Vitrification of stallion sperm using 0.25 ml straws: Effect of volume, concentration and carbohydrates (sucrose/trehalose/raffinose)

C Consuegra[a], F Crespo[b], J Dorado[a], M Diaz-Jimenez[a], B Pereira[a], I Ortiz[a], M Hidalgo[a],*

[a] Veterinary Reproduction Group, Department of Medicine and Animal Surgery, Faculty of Veterinary Medicine, University of Cordoba, Cordoba, Spain
[b] Department of Reproduction, Centro Militar de Cría Caballar (CCFAS-Ministry of Defense), Ávila, Spain

ARTICLE INFO

ABSTRACT

Sperm vitrification is a rapid freezing method in which carbohydrates are used as cryoprotectants. The aim of this study was to determine the optimal volume, concentration and type of carbohydrates for stallion sperm vitrification using 0.25 ml straws in comparison to conventional freezing. Ejaculates ($n = 54$) were collected from six stallions. For vitrification, straws were filled with different volumes (30, 70, 100 μl), sperm concentrations (50, 100, 200×10^6 sperm/ml) and extenders containing sucrose (20, 100, 200 mM), trehalose (50, 100, 200 mM) and raffinose (50, 100, 200 mM) and plunged into LN_2. Conventional freezing was performed in 0.5 ml straws frozen in LN_2 vapors. Sperm motility, plasma and acrosome membrane integrities and DNA fragmentation were compared among treatments. The use of straws filled with 100 μl at 100×10^6 sperm/ml with the extender containing 100 mM trehalose resulted in greater values for sperm quality than the other concentrations, volumes and carbohydrates. With vitrification, there were greater values (mean ± SEM; $P < 0.05$) than freezing for progressive motility (48.2 ± 2.3 compared with 37.3 ± 2.2%), plasma membrane integrity (82.8 ± 1.5 compared with 74.1 ± 1.9%), and intact acrosomes (50.2 ± 1.2 compared with 43.1 ± 1.4%); and less DNA fragmentation (6.4 ± 0.7 compared with 8.2 ± 0.3%). In conclusion, stallion sperm can be vitrified in 0.25 ml straws filled with 100 μl of sperm at 100 x 10^6 sperm/ml using an extender with 100 mM of trehalose, obtaining better sperm quality after warming than conventional freezing.

1. Introduction

Cryopreservation is used in the horse industry for long-term storage of stallion sperm. In general, slow cooling rates and extenders containing permeable cryoprotectants are used for storage of stallion semen. This procedure results in an osmotic imbalance for sperm because of the use of these processes as well as from the addition of cryoprotectants (Peña et al., 2011). Sperm vitrification is an alternative method of cryopreservation, performed by plunging small volumes of semen directly into liquid nitrogen (LN_2), without using any permeable cryoprotectant. Nevertheless, carbohydrates and proteins are added as non-permeable agents

(Isachenko et al., 2017).

There are important factors for vitrification success, such as the semen volume, vitrification media or the equilibration period (Arav et al., 2002; Hidalgo et al., 2018). In previous studies of different species, including stallions, vitrification has been conducted with success using the spheres method (Isachenko et al., 2008; Sanchez et al., 2011; Pradiee et al., 2015; Hidalgo et al., 2018). This, however, is a non-aseptic technique, where a smaller volume of semen is utilized, and as a result, compromises the procedures used for artificial insemination of mares (Govaere et al., 2014). The straw method has been used as an alternative to this technique. This procedure provides for the opportunity to use a larger volume and concentration of sperm as well as for isolating sperm from LN_2, avoiding the risk of cross-contamination (Isachenko et al., 2017). The optimal volume and sperm concentration for stallion sperm vitrification using straws have yet to be determined. The addition of carbohydrates to the extender results in a greater viscosity of the solution that suppresses ice crystal formation, therefore, enhancing the vitrification process (Arav et al., 2002). For this purpose, sucrose has been the carbohydrate most commonly used for sperm vitrification in different species (Isachenko et al., 2008; Sanchez et al., 2011; Bóveda et al., 2018). Recently, other carbohydrates, such as trehalose and raffinose, have been also evaluated for sperm vitrification (Horta et al., 2017; Schulz et al., 2017; Caturla-Sánchez et al., 2018). The optimal concentration of each sugar appears to be species-specific. In this regard, Iberian ibex sperm (Pradiee et al., 2015) appear to be more sensitive to relatively greater concentration of carbohydrates than dog (Caturla-Sánchez et al., 2018) or donkey (Diaz-Jimenez et al., 2017, 2019) sperm. This fact is particularly relevant when considering cryopreservation of stallion sperm, which have previously been found to have poor resistance to the osmotic stress when there is greater molarity of carbohydrates, not only for conventional freezing (Consuegra et al., 2018a) but also for vitrification (Hidalgo et al., 2018).

The aim of the present study is to evaluate the effect of sperm concentration and volume as well as use of different molarities of sucrose, trehalose and raffinose, for stallion sperm vitrification in 0.25 ml straws as an alternative to conventional freezing.

2. Materials and methods

This study was approved by the Ethical Committee for Animal Experimentation of the University of Cordoba, in compliance with the Regional Government of Andalusia (project no. 31/08/2017/105) and the Spanish law for animal welfare and experimentation (RD 53/2013).

All chemicals used were purchased from Sigma Aldrich (Sigma-Aldrich, USA) unless stated otherwise. The basic medium used for sperm processing was a commercial milk fraction-based extender (INRA96, IMV Technologies, France) added to bovine serum albumin (BSA) and with inclusion of different concentrations of carbohydrates for sperm vitrification.

2.1. Animals

Six stallions of different breeds (five Purebred Spanish Horse and one Hispano-Arab) aged from 10 to 23 years, clinically healthy and with known fertility (> 60%) were used as semen donors. Animals were housed in individual paddocks placed at the Equine Breeding Centre of the Spanish Army located in Avila, Spain (40.66 °N, 4.70 °W).

