Acta Scientiae Veterinariae, 2019. 47: 1633.

RESEARCH ARTICLE
Pub. 1633

ISSN 1679-9216

Functional Assessment of Diluent Choice for Semen Cryopreservation from Stallions with High and Low Freezability

Heder Nunes Ferreira[1], José Carlos Ferreira-Silva[1], Jorge Motta Rocha[2], Pamela Ramos-Deus[1], Joane Isis Travassos Vieira[1], Marcelo Tigre Moura[1], José Pompeu Santos Filho[1] & Marcos Antonio Lemos Oliveira[1]

ABSTRACT

***Background*:** Fertility using horse frozen-thawed semen remains lower than in other livestock species. This fact suggests that horse semen hold intrinsic sensitivity to cryoinjury that must be investigated. Moreover, there is substantial evidence of genetic factors upon horse cryopreservation outcome. Nonetheless, diluent and cryoprotectant choice for horse semen cryopreservation are under intense research. Thus these factors could be explored to identify conditions that may increase semen viability after thawing. The aim of this work was to evaluate the effect of diluents Botu-Crio®, Lactose-EDTA®, and INRA-82® on cryopreserved semen from stallions with high (HFA) and low freezability (LFA).

***Materials, Methods & Results*:** Frozen-thawed semen was evaluated for motility and membrane integrity using computer-assisted semen analysis (CASA), and also inferred for sperm DNA fragmentation by sperm chromatin structure assay during the thermoresistance test (TRT). Comparisons for each parameter were done in a pair-wise fashion between HFA and LFA semen at one-hour intervals during the TRT (0 h - 4 h). Sperm motility in HFA, regardless of the diluent, was larger ($P < 0.05$) than LFA, both on 0h and 1h. In the 2h evaluation, sperm motility using Botu-Crio® and Lactose-EDTA® was greater ($P < 0.05$) for HFA. Analysis of sperm membrane integrity was similar between HFA and LFA semen ($P > 0.05$) at 0 h and 3 h. Sperm DNA fragmentation was lower ($P < 0.05$) in HFA semen at 0 h and 1 h.

***Discussion*:** Artificial insemination in horses using frozen-thawed semen is gaining wider acceptance under commercial settings, although its current limited outreach due to low semen viability after thawing. Therefore, several efforts were made toward modifying horse semen cryopreservation methodologies to meet the market demand, particularly for breeds that display low semen freezability under a regular basis. Frozen-thawed semen from stallions of high freezing ability showed greater motility at all analysis, irrespectively of diluent choice, suggesting a strong influence of genetic factors on cryopreservation outcome under the experimental conditions described in the text. Nonetheless, the diluent choice did affect motility for LFA stallions, and cryopreservation protocols including lower glycerol concentrations seem more promising. Membrane integrity was similar immediately after thawing but did differ later on other TRT time-points, irrespectively of diluent choice. As observed for motility, it was expected that sperm cells of stallions of HFA would show higher membrane integrity than their LFA counterparts. Hence, the diluent choice is relevant for semen cryopreservation from LFA stallions. This fact is supported by the well-described correlation between sperm membrane viability and motility. Sperm DNA fragmentation was quite low for both groups, as generally described in horses. Surprisingly, sperm DNA fragmentation incidence was constant throughout the analysis for both HFA and LFA. It was initially envisioned that increased DNA fragmentation would be found in semen from LFA stallions since it is caused by multiple origins such as genetic factors. Therefore, it can be suggested that sperm DNA integrity was more influenced by the balance of genetic and environmental factors during gametogenesis than the TRT assay. In conclusion, the semen diluent affects horse sperm motility after thawing, particularly from stallions with lower semen freezability.

Keywords: reproduction, male, cryopreservation, diluent.

