FULL ARTICLE

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Effect of egg yolk-based extender and seminal plasma removal on sperm viability of cooled donkey semen

Efeito do diluente à base de gema de ovo e remoção do plasma seminal na viabilidade espermática do sêmen refrigerado de jumento

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ABSTRACT

Developing effective cooled semen protocols is essential to increase pregnancy rates and reproductive efficiency in donkeys. This study aimed to evaluate the effect on sperm kinetic parameters and membrane integrity in cooled donkey semen diluted with defined milk proteins extender with 1% or 2% of egg yolk and the removal of seminal plasma. Twenty-four ejaculates from six jackasses were collected. Each ejaculate was divided into four aliquots that were diluted in extender with 1% (EY1) or 2% (EY2) egg yolk. One sample from each group was centrifuged, seminal plasma was removed (CEY1, CEY2 groups, respectively), and the samples were then refrigerated at 5 °C for 24 h. Fresh and cooled semen samples were assessed for sperm motility, morphology, and plasma membrane integrity. Total motility, progressive motility, sperm kinetic parameters, or live sperm cells were not statistically different when semen was cooled with an extender supplemented with 1% or 2% of egg yolk. Seminal plasma removal does not affect total motility or sperm kinetic parameters. However, progressive motility decreased (P<0.05) when semen was extended with 2% of egg yolk and seminal plasma was removed. Membrane integrity was affected (P<0.05) in centrifuged samples. In conclusion, the obtained results suggest that there is no difference in sperm kinetics and membrane integrity when 1% or 2% of egg yolk was added to the Equiplus® extender. Also, the removal of seminal plasma by centrifugation did not have any beneficial effect on cooled donkey semen. Further studies are needed to relate these results with in vivo fertility tests with cooled donkey semen.

Keywords: Donkey. Cooled semen. Semen preservation. Seminal plasma. Egg yolk extender.

RESUMO

O desenvolvimento de protocolos de sêmen resfriado eficazes é essencial para aumentar as taxas de prenhez e eficiência reprodutiva em jumentos. O objetivo desse estudo foi avaliar o efeito do diluente à base de proteínas do leite com 1 ou 2% de gema de ovo sobre os parâmetros cinéticos do sêmen e integridade da membrana em sêmen resfriado de jumento, com ou sem a remoção do plasma seminal. Vinte e quatro ejaculados de seis jumentos foram coletados. Cada ejaculado foi dividido em quatro alíquotas e diluído em diluente com 1% (EY1) ou 2% (EY2) de gema de ovo. Uma amostra por grupo foi centrifugada e o plasma seminal removido (grupos CEY1 e CEY2, respectivamente). Os *pellets* foram novamente ressuspendidos nas mesmas concentrações e diluentes. Em seguida, as quatro alíquotas foram refrigeradas a 5°C por 24 horas. Amostras de sêmen fresco e refrigerado foram avaliadas quanto à motilidade espermática e integridade da membrana plasmática. Motilidade total, motilidade progressiva, parâmetros de cinética espermática ou células espermáticas vivas não apresentaram diferença significativa quando o sêmen foi resfriado com diluente suplementado com 1% ou 2% de gema de ovo. A remoção do plasma seminal não afetou a motilidade total ou os parâmetros de cinética espermática; entretanto, a motilidade progressiva diminuiu (P<0,05) quando o sêmen foi diluído com 2% de gema de ovo e o plasma seminal removido. Nas amostras centrifugadas, a integridade da membrana foi afetada (P<0,05). Em conclusão, os resultados sugerem que não há diferença na cinética espermática e na integridade da membrana quando 1% ou 2% de gema de ovo são adicionados ao diluente Equiplus® e a remoção do plasma seminal por centrifugação não teve nenhum efeito benéfico no resfriamento de sêmen de jumento. Mais estudos são necessários para relacionar esses resultados com testes de fertilidade in vivo com sêmen resfriado em jumentos.

Palavras-chave: Jumento. Sêmen resfriado. Preservação de sêmen. Plasma seminal. Diluente de gema de ovo.

