Acta Scientiae Veterinariae, 2017. 45: 1482.

RESEARCH ARTICLE
Pub. 1482

ISSN 1679-9216

Viability of Pony Stallion Semen in Different Temperature and Dilution

Janislene Mach Trentin[1], Murilo Farias Rodrigues[1], Gilson Antonio Pessoa[1], Mariani Farias Fiorenza[2], Ricardo Olimpio Schenatto[1], Laurence Boligon de Araujo[2], Karine Vargas Aires[2] & Mara Iolanda Batistella Rubin[3]

ABSTRACT

***Background*:** Artificial insemination and transport of cooled semen has been routinely used in equine industry in the past 20 years. However, more investigations are needed regarding the methods for long time storage in pony stallion semen. The effect of dilution and cooling temperature on pH, sperm motility, membrane integrity and mitochondrial activity were investigated before and after cooling of stallion semen. ***Materials, Methods & Results*:** Two ejaculates each from nine Brazilian ponies were diluted in a non-buffered powder milk extender cooled at 5°C or 15°C for 48 h using three different dilutions (1:1, 1:2 or 1:3). Data were assessed by analysis of variance and the rate comparison was performed using the Duncan test. Samples diluted 1:1 at 5ºC or 15°C showed higher pH values (7.63 ± 0.34 e 7.57 ± 0.27) and lower progressive motility (10.3 ± 11.05, 17.08 ± 9.95). All samples cooled at 15°C also showed lower incidence of morphologically altered spermatozoa (1:1 = 55.84%; 1:2 = 51.84%; 1:3 = 49.95%) [$P < 0.01$]. Mitochondrial activity was higher on the 1:3 dilution (0.86 ± 0.19 nm) at 5°C and on the 1:1 (0.89 ± 0.23 nm), 1:2 (0.93 ± 0.2 nm) and 1:3 (0.92 ± 0.2 nm) dilutions at 15°C. Progressive motility was higher when semen was diluted 1:3 and cooled at 15°C (42.22 ± 12.38; $P < 0.05$). Considering mitochondrial activity, similar results were observed when different dilutions of semen were used ($P > 0.05$) despite time and temperature. The pH, progressive motility, mitochondrial activity and membrane integrity remained similar ($P > 0.05$) on fresh semen samples independent of the dilution grade used. The best results were obtained when semen was diluted 1:3 and cooled at 15°C. All dilution grades were safe for fresh semen and pH wasincreased when semen was diluted and cooled for 48 h. ***Discussion*:** The methodology used to collect and process equine semen and semen from ponies is practically the same. Equine semen when sent for artificial insemination is usually cooled to 5°C. Our results showed that cooling reduces sperm viability, which has also been demonstrated by other studies. In contrast, the best cooling temperature was at 15°C. However, it is easier to keep the temperature at 5°C during transport, due to the large temperature oscillation that may occur during transportation. The semen of ponies can tolerate cooling at both 5 and 15°C. The 1:3 dilution cooled to 15°C provided better viability of pony sperm, and more stable pH during 48 h of cooling. Dilution 1:1 should not be used for cooling in powdered skim milk extender.

Keywords: concentration, cooling, equine, extender, semen.

Received: 8 May 2017 Accepted: 27 August 2017 Published: 15 September 2017

[1]Programa de Pós-graduação em Medicina Animal: Equinos (PPGMAE), Faculdade de Veterinária (FaVet), Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil. [2]Laboratório de Embriologia Animal, Departamento de Clinica de Grandes Animais & [3]Departamento de Clinica de Grandes Animais, Universidade Federal de Santa Maria (UFSM), Santa Maria, RS. CORRESPONDENCE: J.M. Trentin [janislenetrentin@yahoo.com.br - Tel.: +55 (55) 996077577]. Programa de Pós-graduação em Medicina Animal: Equinos (PPGMAE), FaVet, UFRGS. Av. Bento Gonçalves n. 9090. Bairro Agronomia. CEP 91540-000 Porto Alegre, RS, Brazil.

INTRODUCTION

Transport of cooled semen has been routinely used in the past 20 years. Today, most equine breeder associations allow the use of artificial insemination, and owners have enjoyed the benefits of this biotechnology [1]. The focus of research for over two decades has been optimization of protocols for equine cooled-shipped semen [11] because temperature, storage time, extender, dilution and cooling curve affect fertilizing capacity [34].

