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# A preliminary study on the use of jenny colostrum to improve quality in extenders for freezing donkey semen

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ABSTRACT

Sperm quality in donkeys (*Equus asinus)* after freezing thawing is still considered lower than that from other animals, including horses. The aim of this study was to test a new freezing extender supplemented with jenny colostrum on donkey sperm. After thawing, we evaluated sperm motility by means of computer-assisted analysis, viability by SYBR-14 and propidium iodide (PI), membrane functional integrity by HOS-test and acrosome integrity by isothiocyanate conjugated with peanut agglutinin (FITC-PNA) and PI. Ejaculates were collected from five fertile Donkeys. Sperm samples were pooled, diluted and cryopreserved into three experimental extender groups: BotuCrio®, lactose-extender supplemented with egg yolk (20%) and lactose-extender supplemented with jenny colostrum (20%). The results demonstrated that lactose-jenny colostrum samples displayed significantly higher values in almost all parameters evaluated ($p < 0.05$) compared with the other two extenders after thawing (BotuCrio® and lactose-egg yolk based extender, respectively) –Total Motility, Viability, HOS test, VCL, VSL and VAP. Acrosome status, LIN, STR and WOB despite showing lower values, none of them were statistically significant ($p > 0.05$). In conclusion, the extender containing jenny colostrum can be successfully used for donkey semen crypreservation and could effectively improve donkey sperm qualities after freezing -thawing.

## 1. Introduction

Global donkey (*Equus asinus*) population is decreasing considerably. Many different breeds of autochthonous donkeys are at high risk of extinction. It is important to make efforts to contribute to the conservation of these animals. The use of artificial insemination (AI) techniques can help in this context [6,42]. Frozen donkey semen, in comparison to horses, shows low conception rates (33%–56% mares vs. 0%–36% jennies). There are only few studies about donkey semen cryopreservation [2,11,45]. Hence, it is necessary to implement different strategies during cryopreservation process in order to improve sperm quality, functionality and fertility [44].

Extenders composition may determinate the sperm quality after thawing. Egg yolk and skim milk have been considered the best non-penetrating cyoprotectants in horse extenders [25].

Previous research has demonstrated that milk contains some factors and substances, as caseinate proteins, that provide semen protection during its storage [7,21]. In addition, milk has been shown to have an antioxidant effect that is responsible for a strong plasma membrane protection [7,8].

With regard to milk properties, it has been demonstrated that donkey milk has similar composition than human milk with analogous antimicrobial and anti-inflammatory properties [27,35,46]. Donkey colostrum, as the first form of milk produced by the mammary glands, has, in general terms, similar composition. However, it differs from milk in several important areas, including higher concentrations of immunoglobulins (Ig) (IgG and IgA) and fat, and lower concentrations of lactose [27,28,34]. It is also composed of a wide range of antimicrobial [35] and immunomodulatory factors, including lactoferrin, lactoperoxidase, lysozyme and oligosaccharides [9].

The uterine reaction of the mares/jennies after insemination is a natural process that aims to eliminate sperm, seminal plasma and other potential pollutants [17]. This inflammation is resolved within 24–36 h [43]. Nevertheless, some mares (and more frequently in jennies), are unable to eliminate inflammation, they consequently develop persistent breeding-induced endometritis, which is incompatible with pregnancy. Some extender components such as glycerol or egg yolk proteins play a major role in this reaction by inducing a greater migration of polymorphonuclear neutrophils (PMNN) in the endometrium [33,41].

We hypothesized that the addition jenny colostrum in an extender for freezing donkey semen could have a protective effect after thawing. Furthermore, if the results obtained are relevant, it may be beneficial in

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the jenny uterus after insemination. The information obtained might help to preserve these species and increase the population of donkeys.

## 2. Materials and methods

### 2.1. Reagents and media

All chemicals were obtained from Sigma-Aldrich Química S.A. (Madrid, Spain), unless otherwise indicated. Dimethylformamide (DMF) was from Panreac Química S.L.U. (Barcelona, Spain) and Equex Paste from Minitub Ibérica S.L. (La Selva del Camp, Spain). The medium used for washing the 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. Jenny colostrum collection, evaluation and preservation

Colostrum was collected from four jennies of different ages, from the Military Horse Breeding Centre (MHBC) in Zaragoza, Spain.