2.2. Semen collection and processing

Semen samples were obtained using a Missouri-model artificial vagina in the presence of a mare in estrus. Semen was collected during the breeding season once or twice a week with nine ejaculates being collected from each stallion (n = 54). In each experiment, three ejaculates per animal were used (n = 18). Sperm quality of ejaculates was evaluated before freezing and had physiological values (mean ± SEM) for: gel-free volume (49.5 ± 2.9 ml), measured in a graduated collector, sperm concentration (269.2 ± 10.8 × 10^6 sperm/ml), assessed with a sperm photometer (Spermacue, Minitube, Germany) and total sperm motility (95.8 ± 0.5%), progressive sperm motility (78.6 ± 0.8%) and plasma membrane integrity (93.5 ± 0.6%), evaluated as subsequently described in this manuscript. Fresh semen was diluted with the milk-based extender at 37°C (1:1/v:v) and centrifuged for 10 min at 600 x *g*. The sperm pellets were re-suspended in different extenders for sperm vitrification or conventional freezing procedures (see experimental design). Osmolalities of all extenders were assessed using a freezing-point digital micro osmometer Type 6 (Löser Messtechnik, Germany).

2.3. Vitrification and warming of sperm

Sperm vitrification was conducted in covered 0.25 ml straws (Consuegra et al., 2018b). The semen samples diluted in the vitrification media were cooled for 1 h at 5°C. After cooling, 0.25 ml French straws were filled with the semen samples using a micropipette, and horizontally inserted in 0.5 ml straws. Subsequently, both ends of the external straw were heat-sealed and horizontally immersed in a styrofoam box containing liquid nitrogen (LN_2). Post-warming analysis was performed after 24 h of storage in LN_2 containers. For warming, 0.25 ml straw was removed from the covering straw and immersed in 3 ml of INRA96 pre-warmed to 43°C with gentle pipetting until the vitrified solution was liquid. The post-thaw sperm suspension was centrifuged (600 x *g* for 10 min) and the sperm pellet was re-suspended in milk-based extender to a final concentration of 25 × 10^6 sperm/ml for sperm evaluation.

2.4. Conventional sperm freezing and thawing

Semen samples were frozen using a standard protocol for stallions with modifications (Consuegra et al., 2018a). Briefly, centrifuged sperm pellets were re-suspended in a freezing extender containing 4% methylformamide and 1% glycerol (Botucrio, Nidacon, Sweden; osmolality = 1162 mOsm/kg) (Macedo et al., 2018), to a final concentration of 50×10^6 sperm/ml (Consuegra et al., 2018a). After that, samples were cooled to 5°C within 2 h, samples were then loaded into 0.5 ml straws (Ortiz et al., 2014). The straws were placed horizontally for freezing in racks that were positioned 4 cm above the surface of LN_2 for 10 min and plunged in LN_2, and subsequently then stored in LN_2 tanks for at least 24 h before further assessment occurred. Straws were thawed using the immersion procedure in a 37°C water bath for 30 s and re-suspended with the basic medium for semen analysis (INRA96).

2.5. Post-thawing/warmed sperm evaluation

Sperm motility was objectively evaluated by CASA system (SCA v5.01, Microptic S.L., Spain). The features of this system have been described previously (Ortiz et al., 2014). The sperm kinematic variables recorded were: total (TM, %) and progressive motility (PM, %), curvilinear (VCL, μm/s), straight line (VSL, μm/s) and average path velocities (VAP, μm/s), linearity (LIN, VSL/VCLx100), straightness (STR, VSL/VAPx100), wobble (WOB, VAP/VCLx100), amplitude of lateral head displacement (ALH, μm) and beat cross frequency (BCF, Hz). Sperm motility was considered progressive when VCL > 90 μm/s and STR > 75%.

The integrity of plasma membrane was assessed utilizing the Vital test (Halotech SL, Spain) using fluorescence microscopy as previously described (Cortés-Gutiérrez et al., 2008). The percentage of sperm with intact plasma membrane was recorded (IMS, %). Acrosome integrity was evaluated using the propidium iodide/peanut agglutinin–fluorescein isothiocyanate double stain (Dorado et al., 2014). At least 200 sperm were evaluated on each slide. Percentages of sperm with an intact acrosome membrane (AIS, %) were recorded.

Sperm DNA integrity was assessed using the Sperm-Halomax kit (Halotech SL, Madrid, Spain) for stallion sperm (López-Fernández et al., 2007) and a commercial DNA stain using fluorescence microscopy (Fluogreen, Halotech SL, Madrid, Spain) following the manufacturer's instructions. At least 200 sperm per sample were evaluated. Sperm with a large halo of chromatin dispersion were recorded as sperm with fragmented DNA (sDF, %). Sperm DNA fragmentation was assessed after 0, 4, 8 and 24 h of incubation at 37°C after thawing/warming.

2.6. Experimental design

2.6.1. Experiment 1. Evaluation of different volumes and concentrations for sperm vitrification using straws

After semen processing, sperm were extended in a milk-based extender adding sucrose at 20 mM (mmol/l) plus 1% BSA (314 mOsm/kg) with the result being three different sperm concentrations: 50, 100 and 200 (x10^6 sperm/ml). Subsequently, the 0.25 ml straws covered with 0.5 ml straws were filled with sperm at each concentration but using different volumes: 30, 70 and 100 μl. Both ends of 0.5 ml straws were subsequently heat-sealed as previously described. The sperm quality variables were assessed following warming and comparisons were made among values obtained using different volumes and concentrations.