The maintenance of sperm viability for at least 24 h is essential in the equine reproduction [31]; in Brazil, however, considering distance and logistics, this period should be extended to 48 h. It has already been proven that there is a decrease in motility, membrane integrity [24] and fertilizing ability [22] of equine spermatozoa during cooled-storage. Also occur during cooled storage, the "cold shock" [5,9] and other changes such as lipid peroxidation [14], modifications in pH [6], ATP depletion [7], oxidative phosphorylation [27], and bacterial contamination [30].

The interval between collection and use leads to a constant search to improve maintenance of sperm viability. Besides these facts, it is well known that there are variability from stallion to stallion and differences between breeds. The search for knowing the reproductive parameters of the breeds is important in the identification of individuals with high and low reproductive potential. The breeding of ponies gained remarkable popularity in recent years becoming an important trade field for breeders and professionals. However, this specie still lacks studies that address issues mainly related to semen quality. In horses, there is extensive literature on reproductive biotechnologies related to handling, cooling, transportation and freezing, which has not occurred with the pony. Because of that, our goal was to test the effect of temperature (5° or 15°C) and different dilutions on semen for 48 h on sperm viability of Brazilian pony stallions.

MATERIALS AND METHODS

Place and Animals

This study was conducted between January and June 2013 at the Laboratory of Animal Embryology of the Federal University of Santa Maria (UFSM), Santa Maria/RS, Brazil. Nine pony stallions of the Brazilian breed, aged 9 to 13 years had their semen collected routinely with an artificial vagina before and during the study.

Semen collection

Two ejaculates from each pony were obtained with a Hannover model artificial vagina. Semen collection was performed as previously described [17]. Ejaculates were filtered with sterile gauze to separate the gel-free portion of the ejaculate. Then the volume, appearance and color were macroscopically evaluated.

Semen extenders

The extender was composed of 2.4 g of powdered skim milk, 4.9 g of glucose[1] and 95 mL of purified water [adapted from 15]. Each ejaculate was divided into six fractions, and two of these fractions were extended 1:1 (semen:extender), 1:2 and 1:3, respectively.

Semen analyses

Immediately after dilution motility, pH, concentration, membrane function (hypoosmotic - HOST), and mitochondrial activity (MTT) of samples were evaluated. After initial analysis, samples were stored at 5°C ± 1.6 in a refrigerator or at 15°C in a special cooling box for semen (Botubox®) for 24 and 48 h.The cooling rate in the refrigerator ranged from 0.6 to 0.8°C/min. Evaluations were repeated after 24 and 48 h of cooling. Sample evaluation was conducted after 15 min of warming on a platinum device adjusted to 37°C. The pH was measured in freshly diluted samples (pH Meter Tec-2, Tecnal) and after 24 h and 48 h of cooling.

Sperm concentration was determined using a Neubauer haemocytometer counting chamber. Sperm motility was evaluated according to described in the literature [33]. Assessment of sperm function was performed following protocol [18]. Sperm cell morphology was classified according to Neild *et al*. [23].

To assess mitochondrial activity [2], a sample of each group was centrifuged 10 min at 600 x *g*, and concentration was adjusted to 100 x10^6 sptz/mL. Two aliquots of 200 µL containing 100 x10^6 sptz/mL were deposited in 2 mL micro centrifuge tubes. Tetrazolium solution, 20 µL (5 mg/mL) thiazolyl blue tetrazolium bromide[2] in saline PBS was added to these tubes and they were incubated for 30 min in a water bath at 37°C. The micro centrifuge tubes were centrifuged at 12,000 x *g* for 5 min after addition of 200 µL of a 0,04N HCl- isopropanol solution. The supernatant was used to measure the mitochondrial metabolic activity by UV visible spectrophotometry with

a wave length of 540 nm. The reference sample used (white) was the skim milk extender solution with glucose, tetrazolium and 0,04N HCl-isopropanol. Aliquots of each stallion were analyzed in duplicate.

Statistical analysis

The SAS® software (version 9.2, SAS Institute Inc., Cary, NC, USA) was used for statistical analysis. Experimental design was completely randomized. Data were analyzed by analysis of variance (ANOVA) and the rate comparison was performed using the Duncan test.

