Spanish Journal of Agricultural Research
16 (4), e0406, 8 pages (2018)
eISSN: 2171-9292
https://doi.org/10.5424/sjar/2018164-13677
Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, O.A, M.P. (INIA)

RESEARCH ARTICLE

OPEN ACCESS

Improvement of cryopreservation protocol in both purebred horses including Spanish horses

Jordi Miró and Marion Papas

Autonomous University of Barcelona, Veterinary Faculty, Equine Reproduction Service, Dept. Animal Medicine and Surgery, 08193 Bellaterra, Spain.

Abstract

There is a widely held belief that the semen of Purebred Spanish Horses (PRE) is of generally poorer quality than that of other breeds, and survives cryopreservation less well. To determine whether this is the case, sperm concentration, viability and morphological abnormalities were examined in a total 610 fresh ejaculates from 64 healthy PRE (N=47) and non-PRE stallions (N=17). Sperm concentration and viability were then re-examined after pre-freezing centrifugation, and once again after freezing-thawing. No differences were observed between the PRE and non-PRE stallions in terms of any sperm quality variable at any observation point. When considering all PRE and non-PRE samples together, differences in sperm viability were observed between fresh and fresh-centrifuged sperm viability (70.1±12.5% compared to 76.3±10.9%; $p<0.01$). After centrifugation the samples were also more homogeneous in terms of the total number of recovered sperm cells. Centrifugation also improved frozen-thawed sperm viability, reducing differences in sperm quality between individual stallions. For all centrifugations, a sperm:extender ratio of 1:5 was used. This would appear to provide better final results than those reported in the literature for the 1:1 ratio commonly used for PRE stallion sperm cryopreservation. In conclusion, obtained results show that the quality and frozen/thawed results of PRE stallion sperm are not lower than that of non-PRE breeds. In addition, using a 1:5 sperm:extender dilution ratio when selecting sperms by centrifugation prior to freezing, seems to provide better results than those usually reported when using a 1:1 ratio.

Additional keywords: equine; semen; centrifugation; freezing process.

Abbreviations used: ANOR (sperm abnormalities); CTR (fresh centrifuged sperm); EFIC CTR (centrifugation efficacy); EJAC (ejaculate); EU (European Union); F (fresh semen); HOR (horse); INJ (injured sperm); PRE (Purebred Spanish Horses); SPZ (spermatozoa); SPZtot (total spermatozoa); VAP (mean progressive velocity); VIAB (sperm viability); VOL (ejaculate volume).

Authors´ contributions: Both co-authors participated in all stages of the work, including the conception and design of the research, the revision of the intellectual content and the drafting of the paper.

Citation: Miró, J.; Papas, M. (2018). Improvement of cryopreservation protocol in both purebred horses including Spanish horses. Spanish Journal of Agricultural Research, Volume 16, Issue 4, e0406. https://doi.org/10.5424/sjar/2018164-13677

Received: 09 Jul 2018. **Accepted:** 05 Dec 2018.

Funding: The authors received no specific funding for this work.

Competing interests: The authors have declared that no competing interests exist.

Correspondence should be addressed to Jordi Miró: Jordi.Miro@uab.cat

Introduction

The storage of stallion sperm is necessary for artificial reproduction technologies such artificial insemination and intracytoplasmic sperm injection. Cryopreservation is currently the only viable method for storing sperm for indefinite periods (Gibb & Aitken, 2016). The Equine Reproduction Service of the Autonomous University of Barcelona, an authorized center for the Production of equine genetic material (ES09RS01E), has a long tradition in the cryopreservation of ejaculates. Though most of the stallions attended to are Purebred Spanish Horses (PRE; a breed with over 500 years of history), the centre deals with many other breeds (non-PRE). The ejaculates of all stallions collected between 2013 and 2016 (inclusive) provided material for the present study.

Among horse breeders and veterinarians there is a widely held belief that the semen of PRE stallions is generally of poorer quality than that of other breeds, and as such survives cryopreservation less well (Benito *et al.*, 2003). However, this belief is anecdotal; the literature contains very little information that might support this. Knowing whether this is true is important since there are 231,003 horses registered in the PRE Stud Book belonging to 31,050 breeders worldwide. Officially PRE horses are found in 65 countries, with

a quarter of all outside Spain (Madeira, 2017), mostly in the Americas (27,638 horses and 2947 breeders) (Vázquez, 2017). Over the last 15 years, the international market for PRE semen has grown strongly.