The colostrum was collected and filtered (cellulose filter, Minitub Ibérica S.L., La Selva del Camp, Spain) from the jennies just after the foal's birth (100 ml of each jenny). Before the collection, an ultrasound study (Mindray DP-3300 VET®, 7.5 MHz) was performed to rule out any possible pathologies [1,28].

The quality of the colostrum was evaluated using a Brix refractometer, after collection. There is a good correlation between the refractometer reading and colostrum IgG ($r^2 = 0.74$), where $r^2$ is the coefficient of determination which ranges from 0 to 1 [28]. In our study, only samples that had at least 23° Brix were accepted (23°Brix > 60 g/L IgG), Cash (1999) [12] considered values ≥ 20 °Brix as good quality for colostrum samples. Then the samples were frozen at −20 °C in 15 ml labelled test tubes [12,13].

Once the samples were thawed and evaluated, they were mixed together and added to the extender for study.

### 2.3. Semen handling and cryopreservation and thawing

Ejaculates were collected from 5 donkeys from the HBMC of Zaragoza, Spain, ages 5–10 years, during the breeding season, using an artificial vagina (Missouri-model, Nasco, Ft. Atkinsosn, WI, USA). Two ejaculates per donkey (n = 10) were used in this experiment.

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

The sperm samples were pooled and diluted INRA 96® (IMV Technologies; L'Aigle, France) to avoid individual factors. Then, samples were centrifuged at 1000 g for 5 min and divided into three representative groups:

1st Group, BotuCrio®; cryopreservation commercial extender.

2nd Group, Lactose– egg yolk based 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 [18] and 5% (v/v) of DMF [5].

3rd Group, Lactose–jenny colostrum extender; containing 50% (v/v) of 290 mM L-lactose, 20% (v/v) of jenny 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% DMF.

The samples were frozen following a slow freezing process, whereby the temperature progressively decreased for 2 h until reaching 4 °C. Then, the samples were packed into 0.5 ml polyvinylchloride straws (IVM technologies, L'Aigle, France), at $100 \times 10^6$ spermatozoa/ml per straw [40], introduced in vapours of liquid nitrogen for 15 min and finally submerged into liquid nitrogen (−196 °C) in a freezer Styrofoam box with neopor insulation block (Minitub Ibérica S.L., La Selva del Camp, Spain). The stabilization step in BotuCrio® samples was performed following the standard protocol for this extender. All samples were thawed in a water bath at 37 °C for 21 s.

### 2.4. Semen quality assessment

#### 2.4.1. Sperm motility analysis

Total motility, as well as kinetic parameters − curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), linearity (LIN), straightness (STR = VSL/VAP) and wobble (WOB) −, were analysed by means of ISAS® [19]. The particles area was fixed between 4 and 75 μm², spermatozoa with VAP < 10 μ/sec were considered as immotile, > 45 μm/s as normal and > 90 μm/s as rapid. And ALH minimum value was 10 images.

#### 2.4.2. Sperm viability

Sperm viability was evaluated using LIVE/DEAD® sperm viability kit (Molecular Probes Europe, Leiden, The Netherlands) [24]. SYBR-14 stain penetrates both intact and damaged spermatozoa membranes and fluoresces green, while propidium iodine (PI) only penetrates a damaged membrane, and stains the sperm red [23]. Two hundred spermatozoa were analysed using a fluorescence phase-contrast microscope.

#### 2.4.3. Membrane and acrosome status

Membrane and acrosome status were assessed by FITC-PNA/PI staining. Sperm samples were incubated at 38 °C during 5 min with FITC-PNA solution (1 mg/ml) and PI solution (500 μg/ml) and fixed in paraformaldehyde (4% [v/v]). Two hundred spermatozoa were counted under a fluorescence microscope. Spermatozoa with plasma and acrosomal membrane status (PNA−/PI−) were analysed.

#### 2.4.4. Plasma membrane functionality

Plasma membrane functionality was assessed using HOS test [22]. Semen samples were incubated in lactose hypo-osmotic solution (100 mOsm) for 30 min at 37 °C. The samples were then fixed in 8% glutaraldehyde buffered solution and spermatozoa with coiled tails were considered as HOST-positive.