2.6.2. Experiment 2. Comparison among sucrose, trehalose and raffinose for sperm vitrification in straws

The results in Experiment 1 from assessing the combination of semen volume and sperm concentration resulting in the greatest values for sperm quality variables were used to determine the procedures performed in Experiment 2. These determinations resulted in using straws filled with 100 μl at 100×10^6 sperm/ml in subsequent experiments. Semen samples were diluted in the base extender with addition of three different carbohydrates occurring at three different molarities as follows: sucrose 20 mM (S1; 314 mOsm/kg) (Hidalgo et al., 2018), 100 mM (S2; 454 mOsm/kg) and 200 mM (S3; 591 mOsm/kg); trehalose 50 mM (T1; 394 mOsm/kg) (Schulz et al., 2017), 100 mM (T2; 465 mOsm/kg) and 200 mM (T3; 587 mOsm/kg) of; and raffinose 50 mM (R1; 382 mOsm/kg), 100 mM (R2; 449 mOsm/kg) and 200 mM (R3; 552 mOsm/kg). The values for post-warming sperm variables were assessed and compared among treatments.

2.6.3. Experiment 3. Evaluation of sperm vitrification in straws in comparison to conventional freezing

The vitrification treatment resulting in the greatest sperm quality in Experiment 2 (T2 = 100 Mm trehalose) was selected and compared with those obtained with conventional freezing. Semen samples were divided into two aliquots: one was frozen using the conventional freezing protocol; the other was vitrified using the extender with 100 mM of trehalose to a final concentration of 100×10^6 sperm/ml, filling the 0.25 ml straws with 100 μl of the sperm suspension and then covered with the 0.5 ml straw. Subsequently, the values for post-thawing sperm variables were assessed and compared among treatments.

2.7. Statistical analysis

An analysis of the data was conducted using SAS v9.0 (SAS Institute Inc., NC, USA). A general linear model (PROC GLM) and the Duncan test with animals and ejaculates as random factors, were performed for the analysis. For each sperm variable, normality and homogeneity of variances were assessed using the Kolmogorov-Smirnov and Levene test, respectively. A linear regression analysis was conducted for values of DNA fragmentation at different incubation times and the slopes (sDF%/hour) were compared among treatments using the GraphPad Prism v.6 for Mac OS (GraphPad Software, CA, USA). Results were expressed as mean ± standard error of the mean (SEM). The level of significance was set at $P < 0.05$.

Table 1
Comparison of different volumes and concentrations (m = million) for stallion sperm vitrification in 0.25 ml straws.

Variables	Sperm vitrification procedures									P-value
	30 µl/50 m	70 µl/50 m	100 µl/50 m	30 µl/100 m	70 µl/100 m	100 µl/100 m	30 µl/200 m	70 µl/200 m	100 µl/200 m	
TM (%)	31.6 ± 2.6[c]	41.2 ± 3.9[abc]	47.1 ± 4.0[ab]	40.0 ± 4.2[abc]	40.5 ± 3.9[abc]	49.8 ± 3.4[a]	36.0 ± 3.5[bc]	40.5 ± 3.2[abc]	47.2 ± 4.2[ab]	< 0.05
PM (%)	17.9 ± 2.9[c]	24.2 ± 3.1[abc]	27.6 ± 3.4[ab]	19.3 ± 2.5[bc]	22.4 ± 2.8[abc]	31.1 ± 2.7[a]	17.8 ± 1.9[c]	24.4 ± 2.3[abc]	29.4 ± 3.4[a]	< 0.01
IMS (%)	63.0 ± 2.8[a]	67.0 ± 2.4[a]	70.8 ± 2.6[a]	64.5 ± 2.9[a]	65.6 ± 2.4[a]	66.5 ± 2.4[a]	66.1 ± 2.2[a]	67.2 ± 2.2[a]	68.1 ± 2.4[a]	> 0.05
AIS (%)	39.2 ± 3.3[a]	46.2 ± 2.5[a]	48.7 ± 2.9[a]	44.9 ± 2.1[a]	44.3 ± 2.8[a]	49.2 ± 2.5[a]	44.4 ± 2.5[a]	48.5 ± 2.8[a]	49.7 ± 2.7[a]	> 0.05
VCL (µm/s)	45.9 ± 2.6[b]	52.1 ± 4.8[ab]	53.3 ± 2.9[ab]	46.8 ± 1.7[b]	48.9 ± 2.9[b]	59.6 ± 3.4[a]	47.6 ± 1.7[b]	55.3 ± 3.2[ab]	52.9 ± 1.9[ab]	< 0.05
VSL (µm/s)	31.2 ± 1.7[c]	35.5 ± 2.3[abc]	39.3 ± 2.0[ab]	33.1 ± 1.4[bc]	36.1 ± 2.6[abc]	41.9 ± 2.3[a]	33.6 ± 1.4[bc]	39.1 ± 2.4[ab]	38.4 ± 2.1[ab]	< 0.01
VAP (µm/s)	35.0 ± 2.2[c]	40.1 ± 2.9[abc]	43.6 ± 2.4[ab]	37.3 ± 1.5[bc]	39.3 ± 2.7[abc]	46.6 ± 2.6[a]	37.3 ± 1.4[bc]	43.4 ± 2.8[ab]	42.0 ± 2.1[abc]	< 0.05
LIN (%)	68.7 ± 2.4[a]	71.1 ± 3.2[a]	74.1 ± 1.7[a]	71.0 ± 2.2[a]	68.9 ± 4.4[a]	70.4 ± 2.1[a]	70.7 ± 1.9[a]	70.5 ± 2.1[a]	71.7 ± 2.0[a]	> 0.05
STR (%)	89.8 ± 1.4[a]	89.2 ± 1.9[a]	90.3 ± 0.7[a]	88.9 ± 1.8[a]	90.6 ± 1.1[a]	89.7 ± 0.8[a]	90.1 ± 1.6[a]	90.0 ± 1.2[a]	90.8 ± 0.8[a]	> 0.05
WOB (%)	76.2 ± 2.0[a]	79.1 ± 2.6[a]	82.0 ± 1.6[a]	79.6 ± 1.4[a]	80.5 ± 1.9[a]	78.4 ± 1.9[a]	78.5 ± 1.4[a]	78.2 ± 1.8[a]	78.8 ± 1.8[a]	> 0.05
ALH (µm)	2.3 ± 0.1[a]	2.4 ± 0.1[a]	2.4 ± 0.1[a]	2.5 ± 0.1[a]	2.4 ± 0.1[a]	2.6 ± 0.1[a]	2.5 ± 0.1[a]	2.6 ± 0.1[a]	2.7 ± 0.1[a]	> 0.05
BCF (Hz)	7.0 ± 0.5[a]	7.2 ± 0.5[a]	6.6 ± 0.3[a]	6.9 ± 0.2[a]	6.8 ± 0.3[a]	7.3 ± 0.5[a]	7.2 ± 0.5[a]	7.3 ± 0.3[a]	7.0 ± 0.3[a]	> 0.05