RESULTS

The average ejaculate volume was 15.69 ± 12.81 mL, and total concentration was 330.83 ± 208.79 x 10^6 sptz/mL. Average concentration and standard deviation of the1:1, 1:2 and 1:3 dilutions was 165.41 ± 104.39 x 10^6 sptz/mL, 110.27 ± 69.59 x 10^6 sptz/mL, and 82.70 ± 52.19 x 10^6 sptz/mL, respectively. Mean sample volume of dilutions 1:1, 1:2 and 1:3 in the graduated tubes was 10.46 ± 8.54 mL, 15.69 ± 12,81 mL and 20.93 ± 17.09 mL.

After semen collection, temperature and dilutions used did not interfere with progressive motility, hypoosmotic test, mitochondrial activity and pH ($P > 0.05$) among the three semen dilutions. Progressive motility was higher (PM, $P < 0.0001$) and pH was lower ($P < 0.001$) when semen was diluted 1:2 and 1:3 at both temperatures (5°C and 15°C) compared to 1:1 dilution. The highest percentage of sperm cells with intact membranes was observed at 15°C, but did not differ between the three dilutions (Table 1). Mitochondrial activity was higher ($P = 0.0006$) at 15°C. Higher pH values were detected on 1:1 dilution at 5°C and 15°C.

Evaluation of three different dilutions of cooled semen after 24 and 48 h showed that the 1:1 dilution, independent of temperature (Table 2) had a higher pH ($P < 0.01$) and a lower percentage of progressive motility. The 1:2 and 1:3 dilutions presented similar progressive motility and pH among treatments at 24 h. Functional membrane integrity and mitochondrial activity results were similar on all three dilutions after 24 h and 48 h of cooling.

Table 1. Means and standard deviation of progressive motility, HOST test, mitochondrial activity and pH on fresh and 48 h cooled (5°C and 15°C) samples of three dilutions of Brazilian pony semen.

Dilution Preservation	1:1	1:2	1:3	*P* Value
		Progressive Motility		
Fresh	68.61 ± 5.63^{a}	68.33 ± 7.07^{a}	68.88 ± 5.3^{a}	
5°C	10.3 ± 11.05^{e}	30.55 ± 14.91^{d}	36.94 ± 14^{cd}	< 0.0001
15°C	17.08 ± 9.95^{b}	35.97 ± 15.80^{cd}	42.22 ± 12.38^{c}	
		Hypoosmotic		
Fresh	62.22 ± 9.4^{c}	61 ± 9^{c}	60.88 ± 11.11^{c}	
5°C	30.16 ± 14.31^{e}	35.55 ± 13.2^{e}	35.75 ± 13.73^{e}	<0.0001
15°C	44.16 ± 14.59^{d}	48.16 ± 13.52^{d}	50.05 ± 11.36^{d}	
		MTT (*nm*)		
Fresh	1 ± 0.26^{e}	0.91 ± 0.18^{ed}	0.93 ± 0.17^{ed}	
5°C	0.74 ± 0.2^{c}	0.84 ± 0.22^{bc}	0.86 ± 0.19^{cde}	0.0006
15°C	0.89 ± 0.23^{de}	0.93 ± 0.2^{de}	0.92 ± 0.20^{de}	
		pH		
Fresh	7.25 ± 0.17^{a}	7.13 ± 0.21^{a}	7.12 ± 0.15^{a}	
5°C	7.63 ± 0.34^{e}	7.46 ± 0.21^{cd}	7.38 ± 0.11^{bc}	< 0.0001
15°C	7.57 ± 0.27^{de}	7.37 ± 0.22^{bc}	7.31 ± 0.15^{c}	

^{a,b,c}Different letters on lines and columns mean $P < 0.05$.

Table 2. Means and standard deviation of progressive motility, HOST test, mitochondrial activity and pH of three dilutions of Brazilian pony semen evaluated after collection (fresh) and 24 h and 48 h after cooling at 5°C or 15°C.

