

Physical characteristics and fertility of fractionated donkey semen cooled at 5°C

[Características físicas e fertilidade do sêmen asinino fracionado e resfriado a 5°C]

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ABSTRACT

The aim of this study was to evaluate the effect of two different extenders (Skimmed Milk Glucose - SMG or Lactose - Egg Yolk - LEY) on physical characteristics and fertility of fractionated donkey semen cooled at 5°C. For this, four Pêga donkeys were used as semen donors. The sperm rich fraction of the ejaculate was diluted preparing insemination doses containing 400×10^6 motile spermatozoa in a volume of 22 mL, cooled to 5°C and stored up to 48 hours in a container proposed by Palhares (1997). Sperm motility and vigor were assessed in fresh semen, after first semen dilution, before insemination, at 24 and 48 hours after storage. For the fertility evaluation, 44 mares were inseminated with semen stored for a period between 12 and 24 hours. The mares were inseminated on fixed days (Mondays, Wednesdays, and Fridays) after the detection of a follicle greater than a 30mm diameter in one of the ovaries through ovulation. Pregnancy diagnosis was performed on day 12 post-ovulation, using transrectal ultrasonography. Semen diluted in SMG showed superior sperm motility than LEY, at the Pre-AI evaluation ($P < 0.05$). At 48 hours of storage, all donkeys had motility values between 45 and 53% for semen diluted in SMG, while only one donkey showed motility greater than 30% in the LEY treatment. The pregnancy rate/cycle for mares inseminated with semen diluted in SMG was superior than that obtained using LEY (56.52% vs 4.76%, respectively).

Keywords: donkey, fractionated collection, cooled semen, fertility

RESUMO

Objetivou-se com o presente experimento avaliar o efeito de dois diferentes diluidores (leite em pó desnatado glicose – SMG ou lactose gema de ovo – LEY) sobre as características físicas e a fertilidade do sêmen asinino coletado de forma fracionada e resfriado a 5°C. Para isso, quatro jumentos da raça Pêga foram utilizados como doadores de sêmen. A fração espermática rica do ejaculado foi diluída preparando-se doses inseminantes contendo 400×10^6 espermatozoides móveis em um volume de 22 mL, resfriadas a 5°C e armazenadas por até 48 horas em contêiner proposto por Palhares (1997). A motilidade e o vigor espermáticos foram avaliados no sêmen fresco, após a pré-diluição, antes das inseminações, às 24 e 48 horas de armazenamento. Para avaliação de fertilidade, 44 éguas foram inseminadas com sêmen armazenado por um período entre 12 e 24 horas, em dias fixos (segundas, quartas e sextas-feiras), após a detecção de um folículo de diâmetro maior ou igual a 30mm em um dos ovários, até a ovulação. O diagnóstico de gestação foi realizado a partir de 12 dias após a ovulação, por meio de ultrassonografia transretal. O sêmen diluído em SMG apresentou motilidade espermática superior à do LEY, já a partir do tempo pré-IA. Às 48 horas de armazenamento, todos os jumentos apresentaram valores de motilidade entre 45% e 53%, quando o sêmen foi diluído em SMG, enquanto apenas um jumento apresentou motilidade superior a 30% no tratamento utilizando LEY. A taxa de concepção/ciclo das éguas inseminadas também foi superior para o sêmen diluído em SMG em relação ao diluído em LEY (56,52% versus 4,76%, respectivamente).

Palavras-chave: jumento, coleta fracionada, sêmen resfriado, fertilidade

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INTRODUCTION

Several factors can influence the sperm longevity and viability of cooled semen, among them the extender used, cooling protocols, methods of transport, physical, morphological, biochemical, and metabolic characteristics inherent to sperm and membranes, as well as individual animal variability.

The extenders most commonly used for stallion and donkey semen dilution and cooling are mainly comprised of macromolecules derived from egg yolk or milk, which provide protection for sperm against damage caused by temperature reduction. Ferreira (1993) and Mello *et al.* (2000) reported better physical characteristics for donkey semen maintained in extenders containing egg yolk versus milk based extender.

Seminal plasma proteins have been identified as the cause of reduced motility and sperm survival in several species (Strzezek *et al.*, 1992; Wishwanath *et al.*, 1992; Carver and Ball, 2002) however, the specific factors present in seminal plasma that are responsible for damage during semen storage remain unknown. For stallion semen Karekoski (2011) observed that seminal plasma removal decreased sperm motility of cooled semen, however, this reduced motility was associated with minor DNA damage during cooled storage. With respect to donkey semen, the effects of seminal plasma removal before cooling semen remain controversial (Ferreira *et al.*, 1991; Dalmau, 2003; Rota *et al.*, 2008).

Aiming to eliminate the deleterious effects of seminal plasma on sperm cells during semen storage and the damage caused by centrifugation, obtaining the sperm-rich fraction of the ejaculate, by fractionated collection, presents as a viable alternative. The sperm rich fraction contributes with up to 94% of the total sperm in 57.6% of the ejaculate volume for donkeys (Mello, 1998). Additionally, Tischner *et al.* (1974) reported minor bacterial contamination of semen when an artificial open vagina was used for semen collection.