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Introduction

In recent years, the demand for donkeys for milk production and industrial derivatives has increased worldwide (Camillo et al., 2018). In consequence, the development of reproductive biotechnologies to preserve semen becomes a commercial requirement. The use of cooled equine semen is widespread due to its similar pregnancy rates to fresh semen (Newcombe & Cuervo-Arango, 2011). Besides, the simplicity and low-cost of the artificial insemination technique contribute to its extensive use in equine breeding programs. In donkeys, a few studies reported a 26% to 40% pregnancy rate per cycle using cooled-extended semen (Alvarez et al., 2004; Rota et al., 2017), suggesting that the development of effective semen cooling protocols are essential to increase pregnancy rates and reproductive efficiency.

Biochemical and physical factors involved in semen preservation may affect sperm functionality. This has been demonstrated in equine semen. Temperature reduction is associated with an increase in oxidative cell damage as a consequence of mitochondrial reactive oxygen species production and membrane potential modifications (Cochran et al., 1984; Aurich, 2005; Ali et al., 2010). The extenders, commonly used for cooling equine semen, are composed of macromolecules derived from non-fat dried milk. They are designed to balance pH and osmolarity, providing thermal resistance, energy source, and dilution or replacement of seminal plasma during cryopreservation procedures (Aurich, 2005). Milk-based extenders supplemented with egg yolk preserved equine sperm motility and fertility for 24 h at 5° to 8 °C (Varner et al., 1989; Jasko et al., 1992). Likewise, egg yolk added to the milk-based extender is frequently used to cool donkey semen, preserving progressive motility, vigor, and acrosome integrity (Cottorello et al., 2002; Rota et al., 2008; Carvalho et al., 2017; Wu et al., 2018). However, their undefined chemical composition constitutes a relevant disadvantage. These biological products have different components that cannot be standardized and may extensively vary between batches. Also, only certain fractions may be needed for beneficial effects on sperm function. It has been shown that casein micelles isolated from milk can protect stallion, goat, ram, and bull sperm during storage at 4° to 5°C (Choong & Wales, 1962; Martin, 1966; Batellier et al., 1997; Leboeuf et al., 2003). However, the mechanism by which casein micelles protect sperm during storage has not been explained. Equine semen extenders with a defined composition, which only contains sperm protective components, would be an advantage (Pagl et al., 2006). Microfiltration, ultrafiltration, diafiltration, and freeze-drying techniques have allowed the preparation of purified milk fractions. Among these fractions, phosphocaseinate, and β- lactoglobulin were found most effective to support the longevity of cooled stored equine spermatozoa (Batellier et al., 1998; Batellier et al., 2001). In stallions, an increase in the quality of cooled semen was observed after semen dilution with an extender containing defined amounts of caseinates and whey proteins (EquiPro®). This was most evident after storage over 24-48 h (Pagl et al., 2006). Di Palma et al. (2020) report that the use of protein-defined extenders improves donkey sperm characteristics compared with milk-based extenders.

In donkeys, seminal plasma composition is still not clarified and the removals in preserved semen do not have conclusive and repeatable results. During semen processing and storage, cholesterol and phospholipid efflux can damage the sperm membrane. Reports revealed the presence of l-binding proteins (BSP) in mammalian seminal plasma that could be beneficial or detrimental to sperm, depending on the concentration and duration of exposure (Calvete et al., 1994; Bergeron et al., 2005; Villemure et al., 2003) In the female reproductive tract, this BSP interacts with oviductal/follicular fluid components as high-density lipoproteins and stimulates cholesterol efflux from the sperm membrane, resulting in capacitation (Bergeron & Manjunath, 2006). In preserved stallion semen, the exposure to BSP proteins from seminal plasma causes continuous cholesterol removal from the sperm membrane, increasing sperm sensitivity, which can render sperm extremely sensitive to cryopreservation (Calvete et al., 1994; Bergeron et al., 2004). In donkey semen, despite some reports described seminal plasma general composition (Talluri et al., 2017; Vyvial et al., 2019), there is not much available information on seminal plasma proteins. Some studies showed that seminal plasma dilution or removal from donkey semen

increases sperm motility and membrane integrity after storage (Serres et al., 2002; Miró et al., 2009). In contrast, Rota et al. (2008) reported that seminal plasma removal during preservation at 5 °C did not offer any advantage over using semen diluted in INRA82® supplemented with 2% of egg yolk or INRA96®. Similarly, in a preliminary study, seminal plasma removal by centrifugation had no beneficial effects on sperm progressive motility, membrane function, or integrity (Alonso et al., 2017).