Unfortunately, in all species, cryopreservation and later thawing negatively affects acrosome integrity, sperm viability and motility, and, as a result, fertility (Bedford *et al.*, 2000; Neild *et al.*, 2003; Brum *et al.*, 2008). Studies on frozen-thawed horse semen during the 1980s recorded *in vivo* fertilization rates for artificial insemination of 17-35% (Cochran *et al.*, 1983; Loomis *et al.*, 1983). Even now, despite the improvement of extenders and cryoprotectants (Loomis *et al.*, 1983; Cochran *et al.*, 1984; Alvarenga *et al.*, 2005; Melo *et al.*, 2007; Roasa *et al.*, 2007; Morillo Rodríguez *et al.*, 2012; Gallardo Bolanos *et al.*, 2014), the quality of frozen-thawed horse sperm is problematic (Alvarenga *et al.*, 2005; Metcalf, 2007; Sieme *et al.*, 2008; Peña *et al.*, 2011; Gibb & Aitken, 2016); its fertilizing capacity remains quite inferior to that of fresh or refrigerated semen (Melo *et al.*, 2007). The problem is not helped by the great individual variation in frozen-thawed sperm quality (Samper *et al.*, 2002; Janett *et al.*, 2003; Metcalf, 2007; Loomis & Graham, 2008; Morrell *et al.*, 2009; Varner *et al.*, 2015).

Sperm selection techniques have been developed to try to improve the fertilizing capacity of both fresh and frozen-thawed horse sperm (Sieme *et al.*, 2003; Varner *et al.*, 2008; Macias Garcia *et al.*, 2009; Morrell *et al.*, 2009, 2010, 2017; Johanisson *et al.*, 2009; Morató *et al.*, 2013). Centrifugation to remove the seminal plasma is the simplest technique (Brinsko *et al.*, 2000; Serres *et al.*, 2002; Rota *et al.*, 2008; Aurich, 2008; Miró *et al.*, 2009), and is commonly used in cryopreservation procedures. The presence of seminal plasma negatively affects sperm conservation, and hinders the freezing of sperm in small volumes (*e.g.*, in 0.5 mL straws). Selection techniques such as wool Sephadex filtration, colloidal centrifugation, and the modified colloid swim-up procedure, are often used with stallion semen prior to freezing (Gutiérrez-Cepeda *et al.*, 2011; Hoogewijs *et al.*, 2011; Hidalgo *et al.*, 2017), specially for 'bad-freezer' stallions. However, in commercial freezing, the semen of 'good-freezer' stallions is simply centrifuged without further ado. For centrifugation, the semen is usually diluted with an extender in the ratio of 1:1 (Dell'Aqua *et al.*, 2001; Sieme *et al.*, 2004). At our facilities, however, the dilution ratio traditionally used has been 1:5 (sperm:extender), the idea being that during centrifugation, fewer collisions between sperm cells will occur, thus reducing the damage they sustain.

The aim of the present study was to compare the quality of PRE and non-PRE sperm subjected to the cryopreservation procedures at our facility to determine whether PRE sperm (fresh, fresh-centrifuged and frozen-thawed) is indeed of generally poorer quality. The results obtained suggest it is not – at any point of observation in the cryopreservation procedure. It may be that diluting sperm 1:5 instead of 1:1 prior to centrifugation assists in maintaining its quality.

Material and methods

Stallions, semen collection and analysis of fresh samples

All the stallions involved in this retrospective analysis were brought to the Equine Reproduction Service of the Autonomous University of Barcelona by their owners for the collection of sperm to be used in artificial insemination. All animals passed the health controls required by the European Union (EU) (equine viral arteritis, equine infectious anemia, and contagious equine metritis), as well as others required for shipping sperm to extra-EU destinations. All animals were in perfect health, both before and during the time they spent at the facility.

A total of 610 ejaculates were obtained from 47 PRE (369 ejaculates) and 17 non-PRE (94 ejaculates) stallions. The first three ejaculates from each were obtained on consecutive days to flush the genital pathway; these ejaculates were discarded. Semen was then obtained every other day as required from each stallion to complete the number of frozen straws required by the owner. Four hundred and sixty-three of these ejaculates were eventually frozen (a mean of 7.8 and 5.5 ejaculates from the PRE and no-PRE stallions respectively). The other 147 were chilled. All ejaculates was obtained using a Hannover artificial vagina (Minitüb, Tiefenbach, Germany) with a nylon filter to remove dirt and separate out the gel fraction.