Dilution Preservation	1:1	1:2	1:3	*P* Value
		Progressive Motility		
Fresh	68.61 ± 5.63^{a}	68.33 ± 7.07^{a}	68.88 ± 5.3^{a}	< 0.0001
24 h	19.16 ± 10.85^{e}	39.72 ± 13.51^{dc}	43.61 ± 11.62^{d}	
48 h	8.22 ± 8.092^{b}	26.8 ± 14.79^{f}	35.55 ± 13.97^{c}	
		Hypoosmotic		
Fresh	62.22 ± 9.4^{a}	61 ± 9^{a}	60.88 ± 11.11^{a}	<0.0001
24 h	43.91 ± 14.81^{de}	46.05 ± 13.33^{e}	46.52 ± 13.56^{e}	
48 h	30.41 ± 14.33^{c}	37.66 ± 15^{cd}	39.27 ± 14.57^{ed}	
		MTT (*nm*)		
Fresh	1 ± 0.26^{a}	0.91 ± 0.18^{abc}	0.93 ± 0.17^{ab}	0.03
24 h	0.86 ± 0.21^{ac}	0.9 ± 0.23^{abc}	0.9 ± 0.21^{abc}	
48 h	0.78 ± 0.24^{c}	0.87 ± 0.2^{abc}	0.89 ± 0.19^{abc}	
		pH		
Fresh	7.25±0.17^{ab}	7.13±0.21^{a}	7.12±0.15^{a}	<0.001
24 h	7.65 ± 0.34^{e}	7.45 ± 0.24^{cd}	7.39 ± 0.12^{bc}	
48 h	7.55 ± 0.27^{de}	7.39 ± 0.2^{bc}	7.3 ± 0.14^{b}	

^{a,b,c}Different letters between lines and columns mean $P < 0.05$.

Furthermore, after 48 h of cooling a higher percentage of cells with progressive motility was observed on dilution 1:3 ($P < 0.05$). Dilutions 1:2 and 1:3 showed lower pH.

Fresh semen showed similar results between dilutions (Figure 1). At 24 h and 48 h, the 1:1 dilution showed lower progressive motility at both temperatures (15°C and 5°C). At 24 h, there was no difference in progressive motility at 5°C or 15°C ($P < 0.005$), motility started to improve at 1:2 dilution. At 48 h, 1:3 dilution at 15°C showed higher progressive motility than at 5°C ($P < 0.001$).

The hypoosmotic swelling test demonstrated that better results were achieved at 15°C after 24 h and 48 h of cooling. Results were similar between dilutions (Figure 2) and fresh semen at this temperature.

Results of the mitochondrial activity evaluation of the diluted sperm samples obtained by absorbance reading over time and temperature (Figure 3), demonstrated similarity between dilutions in fresh semen and cooled samples (24 h and 48 h).

The pH of fresh semen was lower than of the diluted samples after 24 h and 48 h of cooling, with no significant difference between dilutions (Figure 4). Dilution 1:1 showed higher pH and similar results were seen on 1:2 and 1:3 dilutions at 5°C or 15°C at 24 h and 48 h of cooling.

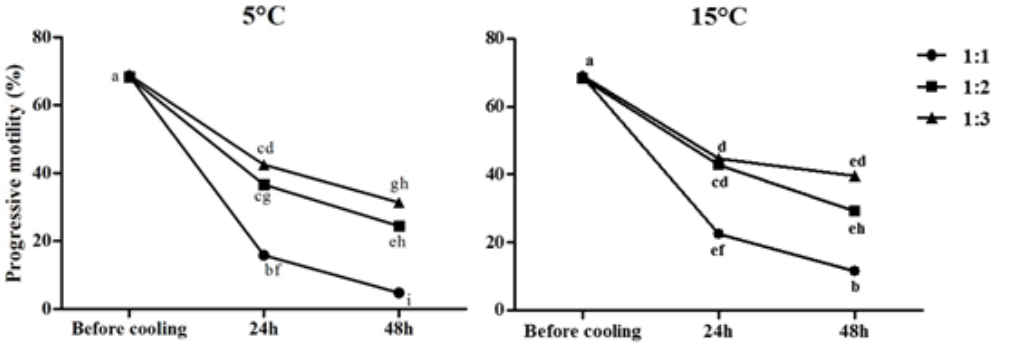

Figure 1. Progressive motility of Brazilian pony sperm diluted with a skim milk-based extender at three different dilutions (1:1, 1:2, 1:3) and cooled at 5°C or 15°C ($P < 0.001$).