In mammalian sperm preservation, an egg yolk extender has been used to prevent sperm damage (Phillips & Lardy, 1940; De Leeuw et al., 1993). Low-density lipoproteins (LDL) present in egg yolk interact with the BSP proteins present in seminal plasma and minimize lipid removal from the sperm membrane, which positively influences sperm preservation (Bergeron & Manjunath, 2006). In cooled donkey semen, some studies have evaluated milk-based extenders with different concentrations of domestic chicken egg yolk (Cottorello et al., 2002; Rota et al., 2008; Wu et al., 2018). Only one study comparing different egg yolk concentrations reported an increase in sperm progressive motility after 48 h of cooling using 1% of egg yolk (Wu et al., 2018). Rota et al. (2008) demonstrated some advantages in kinetic parameters when 2% of egg yolk was added in the milk-based extender, similar to the results obtained in stallion semen (Rota et al., 2004). Despite these results, there is no consistent protocol determining egg yolk concentration or extender formulation to be used in cooled donkey semen. The use of protein-defined extenders without egg yolk improves donkey sperm characteristics compared with milk-based extenders (Di Palma et al., 2020). Similarly, in a preliminary study, an improvement in sperm progressive motility, membrane integrity, and function were observed in cooled semen diluted in a commercial caseinate-base extender supplemented with 2% of egg yolk (Alonso et al., 2017).

The objectives of this study were to determine sperm kinetic parameters and membrane integrity in donkey semen diluted in a defined caseinate-based extender supplemented with egg yolk and evaluate the effect of seminal plasma removal.

Materials and Methods

The experiment was conducted at the Equine Production Laboratory of Rio Cuarto State University, Cordoba, Argentina, from April to June 2019. To prove the hypothesis, an experimental randomized block design was made with six donkeys and four ejaculates per donkey. Each ejaculate was divided into four groups to compare the four treatments on cooled donkey semen.

Donkeys and semen collection

Semen from six donkeys of different breeds and proven fertility, from 4 to 10 years old, was collected using a Botucatu® model artificial vagina (Botupharma, Botucatu, Brazil). Animals were housed in paddocks. Four ejaculates per donkey were collected for a total of 24 ejaculates. Samples were collected at least once a week.

Egg yolk extender preparation

To obtain 20% of egg yolk extender, 2 ml of fresh chicken egg yolk was added to 8 ml of defined caseinates and whey proteins equine semen extender (Equiplus®, Minitube, Germany; commercial name today, EquiPro®), mixed and centrifuged at 2800 g for 60 min. The supernatant was aspirated, and the sediment was discarded. To prepare 1% and 2% Equiplus-yolk final extender, 1 and 2 ml of Equiplus 20% egg yolk solution was added into 17 and 16 ml of Equiplus, respectively. This medium was stored only for three days at 5°C and then discarded.

Semen processing

After semen collection, sperm concentration was evaluated adding 20 µl in 4 ml of buffered formol saline (BFS) and counted using Neubauer chamber (Fisher Scientific Hemacytometer®, USA) by the double-blind procedure.

Semen dilution

Four treatment groups were made. We proved two egg yolk concentrations (EY 1% and 2%), with and without centrifugation (C) CEY1, CEY2, EY1, and EY2, respectively. The control (non-treatment) group was assessed in a previous experiment (Alonso et al., 2017).

