Improvement of cryopreservation protocol in both purebred horses including Spanish horses

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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.

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 Rodriguez 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; Johannisson 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 (Minüt, 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
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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 20°C. 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), and immediately 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 -120°C at 11.11°C/min for 2.70 min. The frozen samples were then stored in liquid nitrogen at -196°C. 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 37°C 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-Smirnov 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 \((76.3±10.9\% \ vs. \ 53.7±10.9\%)\).

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 \(^1\) | 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 \(^1\) | 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 \((i.e.,\ fresh,\ fresh-centrifuged\ or\ frozen-thawed)\). \(^1\)In this row, values with an asterisk are significantly different \((p<0.01)\).
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.

| Variable | 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.
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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.

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