### 2.5. Statistical analysis

In order to determine whether the results were statistically valid, a detailed analysis of all the thawed semen samples was performed (R version: 3.1.3 Platform x86_64 Apple-Darwin 13.4.0 (64bit). The statistical procedure began with a visual exploration of data (boxplots), a summary of centrality estimates (mean and median) and variability (standard deviation). Shapiro-Wilk test was used to confirm a normal distribution. For extenders contrast a non-parametric test (Mann-Whitney-Wilcoxon test) was used. All tests were assessed at the conventional significance level of 0.05. Data are expressed as mean ± standard deviation (SD). This procedure was consistently repeated for all values − motility, kinetic parameters, viability, HOST and acrosome − in all extender samples.

## 3. Results

The values obtained from the four colostrum samples after refractometry analysis were 24.5 ± 1° Brix.

Lactose-based extender with jenny colostrum showed the best results in almost all the compared variables evaluated. Data for comparison of semen quality analysis with different extenders are provided in Fig. 1 and Fig. 2.

Shapiro-Wilk and Mann-Whitney-Wilcoxon tests were performed,

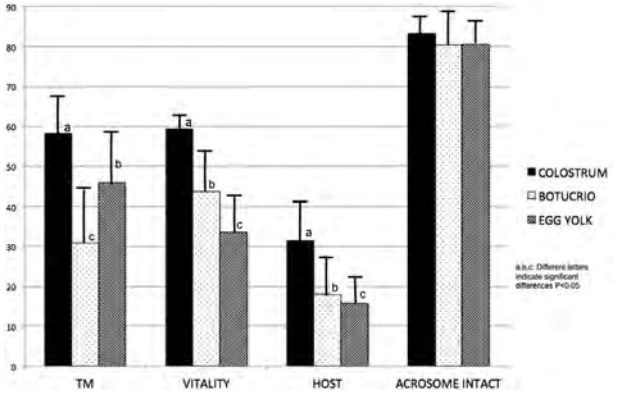


**Fig. 1.** Post-thawing values for Total Motility, Vitality, HOST and Acrosome intact. Total motility, vitality and HOS test show better results for lactose-Jennie colostrum extenders. Superscripts ($^{a,b,c}$) indicate significant differences ($p < 0,05$).

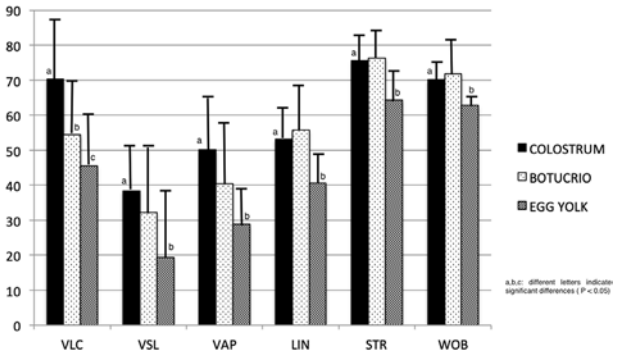


**Fig. 2.** Post-thawing values for Kinetic parameters. Colostrum was statistically better than the other two extenders for VLC. Superscripts ($^{a, b, c}$) indicate significant differences ($p < 0,05$). For the rest of the parameters analysed, there were only significant differences ($p < 0,05$) between colostrum and egg yolk samples (Superscripts $^{a, b}$). For BotuCrio® samples, in spite of showing higher values for LIN, STR and WOB, no significant differences were shown.

comparing the means of all values (total motility, velocity parameters: VCL, VSL, VAP, LI, STR and WOB, viability, HOST and acrosome integrity).

Cryopreservation affected significantly almost all the sperm parameters assessed ($p < 0.05$) regardless of the extender used. Lactose-jenny colostrum extender was significantly better ($p < 0.05$) for all the samples than Lactose-egg yolk, except for acrosome status, in which there was no significant differences ($p > 0.05$) (Fig. 1).

Different results were showed between jenny-colostrum and BotuCrio® extenders, depending on the evaluated parameter. Lactose-jenny colostrum improved the percentage of sperm total motility, viability, HOST, and VLC ($p < 0.05$). However, the rest of the parameters evaluated did not show significant differences between both extenders.

## 4. Discussion

In this study, we have demonstrated that jenny colostrum, once thawed and mixed, did not lose its properties as described by Cash (1999) [12]. IgG quantity and quality were analysed by Brix refractometer and we obtained values superior to 23° Brix. This is a standard method to determine reliable results of colostrum quality [15].

