBC) in Zaragoza (Spain). The colostrum was collected from the mare immediately after parturition (a total of 120 ml was collected from each mare). The udder region of each mare was meticulously cleaned with warm water before collection, and an ultrasound study (Mindray DP-3300 VET®, 7.5 MHz) was performed to rule out any possible pathologies [13,22]. Once colostrum was obtained, it was filtered by cellulose filter (Minitube Ibérica S.L., la Selva del Camp, Spain).

The quality of the colostrum was evaluated using a Brix refractometer [13]. Only samples that showed at least 23º Brix were accepted (23ºBrix >60 g/L IgG [23]. Colostrum samples were frozen at -20ºC in 15 ml labelled test tubes [23,24]. Before each experimental study, colostrum was thawed in a water bath at 37ºC [13,25] and all the samples were mixed together. The composition of colostrum was analysed by means of anIR spectroscopy MilkoScan™ 4000 Foss Electric (Hilleroed, Denmark).

2.3. Semen handling and cryopreservation

Fifteen ejaculates were collected from nine stallions –good freezers− (aged from 7-12 years) during the breeding season from the MHBC in Zaragoza (Spain) using an artificial vagina (Missouri-model, Nasco, Ft. Atkinsosn, WI, USA).

Macroscopic and microscopic assessments were performed immediately after their extraction. The initial concentration of spermatozoa was determined using a Spermacue® 12300/0500 (Minitube Ibérica S.L., La Selva del Camp, Spain). Motility and kinematic parameters were analysed using the Integrated Semen Analysis System (ISAS®, Projectes I Serveis R+D S.L., Valencia, Spain).

Only semen with at least ≥35% progressive motility was accepted[26] for cryopreservation. Semen samples were diluted in INRA® medium and centrifuged at 1000 g for 5 min [2].

Semen samples from every three ejaculates were pooled to avoid individual factors and obtain a more homogeneous group. **Every pooled sample was** divided into three experimental extender groups, **and from each experimental group 5 samples were frozen**.

Experimental groups:

1st Group, Lactose–mare colostrum extender containing 50% (v/v) of 290 mM L-lactose, 20% (v/v) of mare colostrum, 25% (v/v) of Glucose–EDTA medium (322.20 mM glucose, 12.58 mM sodium citrate, 9.93 mM disodium EDTA, 14.28 mM sodium bicarbonate) **and 5%(v/v) of DMF** [27].

2nd Group, Lactose– egg-yolk extender containing 50% (v/v) of 290 mM L-lactose, 20% (v/v) of egg yolk, 25% (v/v) of Glucose–EDTA medium (322.20 mM glucose, 12.58 mM sodium citrate, 9.93 mM disodium EDTA, 14.28 mM sodium bicarbonate), 0.5% (v/v) of Equex Paste [28] and 5%(v/v) of DMF[27].

3rd Group, BotuCrio®, standard cryopreservation extender.

The samples were frozen using a stabilization step process with a slow cooling rate of approximately 0.5ºC per min. The semen samples were cooled down to 4 ºC in 2 hours for the lactose extenders, and during 20 min for BotuCrio® extender samples as instructed in the information leaflet. Then, sperm samples were packed into 0.5 ml polyvinylchloride straws (IVM technologies, L´Aigle, France), which contained100 x 10^6 spermatozoa/ml [12]. Finally, before submerging the samples, they remained for 20 min at 5 cm above liquid nitrogen vapors in a Styrofoam freezer box with neopor insulation block (freezing rate of -60ºC) (MinitubIbérica S.L., La Selva del Camp,

Spain). **The spermatozoa of the five freezing batches from the pooled ejaculates were analysed.**

2.4. Semen quality evaluation

Frozen semen samples were thawed in a water bath at 37ºC for 21 sec. Sperm motility, viability, plasma membrane functionality, acrosome integrity and DNA fragmentation were assessed. Also, a resistant test –2 hours at 37ºC− was performed.

2.4.1. Sperm motility analysis: Total motility, as well as velocity parameters were analysed by means of ISAS® (Projectes I Serveis R+D S.L., Valencia, Spain) [29]. The parameters established for the analysis were 25 consecutive digitalized images per sec. and the particles area was 4-75μ^2. With regard to the setting parameters for the program, sperm, values of VAP <10 μ/sec were considered as slow and >90 μ/sec were considered as fast. Spermatozoa with 75% of the STR were designated as progressive motile. ALH value ≥10μm (minimum number of images calculated). **After 2 hours at 37ºC, a *Resistant Test* (RT) was performed in order to evaluate the motility** [30].