The volume and appearance (color and density) of each ejaculate was immediately assessed, and a 10 µL aliquot of each gel-free ejaculate was diluted in 990 µL of citrate to determine the sperm concentration using a Neubauer chamber. The remaining gel-free semen was then diluted (1:5 vol/vol) with a skimmed milk extender (Keeney *et al.*, 1975), placed in 50 mL air-free conical tube, and warmed to 37ºC. All samples were stored in a polystyrene box and transported to the laboratory for processing and analysis within 8–10 min. Upon arrival, all were kept in a water bath at 20ºC. Aliquots were then taken to assess sperm viability and morphological abnormalities by eosin-nigrosin staining (Bamba, 1988). Sperm motility was assessed using the CASA computerized system (ISAS®, Valencia, Spain). At least 1000 spermatozoa were counted in

each analysis (performed in triplicate). Total sperm motility was defined as the percentage of spermatozoa that showed a mean progressive velocity (VAP) of >15 μm/s. Progressive sperm motility was defined as the percentage of spermatozoa with a VAP >70 μm/s.

Pre-cryopreservation centrifugation and post-centrifugation sperm analysis

Samples of 1:5 diluted semen were subjected to centrifugation in a Medifriger BL-S centrifuge (JP Selecta, Barcelona, Spain) at 600 × *g* for 15 min at 20ºC. The supernatant was then removed and the remaining pellet resuspended in 2 mL of INRA-Freeze® commercial extender (INRA, Paris, France). Sperm viability and concentration were then re-assessed as for the fresh samples.

Cryopreservation and analysis of frozen-thawed sperm

INRA-Freeze® was added to the above resuspended centrifuged samples to reach a final concentration of 200 × 10^6 viable spermatozoa/mL. These samples were then packaged in 0.5 mL plastic straws using semi-automated equipment (Minitüb, Tiefenbach, Germany), and immediately placed in an Ice-Cube 14S freezer (Minitüb, Tiefenbach, Germany) in which they were subjected to a cooling phase from 20°C to 5°C at a rate of 0.25°C/min for 60 min. This was immediately followed by cooling from 5°C to -90°C at 4.75°C/min for 20 min, and then from -90°C to -120ºC at 11.11ºC/min for 2.70 min. The frozen samples were then stored in liquid nitrogen at -196ºC. The total number of straws frozen was 24,394.

At 24 h post-freezing, one straw per ejaculate was thawed by immersion in a water bath at 37ºC for 30 s. Frozen-thawed sperm viability, concentration and motility were then re-evaluated as above.

Statistical analysis

Normality in the distribution of the means of quantitative variables was checked using the Kolmogorov-Smirnof and Shapiro-Wilk tests. Means for PRE and non-PRE sperm variables were compared using the chi-square test. Since no differences were found between the PRE and non-PRE results at any single observation point, differences between fresh, fresh-centrifuged and frozen-thawed sperm variables for all stallions together were examined using the Wilcoxon test. Spearman correlation coefficients were determined to examine possible relationships between variables. The scatterplot procedure was used for making the graphical representations of these correlations. All calculations were made using the IBM® SPSS® Statistics Program v.20.0 (IBM SPSS Predictive Analytics, Chicago, IL, USA).

Results

Table 1 (rows 1 & 2) shows the means±standard deviations (SD) for PRE and non-PRE fresh, fresh-centrifuged and frozen-thawed sperm. No significant differences were observed between the PRE and non-PRE stallions for any variable at any observation point.

Given the above lack of differences, Table 1 (line 3) compares the means±SD for the sperm variables of all stallions taken together, as measured at the different observation points. A highly significant increase (p<0.01) in sperm viability was seen after centrifugation compared to the fresh samples (76.3±10.9% *vs.*

Table 1. Comparison of sperm quality variables (mean±SD) for fresh, fresh-centrifuged and frozen-thawed semen from PRE and non-PRE stallions and from all the stallions (PRE and non-PRE).

Breed	HOR (n)	EJAC (n)	VOL (mL)	SPZ/mL F (×10^6)	SPZtot F (×10^6)	ANOR (%)	ViaB F (%)
PRE	47	369	51.3±21.1	215.6±112.4	9.164.9±4.081.5	34.6±12.4	69.4±13.5
Non-PRE	17	94	47.7±30.1	244. 8±193.4	8.948.4±5.929.5	34.0±9.0	72.3±9.0
PRE and Non-PRE†	64	463	50.3±23.6	223. 3±137.5	9.107.5±4.593.7	34.5±11.5	70.1±12.5*
Breed	**ViaB CTR (%)**	**SPZtot CTR (×10^6)**	**EFIC CTR (%)**	**SPZ INJ (%)**	**ViaB FT (%)**	**STRAWS (n)**	
PRE	76.5±11.4	8.18.5±3.947.5	89.9±8.1	0.43±3.27	53.7±10.9	20.080	
Non-PRE	75.8±10.1	7.644.0±5.905.2	88.6±7.8	1.48±1.73	53.9±6.3	4.314	
PRE and Non-PRE†	76.3±10.9*	8.036.5±4.504.5	89.6±7.9	0.69±2.99	53.7±9.8	24.394	

HOR: Horse; EJAC: Ejaculate; VOL: Ejaculate volume; SPZ: Spermatozoa; F: Fresh sperm; SPZtot: Total spermatozoa; ANOR: Sperm abnormalities; ViaB: Sperm viability; CTR: Fresh-centrifuged sperm; EFIC: Centrifugation efficacy; INJ: Injured spermatozoa; FT: Frozen-thawed sperm; STRAWS: Obtained straws. No significant differences were found between the PRE and non-PRE sperm variable values at any single observation point (*i.e.*, fresh, fresh-centrifuged or frozen-thawed). †In this row, values with an asterisk are significantly different (p<0.01).