DOI: 10.22456/1679-9216.89906

Received: 12 August 2018 Accepted: 28 January 2019 Published: 16 February 2019

[1]Laboratório de Biotécnicas Reprodutivas, Departamento de Medicina Veterinária, Universidade Federal Rural de Pernambuco (UFRPE), Recife, PE, Brazil. [2]Universidade Federal do Rio Grande do Norte (UFRN), Natal, RN, Brazil. CORRESPONDENCE: J.C. Ferreira-Silva [ferreirasilva.jc@gmail.com - Tel.: +55 (81) 33206414]. Rua Dom Manoel de Medeiros s/n. Bairro Dois Irmãos. CEP 52171900 Recife, PE, Brazil.

INTRODUCTION

The approval of artificial insemination usage in horse breeding programs by most breed associations intensified research on this biotechnology, particularly due to its potential for semen storage from valuable stallions for unlimited periods of time. However, fertility rates using horse frozen-thawed semen is consistently lower than in other species [4,30], thus suggesting that horse semen is particularly sensitive to cryoinjury [19,33].

Semen cryotolerance in horses depends upon genetic factors and cryopreservation protocol [2,38], such as diluent and cryoprotectant choice [2,28]. For these reasons, the effect of individual stallions has been extensively investigated throughout the years, aiming to identify factors associated to semen cryotolerance in horses, especially for stallions classified as holding low semen freezability potential [12,15,18,32,33,39].

Diluent and cryoprotectant choice for horse semen cryopreservation is under intense investigation [12,18,30,38], aiming to reduce toxicity and osmotic stress during cryopreservation [12,14,26]. Several parameters are used to measure horse semen cryotolerance, such as sperm motility [1,2], sperm membrane integrity and acrosome and mitochondria viability [8,30], zona pellucida binding assays [27,36], and incidence of DNA fragmentation [20,32]. Based on the facts above, the interplay between horse breed and cryopreservation protocol plays a major role on semen cryotolerance in horses.

Thus, the work aimed to compare the efficiency of semen diluent choice on horse semen cryopreservation using stallions of high freezability (HFA) and low freezability (LFA) evaluating sperm motility, membrane integrity, and DNA fragmentation during a thermoresistance test.

MATERIALS AND METHODS

Stallions and semen collection

A total of 13 stallions were used throughout the experiment, where four stallions from Westfalen, Arabian, Hanoverian and Quarter Horse breeds formed the HFA group (one of each breed). In contrast, nine stallions from the Mangalarga Marchador breed were used for the LFA group. Stallions that display semen with post-thaw motility lower than 30% over multiple collections were considered as LFA [11]. All stallions had four to twelve years of age, of proven fertility, known cryotolerance potential and were further subject to semen collection regimen before the experiment.

Semen collection was performed with artificial vagina warmed at 42°C, using a phantom or mare in estrus. The ejaculate was initially filtered to remove gel and final semen volume was than measured. A semen aliquot was immediately used for analysis (fresh sample), while the other sample was diluted in Botu-Sêmen® diluent1 in a 1 : 2 dilution and divided into three even samples before centrifugation at 600 *g* for 10 min. After removing the supernatant, semen pellets were resuspended in three diluents, namely Botu-Crio®[1] [5,32], INRA-82®[2] [3], and Lactose-EDTA®[3] [9,23]. Semen was further diluted in order to obtain a final concentration of 100 x 10^6 viable sperm cells mL-1 and then loaded into 0.5 mL straws. Moreover, semen straws were cooled at 5ºC for 20 min for Botu-Crio® straws, 90 min for INRA-82® straws, while Lactose-EDTA® straws were not subjected to this step, according to manufacturer recommendations [9,23]. After stabilization at 5ºC, all straws remained in nitrogen steam for 15 min and immersed in liquid nitrogen at -196°C for long-term storage.

Semen analysis

Frozen-thawed semen was initially evaluated immediately after thawing at 46°C for 20 s, subject to TRT test at 37ºC for 4 h and evaluated at one-hour intervals. Frozen-thawed semen was evaluated for sperm motility on computer analysis sperm assay (CASA), as described previously [25]. Membrane integrity was carried out by fluorescence microscopy using 6-carboxifluorescein diacetate probes associated to propidium iodide [17]. Sperm DNA fragmentation was performed by sperm chromatin structure assay (SCSA), as reported by others [35].