TM = total motility; PM = progressive motility; IMS = plasma membrane integrity; AIS = acrosome-intact sperm; VCL = curvilinear velocity; VSL = linear velocity; VAP = average path velocity; LIN = linear coefficient; STR = straightness coefficient; WOB = Wobble coefficient; ALH = lateral head displacement; BCF = beat cross frequency. Different superscripts (a, b, c) indicate significant differences (P < 0.05); Values are expressed as mean ± SEM.

3. Results

3.1. Experiment 1. Evaluation of different volumes and concentrations of sperm for vitrification in straws

The lowest values ($P < 0.01$) for PM, VCL, VSL and VAP were obtained using a volume of 30 μl at any sperm concentration. There were no differences for IMS, AIS, LIN, STR, WOB, ALH and BCF among the volumes and concentrations assessed. When vitrification was performed in straws filled with 100 μl at 100×10^6 sperm/ml, values for TM, PM, VCL, VSL and VAP were greater in comparison to values of the other treatment groups (Table 1); therefore, this procedure was selected for use in the subsequent experiments.

3.2. Experiment 2. Comparison among sucrose, trehalose and raffinose for sperm vitrification in straws

There were greater values for PM and IMS using a concentration of 100 mM of trehalose in comparison to 200 mM sucrose and 100 mM or 200 mM raffinose ($P < 0.05$). With respect to AIS, when 50 and 100 mM of trehalose were used, there were greater values ($P < 0.05$) in comparison to raffinose at any of the concentrations assessed ($P < 0.05$). There were no differences in values among treatment groups for the other variables that were assessed (Table 2). A concentration of 100 mM trehalose was, therefore, selected for the subsequent experiments.

3.3. Experiment 3. Evaluation of sperm vitrification in straws in comparison to conventional freezing

With vitrification using 100 mM of trehalose, there were greater values ($P < 0.05$) than with conventional freezing for PM (48.2 ± 2.3 compared with 37.3 ± 2.2%), IMS (82.8 ± 1.5 compared with 74.1 ± 1.9%), AIS (50.2 ± 1.2 compared with 43.1 ± 1.4%) and most of the values for other kinetic variables assessed (Fig. 1). Additionally, values for sDF at 0 h were greater ($P < 0.05$) with use of conventional freezing (8.2 ± 0.3%) in comparison to vitrification (6.4 ± 0.7%) (Fig. 2). There were no differences in the slopes when sDF values were assessed among treatment groups (Fig. 3).

4. Discussion

Kinetic sperm vitrification performed in straws is a relatively recently developed procedure (Sanchez et al., 2012). In the present study, the smallest French straws available for sperm freezing, 0.25 ml, were used. The straws were filled with different semen volumes ranging from 30 μl, which was previously used for stallion sperm vitrification in spheres (Hidalgo et al., 2018), to 100 μl, volume used in other species for sperm vitrification in 0.25 ml straws (Diaz-Jimenez et al., 2017; Schulz et al., 2017). The lesser volume was applied successfully using the spheres method in dog, human and stallion sperm vitrification (Isachenko et al., 2008; Sanchez et al., 2011; Hidalgo et al., 2018), so the use of this procedure was expected to result in a greater sperm quality among the volumes evaluated in the present study. The use of the greatest volume assessed (100 μl), however, resulted in the greatest total and progressive sperm motility. This could be attributed to differences between methods (spheres compared with straws). When straws are used instead of spheres, there is a larger surface area of sperm exposed to LN₂, resulting in a more rapid cooling rate, as previously reported by Diaz-Jimenez et al. (2018). In this regard, it would be interesting to perform further studies using greater volumes of sperm for vitrification, because straws containing 0.25 ml can be filled with more than 100 μl.

Vitrification has been traditionally performed with human sperm using samples with small concentrations of sperm (Schulz et al., 2017). Semen doses used for regular artificial insemination techniques, however, need to contain spermatozoa in greater sperm concentrations (Sieme et al., 2004). In this regard, previous studies of sperm vitrification in different species have included relatively greater concentrations of sperm (Pradiee et al., 2015; Diaz-Jimenez et al., 2017). Unfortunately, there is no consensus as to what the optimal sperm concentration is for vitrification in animal species, varying from 2 to 200×10^6 sperm/ml (Sanchez et al., 2011; Diaz-Jimenez et al., 2017). In the present study, the sperm concentrations used ranged from 50×10^6 sperm/ml, a concentration which was previously used for stallion sperm vitrification in spheres (Hidalgo et al., 2018) to 200×10^6 sperm/m, that was previously used for donkey sperm vitrification in straws (Diaz-Jimenez et al., 2017). Based on results from the present study, the use of the intermediate concentration of 100×10^6 sperm/ml, resulted in the greatest motility values after warming. This finding is consistent with previous findings for sperm freezing where intermediate concentrations were recommended [26]. It has been proposed that cryopreservation using relatively greater concentrations of sperm (200 and 400×10^6 sperm/ml) could result in a reduction in sperm motility and viability (Nascimento et al., 2008). Additionally, lesser sperm concentrations could be associated with the deleterious "dilution effect" (Hayden et al., 2015). Either of these factors could result in the loss of motility observed in the sperm samples vitrified at relatively lesser and greater sperm concentrations.