70.01±12.5%). The viability of the frozen-thawed sperm was 53.7% – an excellent result. The percentage of recovered sperm following centrifugation using the 1:5 sperm:extender ratio was 89.6%.

Table 2 shows the Spearman correlation coefficients between sperm variables (taking PRE and non-PRE results together). The viability of frozen-thawed sperm correlated significantly ($p<0.05$) with the total number of spermatozoa in fresh semen, the viability of fresh-centrifuged semen, and the total number of spermatozoa after centrifugation. It also correlated inversely with the percentage of sperm abnormalities in fresh semen.

The fresh sperm concentration significantly influenced the concentration and total number of spermatozoa in the fresh-centrifuged sperm ($p<0.05$), and correlated negatively with the percentage of sperm abnormalities and the number of spermatozoa injured by centrifugation (*i.e.*, the difference between the number of live sperms in fresh and fresh-centrifuged sperm).

The viability of the fresh semen significantly influenced that of the fresh-centrifuged sperm ($p<0.01$). Fresh-centrifuged sperm viability correlated positively with the total number of spermatozoa recovered after centrifugation ($p<0.05$), and negatively with the number of spermatozoa injured by centrifugation. Finally, the percentage of recovered sperm correlated negatively with the number of spermatozoa injured by centrifugation ($p<0.05$).

Figures 1 shows the most important correlations regarding frozen-thawed sperm viability and quality. The percentage of morphological abnormalities in fresh semen correlated negatively with frozen-thawed sperm viability ($p<0.01$). The number of spermatozoa in fresh semen, the number of spermatozoa in fresh-centrifuged semen, fresh sperm viability, and centrifuged sperm viability, all showed positive correlations with frozen-thawed sperm viability ($p<0.01$).

Discussion

The present results show the widely-held belief that the semen of PRE stallions is of poorer quality than that of other breeds (Benito *et al.*, 2003) to be false. No differences were seen in the sperm quality of the present PRE and non-PRE stallions at any point of observation - fresh, fresh-centrifuged or frozen-thawed (Table 1). It may be that some inbred PRE lines produce sperm of lower quality, but the present results reveal that, overall, the sperm of PRE stallions is no worse than that of non-PRE stallions.

When the results were pooled (given the lack of differences between the PRE and non-PRE breeds), a significant increase in sperm viability was recorded after centrifugation compared to fresh semen (Table 1). The percentage of recovered sperm was 89.6% compared to the normally obtained <75% when 1:1 dilutions are used (Dell'Aqua *et al.*, 2001; Sieme *et al.*, 2004). This supports the idea that centrifugation using a 1:5 semen: extender dilution helps improving equine sperm quality for cryopreservation, as previously suggested by Miró *et al.* (2009). Perhaps, increasing the dilution rates the interaction between sperm cells decrease, and as a consequence the sperm damage decrease and the recovery efficacy increase. In addition, the smaller standard

Table 2. Spearman correlation coefficients between frozen-thawed sperm viability and other variables, and between the former and fresh and fresh-centrifuged sperm quality variables.

	ViaB FT	VOL	SPZ/ mL F	SPZtot F	ANOR	ViaB F	ViaB CTR	SPZ/ mL CTR	SPZtot CTR	EFIC CTR	SPZ INJ
VOL	0.14	-									
SPZ/mL F	0.19	-0.42**	-								
SPZtot F	0.35**	0.46**	0.54**	-							
ANOR	-0.46**	-0.10	-0.23	-0.34**	-						
ViaB F	0.47**	0.03	0.06	0.07	-0.45**	-					
ViaB CTR	0.58**	0.22	0.05	0.29*	-0.43**	0.62**	-				
SPZ/mL CTR	0.05	-0.16	0.66**	0.47**	-0.09	-0.02	0.07	-			
SPZtot CTR	0.34**	0.38**	0.48**	0.85**	-0.36**	0.04	0.28*	0.60**	-		
EFIC CTR	-0.17	0.00	-0.12	-0.25	0.14	-0.15	-0.05	0.03	-0.17	-	
SPZ INJ	-0.20	-0.15	-0.11	-0.28*	0.14	0.14	-0.41**	-0.00	-0.19	-0.29*	-

ViaB: viability; FT: frozen-thawed sperm; VOL: ejaculate volume; SPZ: spermatozoa; F: fresh sperm; SPZtot: total spermatozoa; ANOR: sperm abnormalities; ViaB F: fresh sperm viability; CTR: fresh-centrifuged sperm; EFIC: centrifugation efficacy; SPZ INJ: injured spermatozoa by centrifugation. * $p<0.05$, ** $p<0.01$. Values with grey backgound show the variables significantly correlated with frozen-thawed sperm viability.