Statistical analysis

The data recorded as percentages was tested for normality using the Kolmogorov-Smirnov test [24] and further subjected to arcsin transformation. The data after transformation as subject to ANOVA. Mean comparisons were determined by Tukey's test with a confidence level of 95%.

RESULTS

The comparisons for sperm motility, sperm membrane integrity, and sperm DNA fragmentation between stallions of HFA and LFA were performed

during a five-time point period on TRT assay with one-hour intervals and among the three diluents.

Table 1 contains the relative percentages for sperm motility immediately after thawing and during the TRT assay. It can be noted that semen from HFA stallions had higher ($P < 0.05$) sperm motility than LFA stallions, irrespectively of diluent choice, both immediately after thawing (0 h) and at 1 h. Similar results were obtained at 2 h, where semen motility using Botu-Crio® and Lactose-EDTA® diluents was higher ($P < 0.05$) on semen from HFA stallions, although INRA-82® diluent did not lead to similar results. For the remaining TRT intervals, no difference was observed within diluents ($P > 0.05$).

Table 1 also describes, in terms of diluent choice, sperm motility immediately after thawing was higher ($P < 0.05$) on Botu-Crio® than other diluents, for both HFA and LFA stallions. On the same analysis, sperm motility on INRA-82® was higher ($P < 0.05$) than Lactose-EDTA® diluent only for HFA stallions. Furthermore, sperm motility using Botu-Crio® was higher ($P < 0.05$) than INRA-82® and Lactose-EDTA® after 2 h and 3 h on TRT on semen of stallions from both HFA and LFA.

The results on Table 2 show sperm membrane integrity analysis. There was no difference ($P > 0.05$) immediately after thawing and at 3 h between HFA and LFA semen. However, for the remaining time points, sperm membrane integrity was higher in the HFA group ($P < 0.05$) than the LFA.

The comparisons among diluents showed that Botu-Crio® was similar to INRA-82® ($P > 0.05$) but more efficient than Lactose-EDTA® ($P < 0.05$) immediately after thawing and at 1h for both LFA and HFA groups (Table 2). Moreover, Botu-Crio® was more efficient ($P < 0.05$) than INRA-82® and Lactose-EDTA® within initial three hours. The INRA-82® and Lactose-EDTA® diluents were similar throughout TRT analysis ($P > 0.05$).

The results on Table 3 show sperm DNA fragmentation, both at 0 h and 1 h of TRT. Irrespectively of diluent choice, DNA fragmentation was lower ($P < 0.05$) on HFA group. For the remaining time points, there was no difference among groups ($P > 0.05$).

DISCUSSION

Artificial insemination in horses using frozen-thawed semen in gaining wider acceptance under commercial conditions, despite its current limited outreach compared to the cattle industry. Thus, several efforts have been made toward modifying horse semen cryopreservation protocols [10,16], particularly for stallions or breeds that display low semen freezability [1,5,14,26].

Frozen-thawed semen from stallions of high freezing ability showed greater motility at all analysis, irrespectively of diluent choice, suggesting a strong influence of genetic factors on cryopreservation outcome [1,38]. These results are in agreement with the hypothesis that yet unidentified factors that cause high freezing ability play a major role on horse semen survival after cryopreservation. This ability may be acquired by differences in sperm membrane composition, that varies across horse breeds and correlates with semen freezability, as proposed by Holt [18].

Botu-Crio® increased the survival of sperm cells from LFA stallions. Frozen-thawed semen should display minimum requirements to be used for artificial insemination, the reason why CBRA [7] established that frozen-thawed semen should display motility of 30% or greater. By taking this requirement into account, Botu-Crio® diluent should be used for both animals of high and low freezability. Under the same reasoning, it is convenient to state that INRA-82® diluent should be used for semen of high freezability, while Lactose-EDTA® should not be recommended for this purpose. The results described here are not integrally in accordance to other researchers [5,12,14], possibly

Table 1. Sperm motility (%) in frozen-thawed semen using different diluents, from stallions of high and low freezability and evaluated by the thermoresistance test at 37ºC.