Considering the importance of artificial insemination use and semen cryopreservation in advance of the equine industry, it is essential that further studies be developed in this direction. In this sense, the aim of this study is to evaluate two

different extenders, skimmed milk glucose or lactose-egg yolk based, in the preservation of the physical characteristics of donkey semen and pregnancy rate in mares inseminated with asinine fractionated semen cooled at 5°C.

MATERIALS AND METHODS

The experiment was conducted at Lagoa Dourada, Minas Gerais, Brazil, in the months of October and November of 2010. For semen donors, four Pêga donkeys, aged between 5 and 17 years, with a history of proven fertility were used. For the fertility test 44 crossbreed mares, aged between 2.5 to 18 years were used. The mares were distributed in a completely randomized design using toss, after grouping by age and reproductive class.

28 ejaculates (Donkey 1 = 8, Donkey 2 = 6, Donkey 3 = 5 and Donkey 4 = 7 ejaculates) were used and subjected to a split plot experimental design, involving the use of two extenders - Skimmed Milk Glucose (SMG – Kenney *et al.*, 1983) and Lactose - Egg Yolk (LEY – Nagase and Graham, 1964) without glycerol.

Thus, from each ejaculate, insemination doses of 22mL containing 400×10^6 motile spermatozoa diluted in extenders SMG or LEY were prepared and subjected to cooling at 5°C in the container proposed by Palhares (1997) for up to 48 hours.

The sperm rich fraction of each ejaculate was obtained with the use of an open model artificial vagina, adapted from Tischner *et al.* (1974), consisting of a thick rubber cylinder, measuring 28 cm in length and 14 cm in diameter. A plastic container with a diameter of greater than 14 cm and holding up to 2.0 L was utilized for collection of the first three jets of ejaculate. The semen collections were performed on fixed days (Sundays, Tuesdays, and Thursdays) in the afternoon, using a phantom, and artificial inseminations were carried out on Monday, Wednesday, and Friday mornings, so that the time of semen storage at 5°C was at least 12 and at most 24 hours.

The pre-sperm fraction was discarded, and the identification of three jets made by visual observation and accompanied by the tactile sense of the pulses in the urethral passage of semen during ejaculation. After collection of the sperm

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rich fraction, the rest of the ejaculate was discarded.

After semen collection, the plastic flask was sent to the laboratory and the semen underwent physical evaluation (motility and vigor) using optical microscope. Sperm motility was evaluated on a scale of 0-100%, and the vigor on a scale of 0-5. After physical evaluation, fresh semen was diluted at 1:1 proportion in skimmed milk glucose or lactose egg yolk without glycerol, and sperm motility and vigor were evaluated again.

To assess sperm concentration, semen aliquot of 20 μ L was dissolved into 8mL of saline formaldehyde buffered solution (1:400), and the number of sperm/mL was determined after counting spermatozoa in a haemocytometer (Neubauer chamber). After the sperm concentration was determined, the semen volume necessary to obtain an insemination dose of 400 x 10⁶ motile spermatozoa and the extender volume required to complete the 22mL insemination dose was calculated. The insemination dose was prepared by transferring the required semen volume into the glass tube (22 mL) using a graduated glass pipette. Next, the appropriate extender volume was transferred, using the same graduated glass pipette.

The glass tubes are then closed using PVC film. Insemination doses were then homogenized gently turning the tubes two or three times. The container was prepared by inserting the refrigerator block, and then the insemination doses, whereupon it was sealed. The container

was identified by donkey name and date of semen collection, remaining closed until the time of insemination. It is noteworthy that in the present experiment, the refrigerator block was changed every 24 hours of sperm storage.

The first physical evaluation was performed in the fresh semen, corresponding to time zero, and this evaluation was independent of treatment. Immediately after the pre-dilution of semen, the second evaluation was performed, corresponding to the period after the pre-dilution (PD). Physical evaluation was also completed for each donkey and treatment prior to inseminations.

The container was opened and a No. 14 urethral catheter was inserted into the tube containing the insemination dose aimed to subsequent evaluations. The container was capped again leaving only the opening necessary for the passage of the catheter. A 6mL syringe was attached to the catheter to homogenize the semen filling and emptying the syringe 10 times. A 2mL sample was transferred to an ependorff and placed in a water bath at 37°C for 5 minutes. Then, a physical assessment of semen (motility and vigor) was done, registering the time of the evaluations. For subsequent evaluations (24 and 48 hours after semen collection), the procedure was performed as described for evaluations pre-insemination. Thus, the containers were opened in the same order of semen collection of donkeys, and the samples of the two treatments of the same animal taken and evaluated jointly. The real range of conducting evaluations of semen is presented in Table 1.