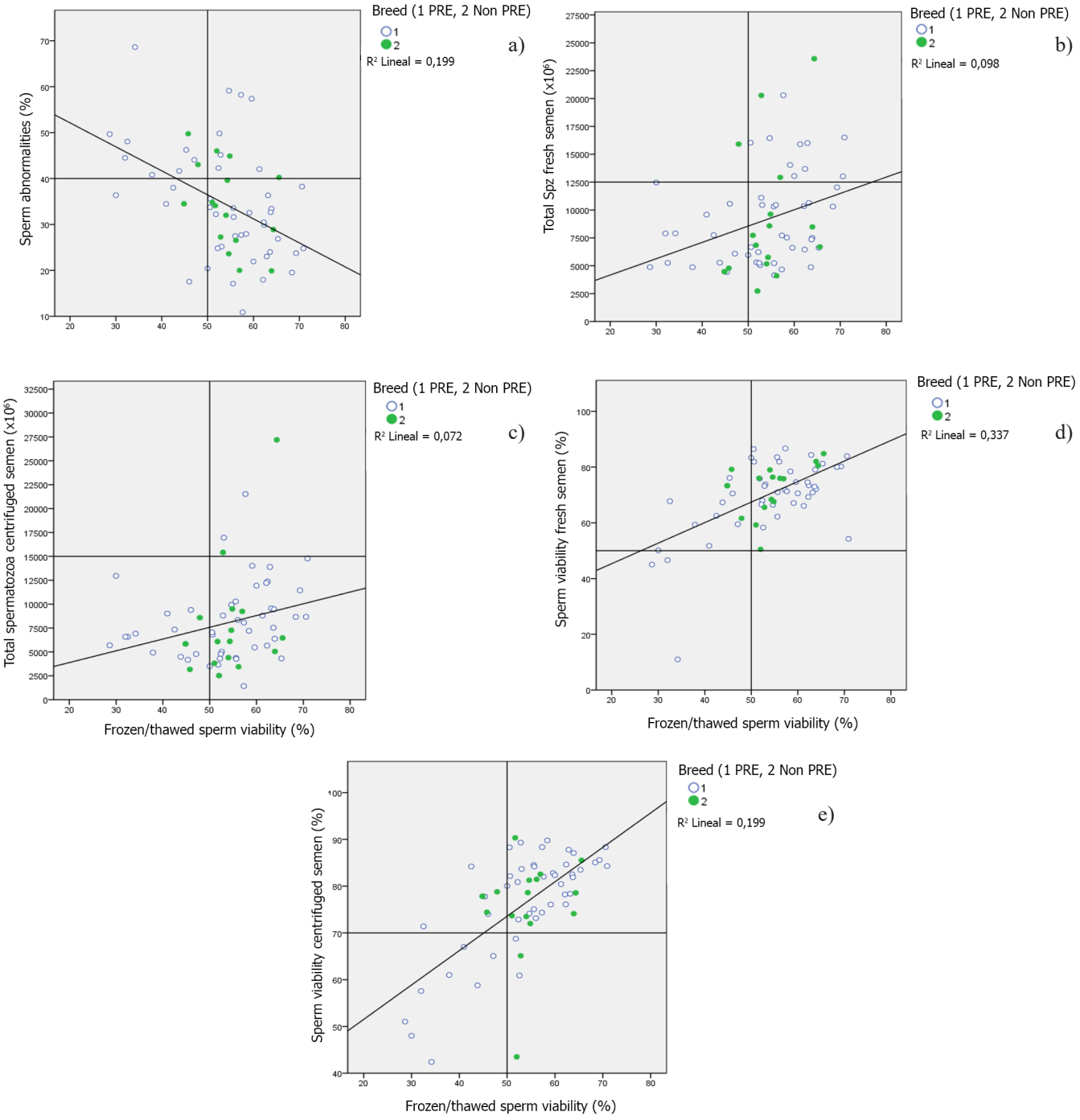

Figure 1. Correlation between frozen-thawed sperm viability and (a) the percentage of sperm abnormalities in fresh semen (R=0.46, p<0.01); (b) the fresh semen total sperm number (R=0.35, p<0.01); (c) the total number of sperm cells in fresh-centrifuged semen (R=0.34, p<0.01); (d) the fresh sperm viability (R=0.47, p<0.01); and (e) the fresh-centrifuged sperm viability (R=0.58, p<0.01).

deviation of the mean for fresh-centrifuged sperm viability compared to that of the fresh sperm (Table 1), suggests this dilution may reduce the differences between individuals.