	High Freezability (HFA)					Low Freezability (LFA)				
	Thermoresistance Test (hours)					Thermoresistance Test (hours)				
Diluent	0	1	2	3	4	0	1	2	3	4
Botu-Crio®	$62.2^{Aa} \pm 11.4$	$31.2^{Aa} \pm 6.5$	$17.7^{Ba} \pm 14.7$	$5.5^{Ab} \pm 6.8$	0^{Aa}	$48.1^{Ba} \pm 15.5$	$12.0^{Ba} \pm 11.8$	$5.3^{Aa} \pm 12.6$	0^{Aa}	0^{Aa}
INRA-82®	$53.0^{Ab} \pm 17.4$	$8.0^{Ab} \pm 7.6$	0^{Ab}	0^{Ab}	0^{Aa}	$21.2^{Bb} \pm 6.7$	$0.4^{Bb} \pm 1.3$	0^{Aa}	0^{Aa}	0^{Aa}
Lactose-EDTA®	$19.3^{Ac} \pm 11.5$	$10.0^{Ab} \pm 7.0$	$3.3^{Bb} \pm 3.0$	0^{Ab}	0^{Aa}	$2.1^{Bc} \pm 3.5$	0^{Bb}	0^{Ab}	0^{Aa}	0^{Aa}

Different superscript letters (A, B) on the same line denote statistical difference ($P < 0.05$) between HFA and LFA groups. Different lowercase superscript letters (a, b, c) on the same column denote statistical difference ($P < 0.05$) among diluents.

Table 2. Sperm membrane integrity (%) in frozen-thawed semen using different diluents, from stallions of high and low freezability and evaluated by the thermoresistance test at 37ºC.

	High Freezability (HFA)					Low Freezability (LFA)				
	Thermoresistance Test (hours)					Thermoresistance Test (hours)				
Diluent	0	1	2	3	4	0	1	2	3	4
Botu-Crio®	$49.7^{Aa} \pm 4.0$	$47.5^{Aa} \pm 5.3$	$47.2^{Aa} \pm 4.8$	$45.7^{Aa} \pm 6.9$	$43.7^{Aa} \pm 5.4$	$42.1^{Aa} \pm 5.7$	$37.3^{Ba} \pm 8.6$	$34.8^{Ba} \pm 5.8$	$37.4^{Aa} \pm 6.0$	$33.6^{Ba} \pm 4.4$
INRA-82®	$44.7^{Aab} \pm 11.8$	$38.2^{Aab} \pm 9.3$	$38.7^{Aa} \pm 6.4$	$34.7^{Ab} \pm 9.7$	$35.0^{Aa} \pm 9.7$	$35.1^{Aab} \pm 8.2$	$32.4^{Bab} \pm 5,0$	$33.7^{Aa} \pm 7.0$	$34.1^{Ab} \pm 5.2$	$34.3^{Ba} + 6.9$
Lactose-EDTA®	$33.3^{Ab} \pm 3.2$	$34.0^{Ab} \pm 2.0$	$29.6^{Aa} \pm 2.1$	$33.6^{Ab} \pm 4.0$	$33.6^{Aa} \pm 3.2$	$33.8^{Ab} \pm 10.7$	$30.6^{Bb} \pm 5.8$	$33.4^{Ba} \pm 5.15$	$31.8^{Ab} \pm 3.8$	$34.1^{Ba} \pm 3.1$

Different superscript letters (A, B) on the same line denote statistical difference ($P < 0.05$) between HFA and LFA groups. Different lowercase superscript letters (a, b) on the same column denote statistical difference ($P < 0.05$) among diluents.

Table 3. Sperm DNA fragmentation (%) in frozen-thawed semen using different diluents, from stallions of high and low freezability and evaluated by the thermoresistance test at 37ºC.