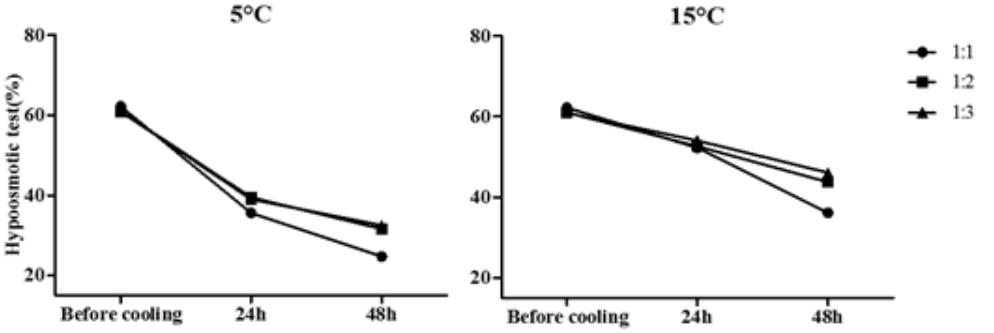

Figure 2. Percentage of swollen sperm cells on three different dilutions of fresh and cooled Brazilian pony semen samples on the hypoosmotic test ($P < 0.001$).

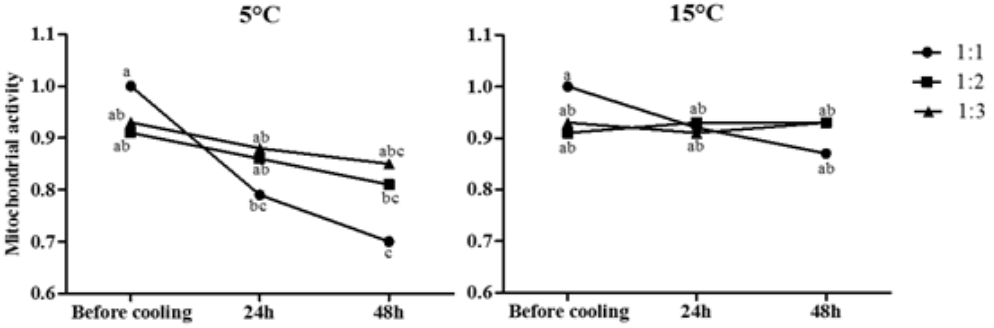

Figure 3. Mitochondrial activity of sperm cells from Brazilian ponies at three different dilutions after collection and cooled at 5°C or 15°C ($P = 0.049$).

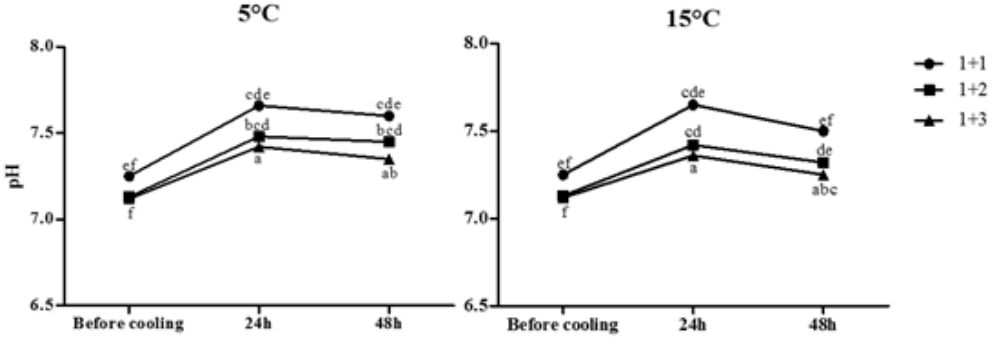

Figure 4. pH of fresh and cooled semen of Brazilian pony sperm on three different dilutions after 24 h and 48 h, at 5°C or 15°C ($P < 0.0001$).

DISCUSSION

Equine semen when stored or shipped is usually held for 12 h to 48 h at 5°C. This is due to the ease of maintaining the temperature around 5°C. The results showed that cooling reduces semen viability. This study; moreover, showed better results when semen diluted in powdered skim milk was maintained at 15°C, agreeing with previous reports [29]. These authors, regardless of extender used, achieved higher motility on semen samples stored at 20°C or15°C than at 10°C or 5°C.

Progressive motility of pony spermatozoa after 24 h of cooling was similar between 5°C and 15°C. However, in equine spermatozoa was demonstrated [32,33] that cooling at 4°C and 5°C for 24 h resulted in greater sperm motility than at 20°C or 25°C, with a pregnancy rate of 73% for both temperatures. Price *et al*. [28] found no difference between progressive motility of diluted equine semen kept at 5°C or 15°C after 48 h of storage. It remains

unclear whether the addition of gentamicin, which blocks bacterial growth, may have favored this result. The hypoosmotic swelling test demonstrated that better results were achieved at 15°C after 24 h and 48 h of cooling. The percentage of sperm with intact membranes may prove to be one of the more important criteria for predicting potential fertility of cool-stored semen in stallions [16].