Table 2 shows that the main fresh and fresh-centrifuged sperm quality variables correlate well with frozen-thawed sperm viability. The total number of sperm cells in fresh semen and the percentage of viable sperms in frozen-thawed sperm also showed a significant correlation. A negative correlation was detected between the percentage of fresh sperm with morphological abnormalities and frozen-thawed sperm viability (Table 2 and Fig. 1a).

Optimizing the quality and fertilizing capacity of frozen-thawed horse semen is of great importance. Both aspects are generally much lower than those of fresh sperm, however there is great individual variation among stallions (Janett *et al.*, 2003, 2012; Metcalf, 2007; Aurich, 2008) – note the large standard deviations associated with the present results for both the PRE and non-PRE-breeds (Table 1). The correlation between the total number of sperm cells in the fresh-centrifuged samples and the percentage viable sperms in the frozen-thawed samples (Fig. 1c) shows, however, that after the centrifugation, the samples became more homogeneous, *i.e.*, the variation in samples from different horses was reduced.

In fresh semen, the crude range of sperm viability was 50-70%, correlating well with the 50-70% for frozen-thawed sperm (Fig. 1d). A post-centrifugation sperm viability of 70-90% is required before cryopreservation (Fig. 1e) if a sperm viability of 50-70% for frozen-thawed semen is to be achieved. Indeed, centrifugation increased fresh sperm viability by about 20% (Fig. 1e).

In conclusion, the present results suggest that the quality of PRE stallion sperm is no lower than that of non-PRE breeds. In addition, using a 1:5 sperm:extender dilution ratio when selecting sperms by centrifugation prior to freezing, seems to provide better results than those usually reported when using a 1:1 ratio. These results seem interesting for breeders, veterinarians and owners of PRE horses.

References

Alvarenga MA, Papa FO, Landim-Alvarenga FC, Medeiros AS, 2005. Amides as cryoprotectants for freezing stallion semen: a review. Anim Reprod Sci 89: 105-113. https://doi.org/10.1016/j.anireprosci.2005.07.001

Aurich C, 2008. Recent advances in cooled-semen technology. Anim Reprod Sci 107: 268-275. https://doi.org/10.1016/j.anireprosci.2008.04.015

Bamba K, 1988. Evaluation of acrosomal integrity of boar spermatozoa by bright field microscopy using an eosin-nigrosin stain. Theriogenology 29: 1245-1251. https://doi.org/10.1016/0093-691X(88)90004-0

Bedford SJ, Varner DD, Meyers SA, 2000. Effects of cryopreservation on the acrosomal status of stallion spermatozoa. J Reprod Fertil Suppl 56: 133-140.

Benito D, Alvarez A, Crespo F, Mateos E, Gómez-Cuétara C, Serres C, 2003. Análisis computerizado del semen de caballo de Pura Raza Española. IV Congreso Ibérico de Reproducción Animal, Las Palmas (España), Jul 11-12. p: 90.

Brinsko SP, Crockett EC, Squires EL, 2000. Effect of centrifugation and partial removal of seminal plasma on equine spermatozoal motility after cooling and storage. Theriogenology 54: 129-136. https://doi.org/10.1016/S0093-691X(00)00331-9

Brum AM, Sabeur K, Ball BA, 2008. Apoptotic-like changes in equine spermatozoa separated by density-gradient centrifugation or after cryopreservation. Theriogenology 69: 1041-55. https://doi.org/10.1016/j.theriogenology.2008.01.014

Cochran JD, Amann RP, Squires EL, Pickett BW, 1983. Fertility of frozen-thawed stallion semen extended in lactose-EDTA-egg yolk extender and packaged in 1.0-ml straws. Theriogenology 20: 735-741. https://doi.org/10.1016/0093-691X(83)90194-2

Cochran JD, Amann RP, Froman DP, Pickett BW, 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: 25-38. https://doi.org/10.1016/0093-691X(84)90470-9

Dell'Aqua JA, Papa FO, Alvarenga MA, Zahn FS, 2001. Effect of centrifugation and packing system on sperm parameters of equine frozen semen. Anim Reprod Sci 68: 324-325.