In the present study, three carbohydrates were used for vitrification: sucrose, trehalose and raffinose. Sucrose has been the carbohydrate primarily used for sperm vitrification (Isachenko et al., 2008) due to its effectiveness in increasing the viscosity of the extracellular medium and stabilizing the sperm plasma membrane (Chen et al., 1993). In addition, trehalose and raffinose can trap the free radicals that lead to the peroxidation of the lipids of the plasma membrane, maintaining its integrity (Aisen et al., 2005; Bucak et al., 2013). Trehalose also enhances the fluidity of the sperm membrane and preserves the lipid bilayer by stabilization of the water structure around the membrane, protecting the sperm against cryodamage (Aboagla and Terada, 2003; Gheller et al., 2019). The concentrations of these carbohydrates are key factors for vitrification success (Isachenko et al., 2003; Hidalgo et al., 2018). Sperm of some species are more sensitive to the osmotic stress caused by relatively greater carbohydrate concentrations than sperm of

Table 2
Comparison of different molarities of non-permeable cryoprotectants (sucrose, trehalose and raffinose) for stallion sperm vitrification in 0.25 ml straws.

Variables	Sperm vitrification treatments (carbohydrates)									P-Value
	S1	S2	S3	T1	T2	T3	R1	R2	R3	
	20 mM sucrose	100 mM sucrose	200 mM sucrose	50 mM trehalose	100 mM trehalose	200 mM trehalose	50 mM raffinose	100 mM raffinose	200 mM raffinose	
TM (%)	52.1 ± 3.7^{ab}	52.8 ± 3.3^{ab}	49.2 ± 3.1^{b}	56.3 ± 3.8^{ab}	60.3 ± 3.0^{a}	55.4 ± 2.6^{ab}	51.5 ± 3.3^{ab}	45.8 ± 3.0^{b}	47.9 ± 3.5^{b}	> 0.05
PM (%)	31.6 ± 2.2^{bc}	34.6 ± 2.9^{abc}	28.7 ± 2.6^{c}	39.1 ± 3.6^{ab}	41.5 ± 3.3^{a}	35.8 ± 9.0^{abc}	32.5 ± 2.3^{bc}	28.6 ± 2.2^{c}	30.5 ± 3.1^{bc}	< 0.01
IMS (%)	75.6 ± 2.3^{abc}	79.2 ± 1.7^{ab}	74.4 ± 2.2^{bc}	77.0 ± 2.3^{abc}	81.1 ± 2.1^{a}	78.4 ± 1.7^{ab}	73.3 ± 2.0^{bc}	72.9 ± 2.0^{bc}	71.2 ± 2.4^{c}	< 0.05
AIS (%)	47.1 ± 2.8^{ab}	49.1 ± 2.0^{ab}	45.5 ± 2.8^{ab}	51.6 ± 2.0^{a}	53.3 ± 2.0^{a}	47.3 ± 2.8^{ab}	41.2 ± 2.3^{b}	43.4 ± 2.4^{b}	43.2 ± 2.9^{b}	= 0.01
VCL (μm/s)	52.1 ± 2.0^{a}	54.3 ± 1.9^{a}	52.5 ± 2.4^{a}	56.4 ± 1.9^{a}	55.4 ± 2.0^{a}	54.3 ± 1.6^{a}	52.4 ± 1.6^{a}	53.5 ± 2.2^{a}	53.2 ± 2.6^{a}	> 0.05
VSL (μm/s)	39.5 ± 1.7^{a}	42.1 ± 2.0^{a}	37.5 ± 1.9^{a}	43.5 ± 1.9^{a}	43.1 ± 1.8^{a}	42.0 ± 1.6^{a}	40.5 ± 1.5^{a}	40.0 ± 1.7^{a}	39.8 ± 1.7^{a}	> 0.05
VAP (μm/s)	42.9 ± 1.9^{a}	45.6 ± 2.1^{a}	42.7 ± 2.2^{a}	47.1 ± 2.0^{a}	46.9 ± 1.9^{a}	45.9 ± 1.6^{a}	43.5 ± 1.5^{a}	44.0 ± 2.2^{a}	44.1 ± 2.2^{a}	> 0.05
LIN (%)	75.6 ± 1.5^{a}	77.3 ± 2.0^{a}	72.7 ± 2.0^{a}	76.9 ± 1.8^{a}	77.7 ± 1.7^{a}	77.2 ± 1.3^{a}	77.2 ± 1.4^{a}	75.0 ± 1.6^{a}	75.6 ± 2.1^{a}	> 0.05
STR (%)	92.0 ± 0.6^{a}	92.2 ± 0.7^{a}	89.3 ± 0.9^{a}	91.9 ± 0.7^{a}	91.9 ± 0.7^{a}	91.4 ± 0.6^{a}	92.8 ± 0.6^{a}	91.3 ± 1.1^{a}	90.9 ± 1.2^{a}	> 0.05
WOB (%)	82.1 ± 1.3^{a}	83.7 ± 1.6^{a}	81.3 ± 1.7^{a}	83.5 ± 1.5^{a}	84.5 ± 1.4^{a}	84.3 ± 1.0^{a}	83.2 ± 1.2^{a}	82.1 ± 1.4^{a}	83.0 ± 1.5^{a}	> 0.05
ALH (μm)	2.5 ± 0.1^{a}	2.4 ± 0.1^{a}	2.6 ± 0.2^{a}	2.5 ± 0.1^{a}	2.4 ± 0.1^{a}	2.4 ± 0.1^{a}	2.4 ± 0.1^{a}	2.5 ± 0.1^{a}	2.4 ± 0.1^{a}	> 0.05
BCF (Hz)	7.0 ± 0.2^{a}	6.9 ± 0.2^{a}	6.9 ± 0.2^{a}	7.1 ± 0.2^{a}	6.8 ± 0.1^{a}	6.8 ± 0.1^{a}	7.1 ± 0.1^{a}	7.2 ± 0.2^{a}	6.7 ± 0.1^{a}	> 0.05