	High Freezability (HFA)					Low Freezability (LFA)				
	Thermoresistance Test (hours)					Thermoresistance Test (hours)				
Diluent	0	1	2	3	4	0	1	2	3	4
Botu-Crio®	$1.6^{Aa} \pm 0.6$	$1.7^{Aa} \pm 0.8$	$1.5^{Aa} \pm 0.7$	$2.1^{Aa} \pm 2.5$	$1.6^{Aa} \pm 0.5$	$2.6^{Ba} \pm 1.1$	$2.6^{Ba} \pm 0.9$	$2.0^{Aa} \pm 1.0$	$2.0^{Aa} \pm 1.1$	$1.9^{Aa} \pm 1.0$
INRA-82®	$1.3^{Aa} \pm 0.6$	$1.3^{Aa} \pm 0.7$	$1.3^{Aa} \pm 0.5$	$2.2^{Aa} \pm 1.4$	$1.9^{Aa} \pm 0.8$	$2.8^{Ba} \pm 1.6$	$2.8^{Ba} \pm 1.6$	$2.1^{Aa} \pm 1.4$	$2.1^{Aa} \pm 1.4$	$1.9^{Aa} \pm 1.5$
Lactose-EDTA®	$1.5^{Aa} \pm 0.5$	$1.9^{Aa} \pm 0.6$	$1.9^{Aa} \pm 0.7$	$2.1^{Aa} \pm 1.4$	$1.9^{Aa} \pm 0.6$	$2.8^{Ba} \pm 1.0$	$2.6^{Ba} \pm 1.0$	$2.2^{Aa} \pm 0.9$	$1.8^{Aa} \pm 0.9$	$1.9^{Aa} \pm 1.0$

Different superscript letters (A, B) on the same line denote statistical difference ($P < 0.05$) between HFA and LFA groups. Different lowercase superscript letters (a, b) on the same column denote statistical difference ($P < 0.05$) among diluents.

due to the numerous traits evaluated in each report, differences in experimental conditions and possibly other unidentified factors.

Membrane integrity was similar immediately after thawing but did differ later on other TRT time-points, irrespectively of diluent choice. As observed for motility, it was expected that sperm cells of stallions of HFA would show higher membrane integrity than their LFA counterparts. This finding suggests the protection exerted by sperm membrane is less pronounced in some animals, a fact that can explain why artificial insemination is lower in stallions of LFA. This hypothesis is supported by Holt [18], since the author mentioned possible differences in membrane composition among horse breeds.

The Lactose-EDTA® diluent was less efficient to maintain membrane integrity than Botu-Crio® and similar to INRA-82®. However, this later diluent was similar to Botu-Crio®. In an independent comparison of semen diluents for stallion semen cryopreservation, Terraciano and co-workers [37] observed that Botu-Crio® showed greater sperm membrane integrity. It was initially hypothesized that Botu-Crio® would be more efficient, based on its composition that includes cryoprotectants of amine groups, thus displaying lower molecular weight, which allows faster penetrance on sperm cells during cryopreservation and ultimately leads to greater sperm membrane protection. According to Ferreira and co-workers [12], the Botu-Crio® is composed of intracellular cryoprotectants, such as the amine derived ones, and may be associated with low glycerol concentrations, probably by using 4% methylformamide and 1% glycerol**.** Therefore, it differs sharply from other diluents that are formulated with 3% glycerol or more, and that should be avoided for cryopreservation of semen from LFA stallions.

Sperm DNA fragmentation was quite low for both groups, as described in horses [29,34], and other species [6,22]. Surprisingly, sperm DNA fragmentation incidence was constant throughout the analysis for both HFA and LFA. It was initially envisioned that increased DNA fragmentation would be found in semen from LFA stallions, since it is caused by multiple origins such as genetic factors [15,21]. Moreover, frozen-thawed horse semen displayed variable resistance to sperm DNA fragmentation under *in vitro* conditions [20,40], possibly due to assay-intrinsic factors.

CONCLUSION

The semen diluent affects horse sperm motility after thawing, particularl y from stallions with lower semen freezability.