Gallardo Bolanos JM, Bolao da Silva CM, Martín Muñoz P, Morillo Rodríguez A, Plaza Dávila M, Rodríguez-Martínez H, Aparicio IM, Tapia JA, Ortega Ferrusola C, Peña FJ. 2014. Phosphorylated AKT preserves stallion sperm viability and motility by inhibiting caspases 3 and 7. Reproduction 148: 221-235. https://doi.org/10.1530/REP-13-0191

Gibb Z, Aitken RJ, 2016. Recent developments in stallion semen preservation. J Equine Vet Sci 43: 29-36. https://doi.org/10.1016/j.jevs.2016.06.006

Gutiérrez-Cepeda L, Fernández A, Crespo F, Gosálvez J, Serres C, 2011. Simple and economic coloidal centrifugation protocols may be incorporated into the clinical equine sperm processing procedure. Anim Reprod Sci 124: 85-89. https://doi.org/10.1016/j.anireprosci.2011.02.001

Hidalgo M, Ortiz I, Dorado J, Morrell JM, Gosálvez J, Consuegra C, Díaz-Jiménez M, Pereira B, Crespo F, 2017. Stallion sperm selection prior to freezing using a modified colloid swim-up procedure without centrifugation. Anim Reprod Sci 185: 83-88. https://doi.org/10.1016/j.anireprosci.2017.08.005

Hoogewijs M, Morrell J, Van Soom A, Govaere J, Johannisson A, Piepers S, Schauwer C, De Kruif A, De Vliegher S, 2011. Sperm selection using single layer centrifugation prior to cryopreservation can increase thawed sperm quality in stallions. Equine Vet J 43 (Supp 40): 35-41. https://doi.org/10.1111/j.2042-3306.2011.00489.x

Janett F, Thun R, Bettschen S, Burger D, Hassig M, 2003. Seasonal changes of semen quality and freezability in Franches–Montagnes stallions. Anim Reprod Sci 77: 213-221. https://doi.org/10.1016/S0378-4320(03)00039-3

Janett F, Sacher K, Hassig M, Thun R, 2012. Quality of raw and of cold-stored semen in Icelandic stallions. J Equine Vet Sci 32: 590-595. https://doi.org/10.1016/j.jevs.2012.02.004

Johanisson A, Morrell JM, Thoren J, Jönsson M, Dalin A, Rodríguez-Martínez H, 2009. Colloidal centrifugation with Andocoll-ETM prolongs stallion sperm motility, viability and chromatin integrity. Anim Reprod Sci 116: 119-128. https://doi.org/10.1016/j.anireprosci.2009.01.008

Keeney RM, Bergman RV, Cooper WL, Morse GW, 1975. Minimal contamination techniques for breeding mares: technique and preliminary finding. Am Assoc Equine Pract, 327-336.

Loomis PR, Amann RP, Squires EL, Pickett BW, 1983. Fertility of stallion spermatozoa frozen in an EDTA-lactose-egg-yolk extender and packaged in 0.5-mL straws. J Anim Sci 56: 687-693. https://doi.org/10.2527/jas1983.563687x

Loomis PR, Graham JK, 2008. Commercial semen freezing: individual male variation in cryosurvival and the response of stallion sperm to customized freezing protocols. Anim Reprod Sci 105: 119-128. https://doi.org/10.1016/j.anireprosci.2007.11.010

Macias Garcia B., Morrell JM, Ortega-Ferrusola C, Gonzalez-Fernandez L, Tapia JA, Rodriguez-Martinez H, Pena FJ, 2009. Centrifugation on a single layer of colloid selects improved quality spermatozoa from frozen-thawed stallion semen. Anim Reprod Sci 114: 193-202. https://doi.org/10.1016/j.anireprosci.2008.08.025

Madeira P, 2017. El Pura Raza Española, el valor de marca en el mundo. El caballo español PRE 237: 34-38.

Melo CM, Zahn FS, Martin I, Orlandi C, Dell'Aqua Jr, JA, Alvarenga MA, Papa FO, 2007. Influence of semen storage and cryoprotectant on post-thaw viability and fertility of stallion spermatozoa. J Equine Vet Sci 27: 171-175. https://doi.org/10.1016/j.jevs.2007.02.008

Metcalf ES, 2007. The efficient use of equine cryopreserved semen. Theriogenology 68: 423-428. https://doi.org/10.1016/j.theriogenology.2007.04.039

Miró J, Taberner E, Rivera M, Peña A, Medrano A, Rigau T, Peñalba A, 2009. Effects of dilution and centrifugation on the survival of spermatozoa and the structure of motile sperm cell subpopulations in refrigerated Catalonian donkey semen. Theriogenology 72: 1017-1022. https://doi.org/10.1016/j.theriogenology.2009.06.012