2.4.2. Sperm viability was evaluated using LIVE/DEAD® sperm viability kit (Molecular Probes Europe, Leiden, The Netherlands). The staining was performed following the instructions of the commercial kit. SYBR-14 penetrated the membrane of all the spermatozoa and provided a green fluorescence. Propidium Iodine (PI) only penetrated the spermatozoa with altered membrane and showed red fluorescence, which overlapped the green one. A total of two hundred cells were counted using a fluorescence phase-contrast microscope.

2.4.3. Membrane and acrosome status were assessed by Fluorescein Isothiocyanate conjugated with Peanut Agglutinin (FITC-PNA) and PI staining [31] **(Molecular Probes, Europe, Leiden, The Netherlands)**. 200 µl of each of the semen samples were supplemented with FITC-PNA solution (1 mg/ml in doubly distilled water) and PI solution (500 µg/ml), kept 5 min at 38ºC, and finally fixed in paraformaldehyde (4% [v/v]). Two hundred of spermatozoa were counted under a fluorescence microscope. Sperm with intact plasma and acrosomal membrane (PNA−/PI−) were determined.

2.4.4. Plasma membrane functionality was assessed using HOS test [32]. This technique consisted of incubating **30 µl of sperm sample with 100 µl of lactose hypo-osmotic solution (100 mOsm/Kg)** at 37 ºC for 30 min. The samples were then fixed in 8% glutaraldehyde buffered solution. Spermatozoa with coiled tails were considered as HOST-positive.

2.4.5. DNA status was assessed by the Acridine Orange (AO) test. [33]. Metachromatic staining can assess chromatin stability with AO, based on the susceptibility of the sperm DNA to acid-induced denaturation in situ. Air-dried semen samples were fixed in Carnoy solution (3:1methanol:glacialaceticacid) for 3 hours, and then rinsed in distilled water and dried. The slides were then covered with 0.1% AO staining solution, according to the method of Tejada [34]. After 5 min, the slides were washed with tap water and covered with a coverslip. Under fluorescence microscope evaluation, a total of two hundred spermatozoa were counted. Two different partners of staining were determined: spermatozoa carrying a normal DNA appeared as green heads, while single-stranded denaturised DNA produced a red-orange fluorescence.

2.5. Statistical analysis

R-version: 3.1.3 Platform x86_64 Apple-Darwin 13.4.0 (64bit) was used to analyse the results.

The statistical procedure used for the thawed samples results, began with a visual exploration of data (boxplots) and a summary of centrality estimates (mean and median) and variability (standard deviation). For extenders contrast a non-parametric test (Mann-Whitney-Wilcoxon test) was used. All tests were performed with a significance level of 95. This procedure was executed for all values - total motility, velocity parameters − curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), linearity (LIN), straightness (STR = VSL/VAP) and wobble (WOB) −, viability, HOST, acrosome intact, DNA integrity and the RT.

3. Results

The colostrum composition determined in this study in relation to fat, protein lactose and dry matter, is shown in *Table 1*.The results obtained for the four samples after refractometry were 24±1ºBrix.

Pre-freezing percentages of total sperm motility, acrosome integrity and viability didn't differ among the different extenders. After thawing, semen samples showed values of total motility > 60%; therefore all stallions selected were classified as "good freezers".

The results in this study for total motility, velocity parameters and the RT for the three groups are shown in *graphic 1*. **There were no significant differences in the percentage of total motility among the extenders after thawing. BotuCrio® showed significantly higher values for kinematics parameters (VCL, VSL, VAP, WOB, LIN and STR) than the others extenders.** In relation to RT, Lactose egg yolk semen samples showed **higher values** for total motility than other extenders, but there were no significant differences (*P*>.059).

The results in this study for viability, HOST positive, acrosome status and DNA denaturation for the three groups are shown in *graphic 2*. **Lactose egg yolk extender showed significantly higher values for sperm viability (*P*<.02) and significantly lower percentages for sperm HOST positive (*P*<.005) in comparison to BotuCrio® and Lactose colostrum extender.** DNA fragmentation did not show significant differences in any analysed sperm samples. Nevertheless, a very low percentage of fragmented DNA was determined in all stained samples. The highest rate of damaged DNA was found in lactose egg yolk samples (3.33%) and the lowest in lactose colostrum samples (2.11%).