MANUFACTURERS

[1]Botupharma Biotecnologia Animal para Equinos e Pet. Botucatu, SP, Brazil.
[2]IMV Technologies. L'Aigle, France.
[3]Animal Reproduction Systems Inc. Chino, CA, USA.

***Acknowledgements*.** Authors would like to acknowledge the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior and Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco for fellowship support during the study.

Ethical approval. This research was performed after evaluation and approval of the Ethics Committee on the Use of Animals, Faculdade Pio Décimo, Aracaju - SE, Brazil (License: 01/17).

Declaration of interest. The authors declare that they have no conflict of interest and are available to provide any clarification.

REFERENCES

1 **Alvarenga M.A., Leao K.M., Papa F.O., Landim-Alvarenga F.C., Medeiros A.S.L. & Gomes G.M. 2004.** The use of alternative cryoprotectors for freezing stallion semen. *Havemeyer Found Monograph Series*. 12: 74-76.

2 **Alvarenga M.A., Papa F.O., Landim-Alvarenga F.C. & Medeiros A.S.L. 2005.** Amides as cryoprotectants for freezing stallion semen: a review. *Animal Reproducion Science*. 89(1-4): 105-113.

3 **Batellier F., Magistrini M., Fauquant J. & Palmer E. 1997.** Effect of milk fractions on survival of equine spermatozoa. *Theriogenology*. 48(3): 391-410.

4 **Bucci D., Giaretta E., Spinaci M., Rizzato G., Isani G., Mislei B., Mari G., Tamanini C. & Galeati G. 2016.** Characterization of alkaline phosphatase activity in seminal plasma and in fresh and frozen-thawed stallion spermatozoa. *Theriogenology*. 85(2): 288-295.

5 **Candeias M.L., Alvarenga M.A., Carmo M.T., Ferreira H.N., Maior M.R.S., Torres Filho R.A., Rodrigues A.L.R. & Brandão F.Z. 2012.** Sêmen cryopreservation protocols of mangalarga marchador stallions. *Revista Brasileira de Zootecnia*. 41(9): 1989-1995.

6 **Carreira J.T., Mingoti G.Z., Rodrigues L.H., Silva C., Perri S.H.V. & Koivisto M.B. 2012.** Impact of proximal cytoplasmic droplets on quality traits and *in vitro* embryo production efficiency of crypreserved bull spermatozoa. *Acta Veterinaria Scandinavica*. 54(1): 1.

7 **CBRA - Colégio Brasileiro de Reprodução Animal. 2013.** *Manual de exame andrologico e avaliação sêmen animal*. 3.ed. Belo Horizonte: CBRA, 104p.

8 **Celeghini C.C.C., Arruda R.P., Silva F.H.A., Faria D.E., Andrade A.F.C.J. & Raphael C.F. 2007.** Utilization of fluorescent probe association for simultaneous assessment of plasmatic, acrossomal and mitochonarial membrane of rooster spermatozoa. *Revista Brasileira de Ciencia Avicola*. 9(3): 143-149.

9 **Cochran J.D., Amann R.P. & Froman D.P. 1984.** Effects of centrifugation, glycerol level cooling to 5°C, freezing rate and thawing rate on the post-thaw motility of equine sperm. *Theriogenology*. 22(1): 25-38.

10 **Córdova A., Strobel P., Vallejo A., Valenzuela P., Ulloa O., Burgos R.A., Menarim B., Rodríguez-Gil J.E., Ratto M. & Ramírez-Reveco A. 2014.** Use of hypometabolic TRIS extenders and high cooling rate refrigeration for cryopreservation of stallion sperm: presence and sensitivity of 5' AMP-activated protein kinase (AMPK). *Cryobiology*. 69(3): 473-481.

11 **Ferreira H.N., Ferreira-Silva J.C., Rocha J.M., Fárras M.C., Calixto M., Moura M.T. & Oliveira M.A.L. 2018.** Variable Inter-Assay Estimation of Sperm Dna Fragmentation in Stallions Classified As Good and Bad Semen Freezers. *CryoLetters*. 39(1): 67-71.