Table 1. Real average interval, between semen collection and evaluation in different periods

| Interval to evaluation from semen collection | Semen evaluation periods | | | |
|--|--------------------------|----------------|-------------|-------------|
| | PD (min) | Pre-AI (hours) | 24h (hours) | 48h (hours) |
| | 3.41 | 16.10 | 23.37 | 46.18 |

PD = after pre-dilution; Pre-AI = before artificial insemination.

The mares were inseminated with diluted and cooled semen of four donkeys featuring two treatments:

- Treatment I (TI): mares inseminated with donkey semen diluted in Skimmed Milk - Glucose extender (SMG), with the insemination dose of 22 mL and 400 x 10⁶ motile sperm, cooled to 5° C and stored for a period between 12 and 24 hours (n = 23);

- Treatment (TII): mares inseminated with donkey semen diluted in Lactose - Egg Yolk extender (LEY) without glycerol, with the insemination dose of 22 mL and 400 x 10⁶ motile sperm, cooled to 5°C and stored for a period between 12 and 24 hours (n = 21).

The mares were subjected to transrectal palpation and ultrasonography every 2-3 days until

detection of 2.5cm diameter follicle in one of the ovaries, whereupon the procedures became daily until ovulation. In the presence of a follicle with a diameter greater than or equal to 3.0 cm, artificial inseminations began on fixed days (Mondays, Wednesdays, and Fridays) until detection of ovulation.

In the presence of a follicle with a diameter greater than or equal to 3.5cm, associated with the presence of intramural uterine edema, 1667 IU of human chorionic gonadotropin (hCG) was intravenously administered as an agent to induce ovulation. Pregnancy diagnosis by ultrasonography was performed from day 12 post-ovulation. Each mare was inseminated for only one cycle.

Statistical analysis was processed using the SAS (Statistical..., 1999), with significance level of 95% ($P < 0.05$) and using the General Linear Models (GLM) for the analysis of variables. Values are presented as mean \pm standard error of the mean. The percentage values were transformed into arcsine before being subjected to analysis. Quantitative variables were analyzed using the GLM procedure. To compare two means Student's T Test was used, for three or more means, the Student-Newman-Keuls (SNK) test was used. For variable sperm vigor, we used non-parametric analysis, and the Kruskal-Wallis test used to compare more than two means and Wilcoxon test for comparison of means, two by two.

The proportional data (conception rate / cycle and cycles / conception) were analyzed by frequency dispersion and application of Chi-square test to detect differences between treatments.

RESULTS AND DISCUSSION

Data on sperm motility and vigor for the different evaluation periods are presented in Tables 2 and 3.

For semen diluted in LEY, there was a reduction in sperm motility in the pre-AI period compared to fresh semen for all donkeys, while for semen diluted in SMG this characteristic remained unchanged during this period. Semen from donkeys 1 and 2 had better motility in the pre-AI

period for the SMG compared to the LEY extender.

At 24 hours, there was a reduction in sperm motility in SMG compared to fresh semen for donkeys 1 and 4. Sperm motility was higher for donkeys 2 and 3 in SMG compared to LEY at 24 hours. In general, after 48 hours, the percentage of motile sperm remained above 45% for fractionated semen diluted in SMG and did not differ from that observed at 24 hours post-collection, regardless of the donkey. However, for semen diluted in LEY, only one donor (donkey 4) showed more than 30% motile sperm cells at 48 hours. Sperm motility within 48 hours of storage was superior for semen diluted in SMG relative to LEY in three of the four donkeys evaluated.

The extender effect on sperm vigor was evident at the time of pre - insemination evaluation, where vigor was better in SMG extender compared to LEY for donkeys 1 and 2. However, at 24 hours of storage, sperm vigor was similar between extenders and donkeys. At 48 hours, the sperm vigor was higher for sperm cells diluted in SMG compared to LEY in donkeys 1 and 3, but was similar between extenders for donkeys 2 and 4.

All samples diluted in SMG maintained sperm vigor values above 3 when measured after 24 hours of storage. For samples diluted in LEY extender and in the same period of storage, only one (Donkey 1) maintained a sperm vigor greater than 3. At 48 hours of sperm storage, 50% of the samples (Donkeys 2 and 4) maintained vigor values above 3 when diluted in SMG versus only one of four, when diluted in LEY.

For donkey total ejaculate, there are reports in the literature of high sperm longevity when diluting donkey semen in extender containing various concentrations of egg yolk. Using an extender containing 3% of egg yolk, Nishikawa (1959) obtained sperm viability for 337 hours (14.04 days) for semen storage at 4°C. Ferreira (1993) observed sperm viability (motility > 10%) for 96 hours using an extender containing 20% egg yolk. Rota *et al.* (2008) compared the effectiveness of three different extenders, two of them from skim milk (INRA96® and INRA82®) and one with 2% added egg yolk (INRA82-Y) for semen preservation from Amiata donkeys.