Along the years, different studies have proposed several freezing extenders of various compositions in an attempt to improve the quality and the use of frozen stallion semen [10,14,26]. Egg yolk and skim milk are the most common protective agents against cold shock injury utilized on stallion sperm cryopreservation [10,25]. The combination of egg-yolk based medium with glycerol is a standard protocol utilized in commercial extenders as BotuCrio®. However, Álvarez et al. (2014) [5] and Acha et al. (2016) [2], demonstrated that DMF at concentration of 2.5% used as cryoprotectant in donkeys showed better sperm quality – greater values ($p < 0.001$) of total motility, progressive motility and membrane status – than other cryoprotectants after cryopreservation.

DMF reduced the osmotic damage on sperm due the low viscosity and molecule weight of amides [4] and increased the stallion sperm quality after cryopreservation.

BotuCrio® contains pasteurized egg yolk and has shown higher percentage of total motility and fertility when is utilized as stallion sperm frozen extender [32]. However, Olacireguiet al. (2014) [31], observed than pasteurized egg yolk did not improve sperm quality on epididymal stallion sperm. In our study, BotuCrio® showed lower percentages of sperm motility, viability and HOST than Lactose-colostrum extender.

Even though no publication has been found in literature that addresses the use of jenny colostrum to preserve donkey's semen, our research showed that the incorporation of colostrum as a new component in extender for freezing donkey semen increased the percentages of total motility, kinetic parameters and sperm plasma membrane functionality after performing a freezing-thawing procedure.

Our results for sperm motility and kinetic parameters in all extenders were low, but yet in accordance with Rí Zková et al. (2017) [37], with similar mean for all kinetic parameters (VCL 63.23 µm/s, VSL 31.02 µm/s, VAP 47.53 µm/s, STR 65.24%, LIN 48.8%). Results also agreed with Rezagholizadeh et al. (2015) [36] in regards to STR (71.20%) and LIN (45.5%). Kinetic parameters for Lactose-jenny colostrum extender showed better results than for Lactose-egg yolk extender ($p < 0.05$).

Sperm motility is an important parameter to evaluate semen quality in equine species. Donkey semen is less efficiently frozen than that of the stallion, and a significant decrease in total and progressive motility is recorded. Values < 25% post-thaw motility are found to result in a lesser pregnancy rate. In our work, besides the good results of the kinetic parameters, lactose-jenny colostrum also improved total sperm motility (58.3%) after cryopreservation, and it might increase the fertility potential of the sperm samples.

Skim milk-based medium maintains sperm motility, plasma membrane and acrosome integrity in ejaculated and epididymal stallion sperm [30,39]. It has specific proteins as caseinate, which protects sperm even at low concentrations [21]. The colostrum composition, which also contains caseinate, might explain the protecting action on sperm membrane.

We found better percentage on sperm viability on lactose-jenny colostrum extender than on egg yolk-based extenders ($p < 0.05$).

The frozen donkey semen is subjected to conditions that seriously affect sperm functionality and viability, leading to reduce sperm longevity in the jenny genital tract. Moreover, the use of frozen semen induces a stronger inflammatory reaction in the uterus than fresh semen in mares [20,38]. The absence of seminal plasma, certain cryoprotectants [29] such as glycerol, and egg yolk proteins favour this reaction by inducing greater PMNN migration to the endometrium [33,41].

A recent study has demonstrated that the inoculation of colostrum stimulated the uterine immune system and reduced postpartum uterine pathologies in the sows [3]. Another study has demonstrated that Ig, lactoferrin and lactoperoxidase present in colostrum might reduce inflammatory response and oxidative stress of mucous [46].

The use of lactoferrin for its anti-inflammatory and bactericidal properties has also recently been studied by Fedorka et al. (2018) [16] as a treatment on breeding-induced endometritis in susceptible mares.

Consequently, the properties of colostrum shown in those studies could suggest a new field of investigation in which colostrum specific compounds, on the one hand provide good results after thawing and on

the other hand could interact in jenny uterus and decrease reactions due to AI.

It is a preliminary study and subsequent fertility testing is necessary to verify the beneficial effects on post-thaw sperm characteristics.

Summing up the results, we concluded that Jenny-colostrum exerted a protective effective against cold shock, since it improved sperm quality after freezing-thawing process. Therefore, it could be a useful alternative as cryoprotectant on sperm plasma membrane in freezing extenders in donkeys. Furthermore, since extant research on this topic is limited, this paper may create new areas for the study of sperm cryopreservation.

## Conflicts of interest

None.

## Acknowledgments

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