12 **Ferreira H.N., Johnson A.E.M. & Coutinho da Silva N.M.A. 2008.** Effects of freezing extenders and cryoprotectants on sperm motility and viability post-thaw. *Animal Reproducion Science.* 107: 319-320.

13 **Ferreira-Silva J.C., Basto S.R.L., Moura M.T., Rocha J.M., Freitas Neto L.M., Santos Filho J.P., Silva Filho M.L. & Oliveira M.A.L. 2018.** Freezing of stallion semen: *In vitro* evaluation of motility and acrosin activity in sperm cells cryopreserved using different semen extenders. *Biopreservation and Biobanking*. 16: 1-6.

14 **Gomes G.M., Jacob J.C.F., Medeiros A.S.L., Papa F.O. & Alvarenga M.A. 2002.** Improvement of stallion spermatozoa preservation with alternative cryoprotectants for Mangalarga Marchador breed. *Theriogenology*. 58(2-4): 277-279.

15 **Gosálvez J., Nuñez R., Fernández J.L., López-Fernández C. & Caballero P. 2011.** Dynamics of sperm DNA damage in fresh versus frozen-thawed and gradient processed ejaculates in human donors. *Andrologia*. 43(6): 373-377.

16 **Guimarães T., Lopes G., Pinto M., Silva E., Miranda C., Correia M.J., Damásio L., Thompson G. & Rocha A. 2015.** Colloid centrifugation of fresh stallion semen before cryopreservation decreased microorganism load of frozen-thawed semen without affecting seminal kinetics. *Theriogenology*. 83(2): 186-191.

17 **Harrison R.A.P. & Vickers S.E. 1990.** Use of fluorescent probes to assess membrane 6 integrity in mammalian spermatozoa. *Jornal de Reprodução e Fertilidade*. 88(1): 343-352.

18 **Holt W.V. 2000.** Basics aspects of frozen storage of semen. *Animal Reproduction Science*. 62(1-3): 3-22.

19 **Klopee L.H., Vaner D.D., Elmore R.G., Bretzlaff K.N. & Shull J.W. 1988.** Effect of insemination timing on the fertilizing capacity of frozen/thawed equine spermatozoa. *Theriogenology*. 29(2): 429-439.

20 **López-Fernández C., Crespo F., Arroyo F., Fernández J.L., Arana P., Johnston S.D. & Gosález J. 2007.** Dynamics of sperm DNA fragmentation in domestic animals II. The stallion. *Theriogenology*. 68(9): 1240-1250.

21 **Love C.C. & Kenney R.M. 1998.** The relationship of increased susceptibility of sperm DNA to denaturation and fertility in the stallion. *Theriogenology*. 50(6): 955-972.

22 **Machado E.R., Kamimura C.F., Beletti M.E. & Jacomini J.O. 2003.** Diagnóstico de alterações na compactação da cromatina em espermatozoides de suíno através de azul de toluidine e alaranjado de acridina. *Revista Brasileira de Reprodução Animal*. 27: 381-382.

23 **Martin J.C., Klug E. & Günzel A.R. 1979.** Centrifugation of stallion semen and its storage in large volumes straws. *Jornal de Reprodução e Fertilidade*. 27: 47-51.

24 **Martinez M.L., Verneque R.S. & Teodoro R.L. 2000.** Correlações entre características da qualidade do sêmen e a circunferência escrotal de reprodutores da raça Gir. *Revista Brasileira de Zootecnia.* 29(3): 700-706.

25 **Maziero R.R.D. 2010.** *Comparação entre diferentes taxas de sêmen na viabilidade espermática em garanhões de alta e baixa congelabilidade*. 73f. Dissertação (Mestrado) - Universidade Estadual Paulista, Faculdade de Medicina Veterinária e Zootecnia de Botucatu.

26 **Medeiros A.S.L., Gomes G.M., Carmo M.T., Papa F.O. & Alvarenga M.A. 2002.** Cryopreservation of stallion sêmen using different amides. *Theriogenology*. 58: 273-276.