4. Discussion

In this research, we demonstrated that freeze-thawed mare colostrum, did not lose its properties as described by Cash(1999) [23]. The determination of IgG quantity and quality was analysed by Brix refractometer. It is a standard technique todetermine the quality of colostrum [24,35],

The colostrum samples remained frozen for a period of 2 months. Once thawed and analysed, colostrum showed good quality (≥ 23ºBrix, an acceptable IgG levels). The rest of the parameters analysed, as shown in table 1, were also within the average ranges for colostrum composition [14]. Mare colostrum quality found to be adequate for our study maintaining the standard quality [14,23].

We obtained positive results of sperm quality after thawing. The threshold for acceptable post-thaw motility is quite different among authors, but in general, results ≥ 35% are considered acceptable [13]. As shown in table 2, post-thawed sperm motility in our study was superior (>60%).

The Intracellular cryoprotectant used in our research was DMF. 5% DMF maintains frozen-thawed stallion sperm quality better than others cryoprotectants including glycerol, improving all seminal parameters evaluated both in ejaculate and epididymis

sperm samples [27]. Previous research has documented that DMF reduced the osmotic damage on sperm due to the low viscosity and molecule weight of amides [36]. **Egg yolk is one of the most common components in the sperm freezing extender because it minimizes cold-shock effect and maintains sperm quality and functionality** [37,38]**.** BotuCrio® **contains DMF and pasteurized egg yolk**, which has shown to increase the percentages of total motility and fertility when utilized as stallion sperm frozen extender [39]. However, Olaciregui*et al.,* (2014) [40] observed than it did not improve sperm quality on epididymal stallion sperm. Different substances as soybean lecithin, cholesterol loaded cyclodextrins, or low density lipoproteins preserved sperm motility and plasma membrane integrity after the freezing process **instead of egg yolk** [8,39,41].**In our study, we tested the effect of a novel substance, mare colostrum, on stallion cryopreservation extender. We determined that colostrum extender samples showed similar percentages of sperm quality parameters as sperm samples cryopreserved with Botucrio®. In addition, colostrum extender significantly increased the percentages of sperm HOS test positive, LIN, STR and WOB in comparison to Lactose egg yolk extender.**

Colostrum includes some molecules as caseinates in its composition. It has been shown that caseinates act as membrane protectors in sperm during the cryopreservation process [1,10,11]. **Thus, colostrum would perform a protective effect, not only in sperm plasma membrane, but in DNA integrity**. **In addition, several molecules with antibacterial and anti-inflammatory properties have been determined in colostrum composition, which could provide a protection against endometritis post-breeding in the mare [35,36].**

Different options have been tested in stallion sperm cryopreservation medium. But, to the authors´ best knowledge, no publication has been found in literature that addresses the use of mare colostrum to preserve stallions' semen.

Summing up the results, **we concluded that the incorporation of colostrum to semen extender protected stallion spermatozoa during the freezing-thawing process.** It may be a new possibility in sperm cryopreservation field and a useful alternative to apply in mares to avoid persistent endometritis processes. Subsequent fertility testing is necessary to verify the beneficial effects on post-thaw sperm characteristics.

Declarations of interest: none

Acknowledgments

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429

430 **Table 1 and figure legend**

431

Composition	Colostrum ranges *	Mare Colostrum sample
Fat %	0.92 – 2.44	1.12
Protein %	8.2 – 22.22	11
Lactose %	0.64 − 4,28	3.88
Dry matter %	14.47 – 24.21	15.28

**Peka et al., 2012*

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435 *Table 1.Comparative of colostrum means and results obtained from our colostrum*

436 *sample after analysis* −IR spectroscopy MilkoScan™ 4000 Foss Electric (Hilleroed,