Morató R, Souza-Soares J, Orero G, Mogas T, Miró J, 2013. Pre-selection by double layer density gradient centrifugation improves the fertilising capacity of frozen-thawed, capacitated stallion. Anim Reprod Sci 139: 62-68. https://doi.org/10.1016/j.anireprosci.2013.04.005

Morillo Rodríguez A, Balao da Silva C, Macías-García B, Gallardo Bolanos JM, Tapia JA, Aparicio IM, Ortega-Ferrusola C, Peña FJ, 2012. Dimethylformamide improves the in vitro characteristics of thawed stallion spermatozoa reducing sublethal damage. Reprod Domest Anim 47: 995-1002. https://doi.org/10.1111/j.1439-0531.2012.02005.x

Morrell JM, Johannisson A, Strutz H, Dalin A, Rodriguez-Martinez H, 2009. Colloidal centrifugation of stallion semen: Changes in sperm motility, velocity, and chromatin integrity during storage. J Equine Vet Sci 29: 24-32. https://doi.org/10.1016/j.jevs.2008.11.006

Morrell JM, Rodríguez-Martínez H, Johanisson A, 2010. Single layer centrifugation of stallion spermatozoa consistently selects the most robust spermatozoa from the rest of the ejaculate in a large simple size. Equine Vet J 42: 579-585. https://doi.org/10.1111/j.2042-3306.2010.00101.x

Morrell JM, Lagerqvist A, Humblot P, Johanisson A, 2017. Effect of single layer centrifugation on reactive oxygen species and sperm mitochondrial membrane potential in cooled stallion semen. Reprod Fertil Dev 29: 1039-1045. https://doi.org/10.1071/RD15440

Neild DM, Gadella BM, Chaves MG, Miragaya MH, Colembrander B, Agüero A, 2003. Membrane changes during different stages of a freeze-thaw protocol for equine semen cryopreservation. Theriogenology 59: 1693-1705. https://doi.org/10.1016/S0093-691X(02)01231-1

Peña FJ. García BM, Samper JC, Aparicio IM, Tapia JA, Ferrusola CO, 2011. Dissecting the molecular damage to stallion spermatozoa: the way to improve current cryopreservation protocols? Theriogenology 76: 1177-1186. https://doi.org/10.1016/j.theriogenology.2011.06.023

Roasa LM, Choi YH, Love CC, Romo S, Varner DD, Hinrichs K, 2007. Ejaculate and type of freezing extender affect rates of fertilization of horse oocytes in vitro. Theriogenolgy 68: 560-566. https://doi.org/10.1016/j.theriogenology.2007.04.057

Rota A, Magelli C, Panzani D, Camilo F, 2008 Effect of extender, centrifugation and removal of seminal plasma on cooled-preserved Amiata donkey spermatozoa. 69: 176-185.

Samper JC, Vidament M, Katila T, Newcombe J, Estrada A, Sargeant J, 2002. Analysis of some factors associated with pregnancy rates of frozen semen: a multi-center study. Theriogenology 58: 647-650. https://doi.org/10.1016/S0093-691X(02)00754-9

Serres C, Rodríguez A, Alvarez AL, Santiago I, Gabriel J, Gómez-Cuétara C, Mateos E, 2002. Effect of centrifugation and temperature on the motility and the plasma membrane integrity of Zamorano-Leonés donkey semen. Theriogenology 58: 329-332. https://doi.org/10.1016/S0093-691X(02)00876-2

Sieme H, Martinsson G, Rauterberg H, Walter K, Aurich C, Petzoldt R, Klug E, 2003. Application of techniques for sperm selection in fresh and frozen-thawed stallion semen. Reprod Domest Anim 38: 134-140. https://doi.org/10.1046/j.1439-0531.2003.00416.x

Sieme H, Katila T, Klug E, 2004. Effect of semen collection practices on sperm characteristics before and after storage and on fertility of stallions. Theriogenology 61: 769-784. https://doi.org/10.1016/S0093-691X(03)00251-6

Sieme H, Harrison RAP, Petrunkina AM, 2008. Cryobiological determinants of frozen semen quality, with special reference to stallion. Anim Reprod Sci 107: 276-292. https://doi.org/10.1016/j.anireprosci.2008.05.001

Varner DD, Love CC, Brinsko SP, Blanchard TL, Hartman DL, Bliss SB, Carroll BS, Eslick MC, 2008. Semen processing for the subfertile stallion. J Equine Vet Sci 28: 677-685. https://doi.org/10.1016/j.jevs.2008.10.012

Varner DD, Gibb Z, Aitken RJ, 2015. Stallion fertility: a focus on the spermatozoon. Equine Vet J 47: 16-24. https://doi.org/10.1111/evj.12308

Vázquez E, 2017. 525 años del Pura Raza Española en América. El caballo español PRE 238: 42-46.