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Progressive motility did not differ among treatments at 24 hours of storage however, motility was better in INRA82-Y after 48 and 72 hours of storage. The results obtained in this experiment showed different behavior for fractionated donkey semen, once higher sperm viability was associated with use of extender without egg yolk.

Dalmau (2003) noted that sperm viability (MP \geq 30%) was maintained for 47.45, 37.76, 26.84 and 4.26 hours, respectively, to donkey semen diluted in extenders INRA82®, skimmed milk - glucose, skim milk and tris-egg yolk. Regardless of the procedure to which semen was submitted (total ejaculate with or without seminal plasma and sperm rich fraction of the ejaculate) the tris-egg yolk containing 20% of egg yolk showed the worst results. These results are lower than those obtained in this experiment, when the sperm rich fraction, diluted in SMG extender, maintained a sperm motility higher than 30% up to 48 hours of

storage, and the semen diluted in LEY maintained their motility exceeding 30% by 24 hours post-collection except for donkey 3 (Table 2).

When evaluating the use of centrifuged donkey semen, Mann *et al.* (1963) observed a motility of 60% on the eighth day of storage at 5°C, when using an extender containing 16.6% of egg yolk. Beker (1997) observed sperm viability (motility > 10%) up to 120 hours for sperm rich fractionated donkey semen diluted in an extender containing 3% egg yolk stored at 5°C. Mello *et al.* (2000) reported better total and progressive motility for Baken modified extender (10% of egg yolk) compared to a skim milk based extender for both total ejaculate and sperm rich fractions of donkey semen. These researchers observed motility of more than 30% by the 7th day of storage at 5°C for sperm rich fraction samples diluted in Baken extender and until the 5th day for semen diluted in milk based extender.

Table 2. Effect of donkey and extender on sperm motility, assessed after collection (Fresh), after pre-dilution (PD), before artificial insemination (Pre-AI) and in different periods of semen storage at 5°C (24 and 48 hours)

| Evaluation period | Donkey | | | | Extender | | | |
|-------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|---------------------------|--------------------------|----------------------------|----------------------------|
| | SMG ^x | | | | LEY ^y | | | |
| | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 |
| Fresh | 89.38±3.35 ^a | 89.17±3.87 ^a | 87.00±4.24 ^{ab} | 80.71±3.59 ^{ab} | 89.38±3.35 ^a | 89.17±3.87 ^a | 87.00±4.24 ^{ab} | 80.71±3.59 ^{ab} |
| PD | 85.63±3.35 ^{ab} | 85.83±3.87 ^{ab} | 83.00±4.24 ^{ab} | 78.57±3.59 ^{abc} | 83.75±3.35 ^{ab} | 85.00±3.87 ^{ab} | 83.00±4.24 ^{ab} | 77.86±3.59 ^{abc} |
| Pre- AI | 70.71±3.59 ^{abcd} | 72.50±3.87 ^{abcd} | 70.00±4.24 ^{abcde} | 65.00±3.87 ^{bcdef} | 45.00±3.59 ^{efg} | 40.00±3.87 ^{fg} | 48.00±4.24 ^{defg} | 47.86±3.59 ^{defg} |
| 24h | 65.00±4.24 ^{bcdef} | 70.00±4.74 ^{abcde} | 67.50±4.74 ^{abcde} | 57.00±4.24 ^{cdefg} | 45.00±4.24 ^{efg} | 36.25±4.74 ^g | 13.33±5.48 ^h | 42.00±4.24 ^g |
| 48h | 45.00±4.74 ^{efg} | 53.33±5.48 ^{defg} | 51.25±4.74 ^{defg} | 50.00±5.48 ^g | 0.00±5.48 ⁱ | 8.75±4.74 ^h | 0.00±5.48 ⁱ | 35.00±5.48 ^g |

a.b.c.d.e.f.g.h.i Means followed by different letters in the row or column differ (p < 0.05).

^x Skimmed Milk - Glucose extender.

^y Lactose - Egg Yolk extender without glycerol.

Table 3. Effect of donkey and extender on sperm vigor assessed after collection (Fresh), after pre-dilution (PD), before artificial insemination (Pre-AI) and in different periods of semen storage at 5°C (24 and 48 hours)