Raw semen was diluted in four samples, two with Equiplus egg yolk 1% and two with Equiplus® egg yolk 2%. CEY1 and CEY2 were centrifuged at 600 g for 10 min. EY1 and EY2 were reserved at room temperature. After centrifugation, sperm concentration was adjusted to 10 million sperm/ml in the four groups. The four samples were placed in Whirl-pack® (Nasco, NY, USA) containers without air and then refrigerated at 5°C for 24 h in Equitainer® (Hamilton Research Inc., NY, USA).

Semen analysis

Sperm kinetic characteristics, including percentages of total motility (TM) and progressive motility (PM), straight-line velocity (VSL, µm/s), mean curvilinear velocity (VCL, µm/s), and average path velocity (VAP, µm/s) were analyzed by iSperm® (version 4.5.2; Aidmics Biotechnology Co., LTD,

Taiwan) software in fresh and cooled semen, as previously reported (Dini et al., 2019).

Plasma membrane integrity in fresh and cooled semen was evaluated using a hypo-osmotic swelling test (HOS-test) and an eosin-nigrosin test (BotuVital®, Botupharma, Botucatu, Brazil). HOS-test was performed incubating l00 µl of semen in 300 µl of bi-distilled water for 45 min at 37 °C (Rota et al., 2010). The evaluation was accomplished using bright field microscopy (400x), a minimum of 100 spermatozoa was evaluated per slide (two slides per sample), and the percentage of spermatozoa showing patterns of swelling was calculated, as proposed by Rota et al. (2010). The eosin-nigrosin test was performed homogenizing 10 µl of semen to 10 µl of BotuVital® on a slide at 37 °C, then the drop was spread and dried at room temperature for evaluation. For each sample, 100 sperm were examined and classified under 1000x magnification of bright field microscopy. Live spermatozoa were those with an unstained membrane structure and those with a damaged membrane (dead) stained pink.

Morphology evaluation was performed adding 1 ml of fresh semen or cooled semen to 1 ml of BSF. Afterward, a smear was performed and stained with Stain 15 (Biopur SRL®, Rosario, Argentina). Two hundred cells were evaluated using a 1000x magnification of bright field microscopy.

Two operators independently counted in all procedures (HOS-test, eosin-nigrosin test, and morphology) and the average of both were recorded.

Statistical analyses

RStudio (RStudio, Inc., Boston, MA, USA) (R Core Team, 2018) was used for statistical analysis.

To analyze TM, PM, sperm kinetic parameters (VSL, VCL, VAP), live or dead sperm cells, and plasma membrane integrity, we used a generalized linear model on a randomized complete block design (using the Gamma, Normal or Binomial family). Differences were considered significant at $P < 0.05$.

Results

Sperm kinetic parameters (TM, PM, VCL, VAP, VSL) evaluated in cooled semen extended with Equiplus® supplemented with 1% or 2% of egg yolk were not statistically different (Table 1). Seminal plasma removal did not affect TM, VCL, VAP, and VSL. However, sperm PM in semen supplemented with 2% of egg yolk without seminal plasma (CEY2) was significantly lower (P=0.021) than the other treatments.

The comparison of sperm plasma membrane integrity, illustrated in Table 2, showed no significant difference between CEY2 and CEY1. However, EY1 and EY2 were statistically different (P=0.000008 and P=0.00001, respectively) in comparison with CEY1.

The percentage of live sperm cells was significantly lower in CEY1 than in EY2 (P =0.000192). Even so, there was no statistical difference between EY1 and EY2, or CEY1 and CEY2.

Semen morphology was not statistically different between groups CEY1 and EY1 or CEY2 and EY2.

Discussion and Conclusions

For cooled sperm preservation, donkey semen needs an environment that is metabolically and physiologically capable of protecting the sperm from cold shock This environment must be provided by an adequate semen extender. There are not enough studies in donkeys comparing semen extenders, but the use of egg yolk-based extenders seems advantageous. Different reports demonstrated that milk-based extenders supplemented with egg yolk preserved total

Table 1 - Effect of different concentrations of egg yolk extender supplementation and plasma removal on the sperm kinetics of cooled donkey semen. Mean ± SE.