TM = total motility; PM = progressive motility; IMS = plasma membrane integrity; AIS = acrosome-intact sperm; VCL = curvilinear velocity; VSL = linear velocity; VAP = average path velocity; LIN = linear coefficient; STR = straightness coefficient; WOB = Wobble coefficient; ALH = lateral head displacement; BCF = beat cross frequency. Different superscripts (a, b, c) indicate significant differences ($P < 0.05$); Values are expressed as mean ± SEM.
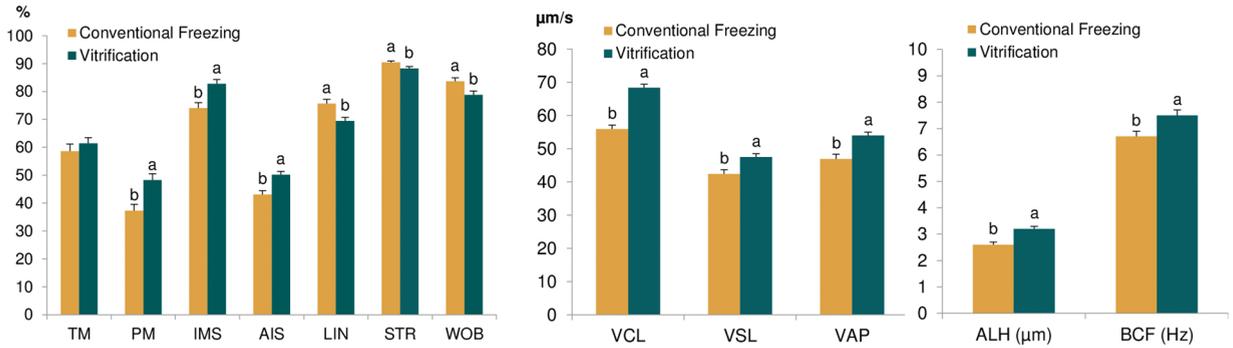
**Fig. 1.** Comparison of conventional freezing and vitrification in 0.25 ml straws filled with 100 μl at 100×10^6 sperm/ml using the extender with 100 mM of trehalose for: TM = total motility, PM = progressive motility, IMS = plasma membrane integrity, AIS = acrosome-intact sperm, LIN = linear coefficient, STR = straightness coefficient, WOB = Wobble coefficient, VCL = curvilinear velocity, VSL = linear velocity, VAP = average path velocity, BCF = beat cross frequency, ALH = lateral head displacement. Different letters (a–b) indicate differences ($P < 0.05$). Values are shown as mean ± SEM.
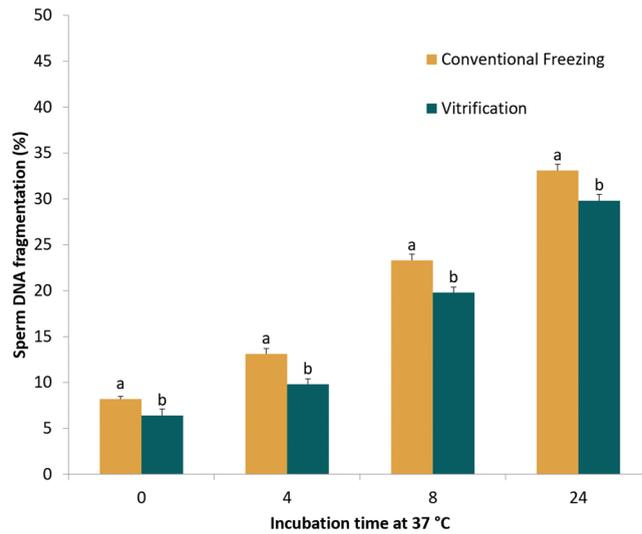
Fig. 2. Comparison of conventional freezing and vitrification on sperm DNA fragmentation (sDF) after 0, 4, 8 and 24 h of incubation; Different letters (a–b) indicate differences ($P < 0.05$). Values are shown as mean ± SEM.
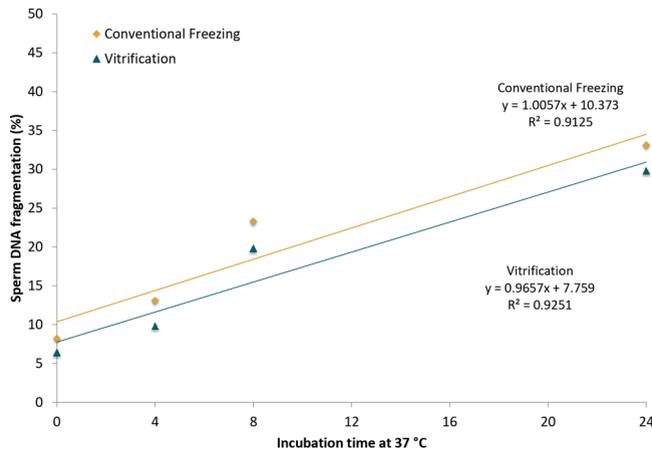
Fig. 3. Effect of vitrification and conventional freezing on the sperm DNA fragmentation rate (sDF%/hour) after 24 h of incubation; no differences were detected in the slopes of sDF between treatment groups ($P > 0.05$).

other species. In this regard, with donkey sperm (Sanchez et al., 2011; Diaz-Jimenez et al., 2017) the optimum molarity is as great as 250 mM of sucrose while with sperm of other species 100 mM molarity is the maximum that can be used, such as those for the Iberian ibex (Pradiee et al., 2015). This is particularly relevant for stallion sperm, which has been previously reported to have a relatively lesser resistance to relatively greater concentrations of carbohydrates (Consuegra et al., 2018a; Hidalgo et al., 2018). In the present study, the molarities of the carbohydrates were selected based on results from previous studies of stallion and human sperm (Schulz et al., 2017; Hidalgo et al., 2018). In the present study, there was the greatest sperm quality with the use of 100 mM of trehalose. This concentration has also been successfully utilized for vitrification of human sperm in straws (Schulz et al., 2017). It should be taken into account, however, that the optimal concentration of carbohydrates differs with use of different vitrification methods, even within the same species. Small volumes of sperm require lesser sugar molarities (20 mM of sucrose) for vitrification when using the spheres method (Hidalgo et al., 2018). This could explain the poor sperm motility obtained in other studies where vitrification in spheres was performed adding molarities above 150 mM (Pérez-Marín et al., 2017; Caturla-Sánchez et al., 2018). Greater carbohydrate concentrations (100 mM) are required for straw vitrification (large volume). This could be attributed to the two main requirements for vitrification: more rapid cooling/warming rates and high viscosity of the solution. When there are larger semen volumes, the cooling/warming processes are slowed down. To compensate for this, a solution of greater viscosity is required for vitrification (Arav et al., 2002), which could be achieved using greater concentrations of carbohydrates (Isachenko et al., 2003).