27 **Mocé E. & Graham J.K. 2008.** *In vitro* evaluation of sperm quality. *Animal Reproduction Science*. 105(1-2): 104-108.

28 **Nascimento J.N., Blume H., Oliveira F.J.G. & Oliveira R.A. 2015.** Utilização de diferentes diluentes na criopreservação de espermatozoides de garanhões mangalarga marchador. *Ciência Animal Brasileira*. 16(3): 324-330.

29 **Naves C.S., Beletti M.E., Duarte M.B., Vieira R.C., Diniz E.G. & Jacomini J.O. 2004.** Avaliação da cromatina espermática em equinos com azul de toluidine e "acridine orange". *Bioscience Journal*. 20(3): 117-124.

30 **Oliveira G.C., Oliveira B.M.M., Celeghini E.C.C., Fernandes C.B. & Mattos C.B. 2013.** Criopreservação do sêmen equino: uma revisão. *Revista Brasileira de Reprodução Animal.* 37(1): 23-28.

31 **Oliveira M.A.L. & Klug E. 1984.** Congelamento do sêmen equino I: efeito da concentração final de glicerina sobre a motilidade espermática e a atividade enzimática acrossomal. In: *Congresso Brasileiro de Medicina Veterin*ária (Belém, Brasil). p.74.

32 **Papa F.O., Melo C.M., FIoratti E.G., Dell'aqua J.R. J.A., Zahn F.S. & Alvarenga M.A. 2008.** Freezing of stallion epididymal sperm. *Animal Reproduction Science*. 107(3-4): 293-301.

33 **Ponthier J., Franck T., Parrilla-Hernandez S., Niesten A., De la Rebiere G., Serteyn D. & Deleuze S. 2014.** Concentration, activity and biochemical characterization of myeloperoxidase in fresh and post-thaw equine semen and their implication on freezability. *Reproduction in Domestic Animal*. 49(2): 285-291.

34 **Silva K.M.G., Moraes T.A.P., Silva E.C.B., Gamboa S.C. & Gerra M.M.P. 2009.** Efeito de adição de Trolox e pentoxifilina na motilidade, integridade de acrossoma e do DNA de espermatozoides equinos após descongelação. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*. 61(1): 42-49.

35 **Simões R., Feitosa W.B., Siqueira A.F.P., Nichi M., Paula-Lopes F.F., Marques M.G. & Assumpção M.E.O. 2013.** Influence of bovine sperm DNA fragmentation and oxidative stress on early embryo *in vitro* development outcome. *Reproduction*. 146(5): 433-441.

36 **Sinowatz F., Wessa E., Neumüller C. & Palma G. 2003.** On the species specificity of sperm binding and sperm penetration of the zona pellucida. *Reproduction in Domestic Animal*. 38(2): 141-146.

37 **Terraciano P.B., Bustamante-Filho I.C., Miquelito L.D.V., Arlas T.R., Castro F., Mattos R.C. & Lima E.O.C. 2008.** Cryopreservation of equine spermatozoa comparing different freezing rates combined with commercial extenders: laboratorial analysis. *Ciência Rural*. 38(7): 1972-1977.

38 **Vidament M., Dupre A.M., Julienne P., Evain A., Noue P. & Palmer E. 1997.** Equine frozen semen freezability and fertility field results. *Theriogenology*. 48(6): 907-917.

39 **Vidament M., Vincent P., Yvon J.M., Bruneau B. & Mar F.X. 2005.** Glycerol in sêmen extender is a limiting factor in the fertility in asine and equine species. *Animal Reproduction Science*. 89(1-4): 302-305.

40 **Yeste M., Estrada E., Rocha L.G., Marin H., Rodriguez-Gil J.E. & Miro J. 2015.** Cryotolerance of stallion spermatozoa is related to ROS production and mitochondrial membrane potencial rather than to the integrity of sperm nucleos. *Andrology*. 3(2): 395-407.