| Evaluation period | Donkey | | | | Extender | | | |
|-------------------|--------------------------|---------------------------|---------------------------|----------------------------|--------------------------|--------------------------|--------------------------|---------------------------|
| | SMG ^x | | | | LEY ^y | | | |
| | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 |
| Fresh | 4.94±0.19 ^{aA} | 4.92±0.22 ^{aA} | 4.80±0.24 ^{aA} | 4.00±0.20 ^{bABC} | 4.94±0.19 ^{aA} | 4.92±0.22 ^{aA} | 4.80±0.24 ^{aA} | 4.00±0.20 ^{bABC} |
| PD | 4.25±0.19 ^{bB} | 4.67±0.22 ^{aAB} | 4.50±0.24 ^{abAB} | 3.93±0.20 ^{bAB} | 4.38±0.19 ^{abB} | 4.33±0.22 ^{aBC} | 4.40±0.24 ^{aAB} | 4.29±0.20 ^{aA} |
| Pre- AI | 3.50±0.20 ^{bC} | 3.92±0.22 ^{aC} | 3.60±0.24 ^{abC} | 3.42±0.22 ^{aCD} | 2.64±0.20 ^{bd} | 3.00±0.22 ^{aE} | 3.10±0.24 ^{aC} | 3.14±0.20 ^{aDE} |
| 24h | 3.40±0.24 ^{aCD} | 3.63±0.27 ^{aCD} | 3.25±0.27 ^{aC} | 3.30±0.24 ^{aCDE} | 3.00±0.24 ^{aD} | 2.75±0.27 ^{aDE} | 1.00±0.31 ^{aCD} | 2.40±0.24 ^{aE} |
| 48h | 2.88±0.27 ^{aD} | 3.17±0.31 ^{aDEF} | 2.88±0.27 ^{aC} | 3.33±0.31 ^{aBCDE} | 0.00±0.31 ^{bE} | 1.25±0.27 ^{abF} | 0.00±0.31 ^{bD} | 3.00±0.31 ^{aDE} |

A.B.C.D.E.F Means followed by different letters within each donkey show effect (p < 0.05) of extender and storage time of semen.

a.b Means followed by different letters on the line, within extender, differ (p < 0.05).

^x Skimmed Milk - Glucose extender.

^y Lactose - Egg Yolk extender without glycerol.

Exceptions are made to methodological differences between experiments, however, the results obtained in this study differ from those obtained by most authors mentioned above, since it was observed that sperm motility and vigor

were better when the sperm rich fraction of Pêga donkey ejaculate was diluted in extender containing skim milk versus extender containing egg yolk during storage. The lowest sperm viability obtained in this experiment related the

sperm rich fraction diluted in LEY extender compared to results obtained by Beker (1997) and Mello (1998), may be related to the higher concentration of egg yolk (20%) present in the composition of modified lactose-egg yolk in relation to Baken (3%) and modified Baken (10%) extender, used by the authors in the order they were cited. Furthermore, it is worth noting that these authors also used the sperm-rich fraction. Comparing the results obtained by Mann *et al.* (1963) and those observed in this

experiment, we highlight the methodological differences in relation to other constituents present in the extender used, besides the egg yolk, though these authors used centrifuged semen and not derived from the sperm-rich fraction. Therefore, it remains unclear whether the behavior of the sperm-rich fraction during storage resembles that observed for the total ejaculate subjected to centrifugation to remove seminal plasma.

Table 4. Control and results parameters of mares inseminated with fractionated semen, diluted and cooled, considering the effect of extender, regardless of donkey used

| Parameters | Extender | |
|---|----------------------------|--------------------------|
| | SMG ^x | LEY ^y |
| N° mares | 23 | 21 |
| Age (years) | 10.07±0.63 | 10.64±0.06 |
| Semen volume (mL) | 0.84±0.06 | 0.82±0.07 |
| Extender volume (mL) | 21.16±0.06 | 21.18±0.07 |
| Insemination dose (x10 ⁶ spztz móveis) | 400.68±0.44 ^b | 402.20±0.63 ^a |
| Collection-pre-dilution interval (min.) | 3.08±0.18 | 3.03±0.20 |
| Collection – dilution interval (min.) | 19.21±1.07 | 17.18±1.09 |
| Collection – cooling interval (min.) | 24.17±1.05 | 21.89±1.14 |
| Collection – AI interval (h) | 18.09±0.23 | 17.91±0.23 |
| N° AIs/cycle | 1.57±0.20 | 1.43±0.21 |
| Number of cycles/pregnancy | 1.77 ^b | 21.00 ^a |
| Pregnancy rate/cycle (%) | 56.52 (13/23) ^a | 4.76 (1/21) ^b |

^{a,b} means in the row followed by different letters differ (P <0.05).

^x Skimmed Milk – Glucose extender

^y Lactose - Egg Yolk extender without glycerol.

To evaluate the fertilizing capacity of fractionated donkey semen diluted in milk powder or egg yolk based extenders, 44 mares were inseminated. There was no donkey x treatment interaction (p > 0.05), therefore it was possible to compare the effect of extender, regardless of donkey on different reproductive characteristics and pregnancy rate of mares inseminated with semen diluted and cooled at 5°C. These parameters are shown in Table 4.

For LEY extender, the physical characteristics of semen were better preserved than fertility, noting that only 4.76% (1/21) of mares became pregnant for semen stored at 5°C for an average time of 17.91 ± 0.23 hours. It should be emphasized further that three of four donkeys maintained sperm motility greater than 30% (36.25 to

45.00%), when evaluated at 24 hours of storage in the LEY extender, after the completion of inseminations.