Furthermore, in the present study a conventional freezing protocol was compared to the vitrification method which resulted in sperm of the greatest quality (straws filled with 100 μl at 100×10^6 sperm/ml with the extender containing trehalose 100 mM). With vitrification, there were greater values than with the conventional freezing protocol for most of the sperm variables assessed. These findings are consistent with those from previous studies of human (Aizpurua et al., 2017) and stallion sperm vitrification using the spheres method (Hidalgo et al., 2018). The lesser sperm motility, and integrity of plasma and acrosome membranes with conventional freezing in the present study could be attributed to the osmotic stress placed on the sperm cells during processing. This stress can result from the freezing process (Peña et al., 2011) as well as from the use of an extender that contains permeable cryoprotectants (Ball and Vo, 2001) for which the osmolality is much greater (1162 mOsm/kg) than the extender used for vitrification in the present study (465 mOsm/kg). The greater sDF obtained after thawing in conventional freezing could also be due to this osmotic stress (Yildiz et al., 2010; Kopeika et al., 2015). The hyperosmotic stress could lead to DNA fragmentation as a result of the activation of scavenging enzymes, increasing the formation of free radicals (Grunewald et al., 2009) with this activation being more pronounced when glycerol is included in the extender (Wündrich et al., 2006). Additionally, when samples were exposed to a stressor (incubation at 37 °C for 24 h; (Urbano et al., 2013), there was greater cryptic DNA damage with use of conventional freezing in comparison to vitrification. These different results within the techniques used could be due to the addition BSA to the vitrification extender, a protein usually combined with carbohydrates, which protects DNA by reducing the amount of ROS (Nang et al., 2012).

Vitrification in 0.25 ml straws is a promising alternative to conventional freezing, but for the horse industry it would also be interesting to perform vitrification using larger volumes (0.5 ml straws), which has occurred with human sperm (Isachenko et al., 2011). Unfortunately, many have failed trying to repeat this technology (Katkov et al., 2012) including with stallion sperm, because of the compromised sperm quality after warming (Consuegra et al., 2019; Restrepo et al., 2019). Consequently, further studies are needed to develop this technology.

5. Conclusion

In conclusion, stallion sperm were successfully vitrified in covered 0.25 ml straws filled with a volume of 100 μl of sperm at 100×10^6 sperm/ml and by adding 100 mM of trehalose, which resulted in greater sperm quality after warming than with conventional freezing.

Conflicts of interest

None

Acknowledgements

References

Aboagla, E.M., Terada, T., 2003. Trehalose-enhanced fluidity of the goat sperm membrane and its protection during freezing. Biol. Reprod. 69, 1245–1250.
Aisen, E., Quintana, M., Medina, V., Morello, H., Venturino, A., 2005. Ultramicroscopic and biochemical changes in ram spermatozoa cryopreserved with trehalose-based hypertonic extenders. Cryobiology 50, 239–249.
Aizpurua, J., Medrano, L., Enciso, M., Sarasa, J., Romero, A., Fernández, M.A., Gómez-Torres, M.J., 2017. New permeable cryoprotectant-free vitrification method for native human sperm. Hum. Reprod. 32, 2007–2015.
Arav, A., Yavin, S., Zeron, Y., Natan, D., Dekel, I., Gacitua, H., 2002. New trends in gamete's cryopreservation. Mol. Cell. Endocrinol. 187, 77–81.
Ball, B.A., Vo, A., 2001. Osmotic tolerance of equine spermatozoa and the effects of soluble cryoprotectants on equine sperm motility, viability, and mitochondrial

membrane potential. J. Androl. 22, 1061–1069.

Bóveda, P., Esteso, M.C., Castaño, C., Toledano-Díaz, A., López-Sebastián, A., Muñiz, A., Prieto, P., Mejía, O., Ungerfeld, R., Santiago-Moreno, J., 2018. Slow and ultra-rapid freezing protocols for cryopreserving mouflon (Ovis musimon) and fallow deer (Dama dama) epididymal sperm. Anim. Reprod. Sci. 192, 193–199.

Bucak, M.N., Keskin, N., Taşpınar, M., Çoyan, K., Başpınar, N., Cenariu, M.C., Bilgili, A., Öztürk, C., Kurşunlu, A.N., 2013. Raffinose and hypotaurine improve the post-thawed Merino ram sperm parameters. Cryobiology 67, 34–39.

Caturla-Sánchez, E., Sánchez-Calabuig, M.J., Pérez-Gutiérrez, J.F., Cerdeira, J., Castaño, C., Santiago-Moreno, J., 2018. Vitrification of dog spermatozoa: Effects of two cryoprotectants (sucrose or trehalose) and two warming procedures. Cryobiology 80, 126–129.

Chen, Y., Foote, R.H., Brockett, C.C., 1993. Effect of sucrose, trehalose, hypotaurine, taurine, and blood serum on survival of frozen bull sperm. Cryobiology 30, 423–431.

Consuegra, C., Crespo, F., Bottrel, M., Ortiz, I., Dorado, J., Diaz-Jimenez, M., Pereira, B., Hidalgo, M., 2018a. Stallion sperm freezing with sucrose extenders: A strategy to avoid permeable cryoprotectants. Anim. Reprod. Sci. 191, 85–91.