437 Denmark).

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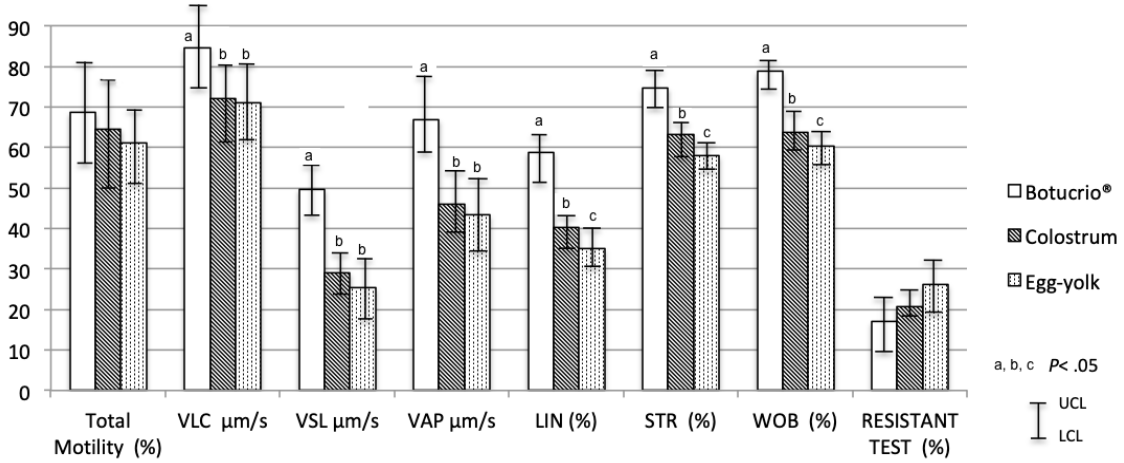
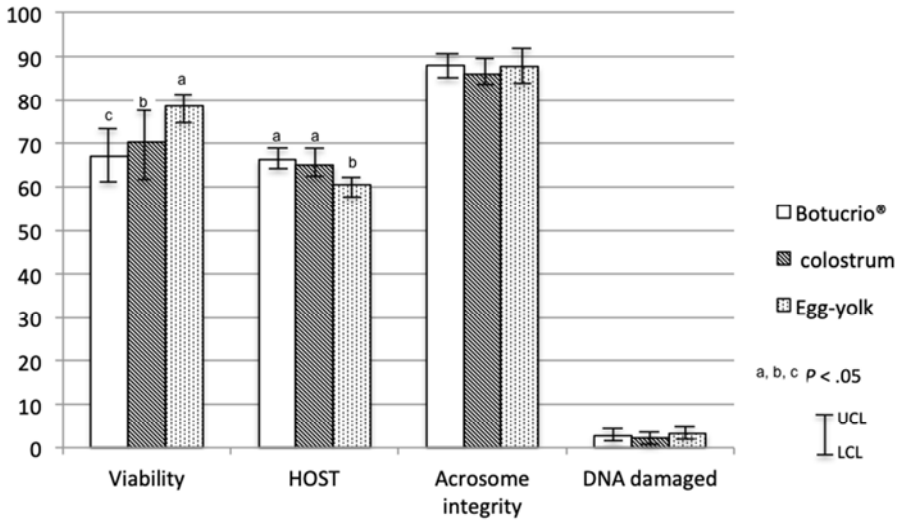
CAPTIONS



Graphic 1. Three different extenders Mean ± SD (UCL: Upper confidence level. LCL: Lower confidence level), for Motility, Kinematic parameters and Resistant Test. (n= 15). Superscript letters (^{a,b,c}) indicate significant differences (P < .05). ***For BotuCrio® samples the results showed higher for all kinetic parameters. For LIN, STR and WOB, colostrum was significant higher than egg yolk extender. For Total Motility there was no significant difference when comparing the three extenders.***



Graphic 2. Three different extenders Mean ± SD (UCL: Upper confidence level. LCL: Lower confidence level), for Vitality, HOST, Acrosome integrity and DNA damaged. (n=15). Superscript letters (^{a,b,c}) indicate significant differences (P < .05).

HIGHLIGHTS

1. The addition of mare colostrum in extenders improved sperm quality and could be effectively used for freezing stallion semen.
2. Lactose- mare colostrum extender decreased the percentages of DNA fragmentation after freezing-thawing process.
3. Mare colostrum is promising cryoprotectant in equine cryopreservation semen

Ethical statement

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The authors declare that the care and the use of animals were in accordance with the Spanish Policy for Animal Protection RD 1201/05 and the directive 2010/63/EU for animal experiments.