Notably, with the exception of the sperm number per insemination dose, the other control parameters capable of interfering with pregnancy rates such as the age of the mare, the semen storage time (AI-collection interval) and the number of inseminations per cycle did not differ between treatments. The small differences observed between treatments, related to concentration per insemination dose, were due to adjustments in determining the volume of the insemination dose at the time of preparation of insemination doses.

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The number of cycles/pregnancy and pregnancy rate/cycle were better for the treatment involving the use of SMG (1.77 and 56.52%, respectively) compared to the modified LEY extender (21.0 and 4.76%, respectively).

Boeta and Quintero (2000) reported a conception rate at the first cycle of 54.5% for mares inseminated with asinine semen diluted in skim milk based extender and cooled for 48 hours. Rossi (2008) achieved conception rates/ cycle similar between treatments involving dilution of Pêga donkey total ejaculate in SMG or glycine-egg yolk extenders, cooled to 5°C for 12 hours, 51% and 49%, respectively. Considering only the pregnancy rate / cycle obtained after insemination of mares with asinine semen diluted in SMG and cooled at 5°C (56.52%), the results obtained in this experiment are similar to those obtained by Boeta and Quintero (2000) and Rossi (2008), but they used total ejaculate.

The modified lactose-egg yolk extender used in this experiment has been used successfully for donkey semen dilution for both fresh and cooled use. Silva (1988) obtained conception rates at the first cycle during three consecutive breeding seasons of 52.4%, 52.2% and 68.5%, when using fresh semen diluted. Using LEY extender for insemination of jennies with fresh donkey semen Palhares *et al.* (1986) obtained a conception rate at first cycle of 57%. The discrepancy between the results mentioned above and those obtained in this experiment, although they involve the use of the same extender, appear to be related to the different methodologies used for semen collection. In this experiment, we used only the sperm rich fraction of the ejaculate, obtained by fractionated collection, while in their experiments Palhares *et al.* (1986) and Silva (1988) used the total ejaculate from donkeys.

In a study involving cooled equine semen, Jasko *et al.* (1992) observed that the addition of 4, 8 or 16% of egg yolk to milk based extender improved total and progressive sperm motility when the seminal plasma was removed by centrifugation. Similar results were obtained by Bedford *et al.* (1995), when the addition of 4% egg yolk to milk based extender was beneficial for maintaining sperm viability of cooled semen *in vitro*. However, there was a decrease in fertility with the addition of egg yolk to the milk based extender demonstrated by an embryo

recovery rate of only 17% for mares inseminated with extender plus 4% egg yolk compared to 50% obtained from mares inseminated with semen diluted in the milk based extender only.

Grouping the results obtained by Palhares *et al.* (1986), Silva (1988), and Rossi (2008), and comparing them to those obtained by Bedford *et al.* (1995) and those presented in this experiment, it is worth considering the existence of an interaction between removal of seminal plasma and the presence of egg yolk in seminal extenders, which additively result in damage to fertility.

In a project involving donkey semen freezing, Jepsen *et al.* (2010) reported a negative effect of the presence of high egg yolk concentrations in the freezing extender on fertility of inseminated mares. In a pre-experiment, they reported that frozen semen extender containing 20% egg yolk associated with the presence of glycerol as cryoprotectant, resulted in 0% of pregnancy. When glycerol was replaced by ethylene glycol, a conception rate of 6.3% for mares inseminated with semen in the presence of 20% egg yolk was reported, whereas there was a conception rate of 46.5% for those inseminated with semen in the presence of 5% of egg yolk. Thus, low fertility obtained in the pre-experiment was associated not only with the use of glycerol as cryoprotectant, but also with the concentration of egg yolk present in the extender. These authors also observed that the negative effect associated with a high percentage of egg yolk was neutralized by the addition of β -cyclodextrin to the freezing extender and obtained a conception rate of 58.3%. The ability of cyclodextrins to bind lipids and preferably cholesterol, suggests that this steroid may be the egg yolk component responsible for the decreased fertility in this species.

Some studies have shown that high cholesterol content can be detrimental to sperm cells (Parks *et al.*, 1981) and contributes to the infertility of human and stallion semen (Sugraroek *et al.*, 1991; Brinsko *et al.*, 2005). It is suggested that their presence could induce a state of decapacitation, inhibiting the acrosome reaction (Davis, 1978). Nevertheless, the presence of 20% egg yolk in seminal extenders used by Silva (1988) and Rossi (2008) did not result in damage to the fertility of mares inseminated with donkey

semen. Perhaps the maintenance of the seminal plasma was responsible for these results.

Studies conducted with bovine indicate that removal of cholesterol from the sperm plasma membrane by seminal plasma proteins is one of the triggering factors of sperm capacitation, and lipid components present in the egg yolk are able to bind to these proteins, and this may provide protection to sperm during storage (Manjunath *et al.*, 2002). The results observed in this experiment, suggest that the proteins present in asinine seminal plasma may also be able to join egg yolk cholesterol and neutralize its negative effects, since the association between fractional collection and extender containing 20% egg yolk resulted in conception rate / cycle unacceptable and seminal plasma removal did not result in damage to donkey sperm when using SMG extender.