Treatment		CEY1	CEY2	EY1	EY2
TM	%	52.88 ± 4.09[a]	43.38 ± 4.17[a]	55.29 ±5.20[a]	52.79± 5.20[a]
PM	%	35.63 ± 2.89[a]	29.67 ± 2.86[b]	37.13 ±3.57[a]	35.88 ±3.35[a]
VCL	µm/seg	160.29 ±3.30[a]	156.79 ±3.48[a]	162.96±3.29[a]	160.29±3.04[a]
VAP	µm/seg	84.71 ±2.45[a]	86.29 ±2.53[a]	87.21 ±2.34[a]	85.63 ±2.18[a]
VSL	µm/seg	75.04 ± 2.28[a]	77.17 ±2.34[a]	76.62 ±2.35[a]	76.25±2.12[a]

Different superscripts in rows indicate a statistical difference (P <0.05). TM: total motility, PM: progressive motility, VSL: straight-line velocity, VCL: curvilinear velocity of motile spermatozoa, VAP: average path velocity. CEY1: Centrifuged Equiplus egg-yolk 1%, CEY2: Centrifuged Equiplus egg-yolk 2%, EY1: Equiplus egg-yolk 1%, EY2: Equiplus egg-yolk 2%.

Table 2 - Effect of different concentrations of egg yolk extender and plasma removal on sperm membrane integrity of cooled donkey semen.

Treatment		EY1	EY2	CEY1	CEY2
HOS +	%	47.07[a]	46.79[a]	40.67[b]	40.96[b]
LIVE	%	75.71[a]	77.96[a]	73.42[b]	73.71[b]

Different superscripts in rows indicate a statistical difference (P <0.05). HOS +: hypo-osmotic swelling test positive, LIVE: Eosin-nigrosine unstained. CEY1: Centrifuged Equiplus egg-yolk 1%, CEY2: Centrifuged Equiplus egg-yolk 2%, EY1: Equiplus egg-yolk 1%, EY2: Equiplus egg-yolk 2%.

and progressive sperm motility (Rota et al., 2008), vigor, and acrosome integrity (Mello et al., 2000; Cottorello et al., 2002). Wu et al., (2018) suggested that 1% of egg yolk addition appears to be the most beneficial concentration because sperm progressive motility was significantly higher after 48 h of cooled storage. Considering this data, our report evaluates sperm kinetics and membrane integrity when 1% or 2% of egg yolk was added to Equiplus®, showing that egg yolk concentration did not affect sperm kinetics or membrane integrity at 24 h of cooled storage. These results are similar to those described by Wu et al. (2018) but introduce a protein-defined extender. In the future, it could be desirable to exclude any biological proteins and replace them with chemically defined extenders. However, more studies are needed to determine the characteristics and functions of different egg yolk proteins and support the relationship between egg yolk concentration and storage time.

Some authors described that removal of seminal plasma by centrifugation has a positive effect on motility and plasma membrane integrity of donkey spermatozoa after preservation in milk-based extenders (Serres et al., 2002; Miró et al., 2009). In contrast, our results indicate that seminal plasma removal did not affect sperm motility or kinetic parameters, and also suggest that plasma removal could be detrimental for membrane sperm integrity and in live sperm cell rate post centrifugation. Rota et al. (2008) found similar sperm kinetic and membrane integrity results after plasma removal. Due to the unexplained disparity of the results, more research is needed to determine a beneficial effect of seminal plasma removal.

In conclusion, our results suggest that the addition of 1% or 2% of egg yolk to Equiplus® extender did not affect sperm motility and membrane integrity. Furthermore, the removal of seminal plasma by centrifugation did not have any beneficial effect and could be detrimental for cooled donkey semen. The use of Equiplus® extender supplemented with 1% of egg yolk without seminal plasma removal seems to be the best option to cool donkey semen. However, further studies are needed to evaluate the relation of these results with the fertility of cooled donkey semen.

Conflict of interest

The authors declare no conflict of interests.

Ethics Statement

This research protocol (# 04/2020) was approved by the Ethics and Animal Use Committee of the Rio Cuarto State University, Córdoba, Argentina.

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