In 1998, significant reduction was observed [3] in fertility when semen was stored for 24 h at 20°C, compared with storage at 5°C. Love *et al*., [19] using refrigerated semen of 18 stallions diluted in a skim milk extender at 5°C or 20°C for 7 h to 46 h, observed higher sperm chromatin integrity at 20°C. Cooling between 4°C and 5°C reduces sperm metabolic activity, bacterial growth, and keeps sperm viable for long periods [13]. Similar results were observed during storage of the testis-epididymis complex at 5°C, which provided better preservation of epididymal sperm than storage at room temperature [21].

In a laboratory setting, equine semen stored for 72 h at 5°C without air exposure or at 15°C with air exposure showed similar fertility results [34]. *In vitro* studies conducted with stallion semen diluted in skim milk diluents and stored for 24 h in different types of dischargeable recipients, and at different temperatures suggest that motility is affected when the internal temperature of the recipient is lower than 2°C and above 20°C [4,20]. Furthermore, equine semen tolerates large cooling rates and storage temperatures [4,34], which was also proved in pony semen in this study.

Progressive motility evaluation of fresh diluted semen was similar between 1:1, 1:2 and 1:3 dilutions. After 24 h and 48 h of cooling, the 1:1 dilution had lower progressive motility ($P < 0.001$). Progressive motility was similar at 1:2 and 1:3 dilutions after 24 h cooling, and at 48 h, the 1:3 dilution resulted in higher progressive motility. Thus, for transportation until 48 h, 15°C and 1:3 dilutions would be more indicated, since these solutions showed the least pH variability, indicating that as the dilution increases cooled sperm viability is favored. Dilution of equine semen in a commercial extender to very low sperm concentrations had only minor effects on sperm quality [10]. Jasko *et al*., [12] using an egg yolk extender, observed that semen dilutions from 1:4 to 1:19 were appropriate to preserve equine semen at 5°C and that increasing the dilution favors sperm viability.

The positive effects of extenders are based on pH control, osmolarity and energy supply [25]. The absence of a buffering component in the extender used in this study may have led to the pH variation observed in the cooled semen. Measured pH clearly indicates that storage time and temperature influence the pH of cooled semen in a skim milk, glucose-based extender. pH increased the first 24 h and declined at 48 h of storage at 5°C and 15°C, and this may have reduced the progressive motility further than the expected reduction in motility due to cooling and time. At 5°C, a higher pH was maintained at 24 h and 48 h, similar to that observed on fresh semen samples. This suggests that the addition of a buffer to the extender is required so that pH does not have a negative influence on the viability of spermatozoa.

The increase in pH observed during cooling may be related to the percentage of viable spermatozoa during storage. Lower storage temperature decreases metabolic rate, slows chemical reactions and reduces bacterial growth, extending the life of fertile spermatozoa [26]. In one research with stallion sperm, pH was not a significant source of variation in motility and live sperm percentage over different cooling periods [6]. In our study, samples with higher motility had lower pH. This was attributed to metabolism reduction induced by cooling. The low pH may be related to the production of hydrogen ions and lactic acid from sperm metabolism. Of our knowledge we support that there are similarities with queries related to *Equus ferus caballus*.

CONCLUSION

Progressive motility, pH, membrane integrity and mitochondrial activity of pre-cooled pony semen diluted in a powdered skim milk-based extender were not affected by dilution 1:1, 1:2 or 1:3. However, the 1:3 dilution cooled at 15°C provided better viability of pony sperm, and more stable pH during 48 h of cooling. Dilution 1:1 should not be used for cooling in powdered skim milk extender. Increasing the dilution does not interfere with mitochondrial activity and promotes sperm viability. Cooling at 5°C increases the pH of diluted semen.

MANUFACTURERS

[1]Merck KGaA. Darmstadt, Germany.

[2]Sigma Aldrich Co. St. Louis, MO, USA.

***Funding*.** Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - CAPES, Brasília, DF, Brazil and Laboratório de Embriologia Animal (EMBRYOLAB), UFSM, Santa Maria, RS, Brazil.

***Ethical approval*.** The Ethical and Animal Research Committee from the Federal University of Santa Maria (UFSM) [protocol number 065/2013] approved all procedures involving the pony stallions used in this study.

***Declaration of interest*.** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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