Given the insemination protocol adopted in the present experiment, there was a maximum interval between insemination and ovulation of 72 hours (last insemination on Friday followed by ovulation on Monday in some instances) accompanied by pregnancy. Assuming an oocyte viability of up to 12 hours (Palhares, 1997; Rossi, 2008) the fractionated donkey semen diluted in SMG, cooled to 5°C and stored for an average of 18 hours, maintained fertilizing capacity for up to 60 hours. The fertility parameters obtained in this study, for the treatment SMG, were similar to those obtained by Silva (1988) for fresh semen diluted, and Rossi (2008), for mares inseminated with asinine semen cooled and stored for up to 12 hours, all utilizing the same insemination protocol. Thus, it is concluded that the dilution of fractionated donkey semen in SMG extender associated to its cooling at 5°C in a container proposed by Palhares (1997) allowed its storage for a mean of 18 and maximum of 24 hours, without a negative effect on fertilization.

CONCLUSION

Donkey semen extended with SMG extender showed sperm motility that was always superior to the LEY, starting at Pre-AI evaluation time. At 48 hours of storage, all the donkeys had motility values above 45% when semen was extended with SMG, while only one donkey showed sperm motility greater than 30% in treatment using

LEY extender. The association of LEY extender to the fractionated collection prevented the use of this protocol for cooling asinine semen. The protocol involving the fractionated collection, semen dilution in SMG, and subsequent cooling at 5°C resulted in a commercially acceptable pregnancy rate.

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REFERENCES

- BEDFORD, S.J.; JASKO, D.J.; GRAHAM, J.K. *et al.* Effect of seminal extenders containing egg yolk and glycerol on motion characteristics and fertility of stallion spermatozoa. *Theriogenology*, v.43, p.955-967, 1995.
- BEKER, A.R.C.L. *Efeitos de diferentes concentrações espermáticas e de antibióticos sobre a longevidade do sêmen asinino (Equus asinus), resfriado a 5°C.* 1997. 91f. Dissertação (Mestrado em Medicina Veterinária) – Escola de Veterinária, Universidade Federal de Minas Gerais, Belo Horizonte, MG.
- BOETA, M.; QUINTERO, L.Z. Utilización de la leche descremada ultrapasteurizada como diluyente de semen refrigerado de burro, destinado a la inseminación de yeguas. *Vet. Méx.*, v.31, p.67-69, 2000.
- BRINSKO, S.P.; LOVE, C.C.; BAUER, J.E. *et al.* Cholesterol and phospholipid analysis in sperm of stallions with unexplained subfertility. *Am. Reprod. Sci.*, v.89, p.217-219, 2005.
- CARVER, D.A.; BALL, B.A. Lipase activity in stallion seminal plasma and effect of lipase on stallion spermatozoa during storage at 5°C. *Theriogenology*, v.58, p.1587-1595, 2002.
- DALMAU, C.S. *Evaluación y conservación del semen en el asno Zamorano-leonés.* 2003. 190f. Thesis (Doctorado) - Facultad de Veterinaria, Universidad Complutense de Madrid, Madrid, ES.

- DAVIS, B.K. Inhibition of fertilizing capacity in mammalian spermatozoa by natural and synthetic vesicles. In: SYMPOSIUM ON THE PHARMACOLOGICAL EFFECTS OF LIPIDS, 1978, St. Louis. *Proceedings...* St. Louis: AOCS, 1978. p.145-157. (Series Monograph, n.5).
- FERREIRA, M.F.L. *Efeito de diluentes e taxa de resfriamento sobre a motilidade espermática e fertilidade do sêmen de jumentos (Equus asinus)*. 1993. 67f. Dissertação (Mestrado em Medicina Veterinária) – Escola de Veterinária, Universidade Federal de Minas Gerais, Belo Horizonte, MG.
- FERREIRA, M.F.L.; JONES, D.N.; HENRY, M. *et al.* Efeito da centrifugação e de diluentes sobre a preservação do sêmen resfriado de jumentos. In: CONGRESSO BRASILEIRO DE REPRODUÇÃO ANIMAL, 9., 1991, Belo Horizonte. *Anais...* Belo Horizonte: Colégio Brasileiro de Reprodução Animal, 1991. p.452.
- JASKO, D.J.; HATHAWAY, J.A.; SACHALTENBRAND, V.I. Effect of seminal plasma and egg yolk on motion characteristics of cooled stallion spermatozoa. *Theriogenology*, v.37, p.1241-1252, 1992.
- JEPSEN, R.J.; EVANS, L.E.; YOUNGS, C.R. Use of direct thaw insemination to establish pregnancies with frozen: thawed semen from a standard jack. *J. Equine Vet. Sci.*, v.30, p.651-656, 2010.
- KAREKOSKI, M. *Components of fractionated stallion seminal plasma and the effects of seminal plasma on sperm longevity*. 2011. 83f. Dissertation (Master) - Faculty of Veterinary Medicine, University of Helsinki, Helsinki, FIN.
- KENNEY, R.M.; HURTTGEN, J.P.; PIERSON, R. *et al.* *Society for theriogenology manual for clinical fertility evaluation of the stallion*. Hastings: Society for Theriogenology, 1983. 100p.
- MANJUNATH, P.; NAUC, V.; BERGERON, A. *et al.* Major proteins of bovine seminal plasma bind to the low-density lipoprotein fraction of hen's egg yolk. *Biol. Reprod.*, v.67, p.1250-1258, 2002.
- MANN, T.; MINOTAKIS, C.S.; POLGE., C. Semen composition and metabolism in the stallion and jackass. *J. Reprod. Fertil.*, v.5, p.109-122, 1963.
- MELLO, S.L.V. *Efeito da coleta fracionada de sêmen e de dois diluentes sobre a longevidade de espermatozoides de jumentos (Equus asinus) resfriados a 5°C*. 1998. 97f. Dissertação (Mestrado em Medicina Veterinária) – Escola de Veterinária da Universidade Federal de Minas Gerais, Belo Horizonte, MG.
- MELLO, S.L.V.; HENRY, M.; SOUZA, M.C. *et al.* Effect of split ejaculation and seminal extenders on longevity of donkey semen preserved at 5°C. *Arq. Bras. Med. Vet. Zootec.*, v.52, p.372-378, 2000.
- NAGASE, H.; GRAHAM, E.F. Pellet semen: comparison of different extenders and processes on fertility of bovine spermatozoa. In: INTERNATIONAL CONGRESS ANIMAL REPRODUCTION AND ARTIFICIAL INSEMINATION, 5., 1964. Trento. *Proceedings...* Trento: [s.n.], 1964. p.387-391.
- NISHIKAWA, Y. *Studies on reproduction in horses*. Tokyo: Japan Racing Association, 1959. 340p.
- PALHARES, M.S. *Adequação de um novo container para o transporte do sêmen equino diluído e resfriado: I. Características termodinâmicas e funcionais; II. Desempenho reprodutivo das éguas inseminadas*. 1997. 246f. Tese (Doutorado em Ciência Animal) – Escola de Veterinária, Universidade Federal de Minas Gerais, Belo Horizonte, MG.
- PALHARES, M.S.; SILVA FILHO, J.M.; OLIVEIRA JUNIOR, E.F. *et al.* Inseminação artificial em jumentas da raça pega com cio induzido pelo dinoprost. *Rev. Bras. Reprod. Anim.*, v.10, p.157-163, 1986.
- PARKS, J.E.; MEACHAM, T.N.; SAACHE, R.G. Cholesterol and phospholipids of bovine spermatozoa. II. Effect of liposomes prepared from egg phosphatidylcholine and cholesterol on sperm cholesterol, phospholipids and viability at 4°C and 37°C. *Biol. Reprod.*, v.24, p.399-404, 1981.
- ROSSI, R. *Comparação de dois diluidores na fertilidade de éguas inseminadas com sêmen asinino a fresco ou resfriado*. 2008. 209f. Dissertação (Mestrado em Medicina Veterinária) – Escola de Veterinária, Universidade Federal de Minas Gerais, Belo Horizonte, MG.

- ROTA, A.; MAGELLI, C.; PANZANI, D. *et al.* Effect of extender, centrifugation and remove of seminal plasma on cooled-preserved Amiat donkey spermatozoa. *Theriogenology*, v.69, p.176-185, 2008.
- SILVA, M.I.F. Viabilidade do uso da inseminação artificial em equinos a nível industrial. In: CONGRESSO BRASILEIRO DE REPRODUÇÃO ANIMAL, 7., 1987. *Anais...* Belo Horizonte: Colégio Brasileiro de Reprodução Animal, 1988. p.47-63.
- STATISTICAL analysis system. Version 5. Cary: SAS Institute, 1999.
- STRZEZEK, J.; KORDAN, W.; KOSTYRA, H. *et al.* Purification and partial characterization of 5700 Da sperm motility inhibiting factor for seminal plasma of boar. *Anim. Reprod. Sci.*, v.29, p.35-52, 1992.
- SUGKRAROEK, P.; MORRIS, K.; LEADER, A. *et al.* Levels of cholesterol and phospholipids in freshly ejaculated sperm and percoll-gradient-pelleted sperm from fertile and unexplained infertile men. *Fertil. Steril.*, v.55, p.820-827, 1991.
- TISCHNER, M.; KOSINIAK, K.; BIELÁNSKI, W. Analysis of the pattern of ejaculation in stallion. *J. Reprod. Fertil.*, v.41, p.329-335, 1974.
- WISHWANATH, R.; SHANNON, P.; CURSON, B. Cationic extracts of egg yolk and their effects on motility, survival and fertilizing ability of bull sperm. *Anim. Reprod. Sci.*, v.29, p.185-194, 1992.