Consuegra, C., Crespo, F., Dorado, J., Ortiz, I., Diaz-Jimenez, M., Pereira, B., Hidalgo, M., 2018b. Comparison of different sucrose-based extenders for stallion sperm vitrification in straws. Reprod. Domest. Anim. 53, 59–61.

Consuegra, C., Crespo, F., Dorado, J., Diaz-Jimenez, M., Pereira, B., Ortiz, I., Arenas, R., Morrell, J.M., Hidalgo, M., 2019. Vitrification of large volumes of stallion sperm in comparison to spheres and conventional freezing: effect of warming procedures and sperm selection. J.Equine Vet. Sci in press.

Cortés-Gutiérrez, E.I., Crespo, F., Gosálvez, A., Dávila-Rodríguez, M.I., López-Fernández, C., Gósalvez, J., 2008. DNA fragmentation in frozen sperm of Equus asinus: Zamorano-Leonés, a breed at risk of extinction. Theriogenology 69, 1022–1032.

Diaz-Jimenez, M., Pereira, B., Ortiz, I., Consuegra, C., Partyka, A., Dorado, J., Hidalgo, M., 2017. Effect of different extenders for donkey sperm vitrification in straws. Reprod. Domest. Anim. 52, 55–57.

Diaz-Jimenez, M., Dorado, J., Pereira, B., Ortiz, I., Consuegra, C., Bottrel, M., Ortiz, E., Hidalgo, M., 2018. Vitrification in straws conserves motility features better than spheres in donkey sperm. Reprod. Domest. Anim. 53 (Suppl. 2), 56–58.

Diaz-Jimenez, M., Dorado, J., Consuegra, C., Ortiz, I., Pereira, B., Carrasco, J.J., Gomez-Arrones, V., Domingo, A., Hidalgo, M., 2019. Optimization of donkey sperm vitrification: Effect of sucrose, sperm concentration, volume and package (0.25 and 0.5 mL straws). Anim. Reprod. Sci. 204, 31–38.

Dorado, J., Acha, D., Ortiz, I., Galvez, M.J., Carrasco, J.J., Gomez-Arrones, V., Calero-Carretero, R., Hidalgo, M., 2014. Effect of extender and amino acid supple-mentation on sperm quality of cooled-preserved Andalusian donkey (Equus asinus) spermatozoa. Anim. Reprod. Sci. 146, 79–88.

Gheller, S.M.M., Corcini, C.D., de Brito, C.R.C., Acosta, I.B., Tavares, G.C., Soares, S.L., Silva, A.C., Pires, D.M., Varela Jr, A.S., 2019. Use of trehalose in the semen cryopreservation of Amazonian catfish Leiarius marmoratus. Cryobiology 87, 74–77.

Govaere, J., Hoogewijs, M., De Schauwer, C., De Vliegher, S., Van Soom, A., Duchateau, L., de Kruif, A., 2014. Effect of artificial insemination protocol and dose of Frozen/Thawed stallion semen on pregnancy results in mares. Reprod. Domest. Anim. 49, 487–491.

Grunewald, S., Sharma, R., Paasch, U., Glander, H.J., Agarwal, A., 2009. Impact of caspase activation in human spermatozoa. Microsc. Res. Tech. 72, 878–888.

Hayden, S.S., Blanchard, T.L., Brinsko, S.P., Varner, D.D., Hinrichs, K., Love, C.C., 2015. The "dilution effect" in stallion sperm. Theriogenology 83, 772–777.

Hidalgo, M., Consuegra, C., Dorado, J., Diaz-Jimenez, M., Ortiz, I., Pereira, B., Sanchez, R., Crespo, F., 2018. Concentrations of non-permeable cryoprotectants and equilibration temperatures are key factors for stallion sperm vitrification success. Anim. Reprod. Sci. 196, 91–98.

Horta, F., Alzobi, H., Jitanantawittaya, S., Catt, S., Chen, P., Pangestu, M., Temple-Smith, P., 2017. Minimal volume vitrification of epididymal spermatozoa results in successful in vitro fertilization and embryo development in mice. Asian J. Androl. 19, 107–112.

Isachenko, E., Isachenko, V., Katkov, I.I., Dessole, S., Nawroth, F., 2003. Vitrification of mammalian spermatozoa in the absence of cryoprotectants: From past practical difficulties to present success. Reprod. Biomed. Online 6, 191–200.

Isachenko, E., Isachenko, V., Weiss, J.M., Kreienberg, R., Katkov, I.I., Schulz, M., Lulat, A.G., Risopatron, M.J., Sanchez, R., 2008. Acrosomal status and mitochondrial activity of human spermatozoa vitrified with sucrose. Reproduction 136, 167–173.

Isachenko, V., Maettner, R., Petrunkina, A.M., Mallmann, P., Rahimi, G., Sterzik, K., Sanchez, R., Risopatron, J., Damjanoski, I., Isachenko, E., 2011. Cryoprotectant-free vitrification of human spermatozoa in large (up to 0.5 ml) volume: A novel technology. Clin. Lab. 57, 643–650.

Isachenko, V., Rahimi, G., Mallmann, P., Sanchez, R., Isachenko, E., 2017. Technologies of cryoprotectant-free vitrification of human spermatozoa: asepticity as criterion of effectiveness. Andrology 5, 1055–1063.

Katkov, I., Bolyukh, V., Chernetsov, O.A., Dudin, P.I., Grigoriev, A.Y., Isachenko, V., Isachenko, E., Lulat, A.G.M., Moskovtsev, S., Petrushko, M.P., Pinyaev, V.I., Sokol, K.M., Sokol, Y.I., Sushko, A.B., Yakhnenko, I., 2012. Kinetic vitrification of spermatozoa of vertebrates: what can We learn from nature? In: Katkov, I. (Ed.), Current Frontiers in Cryobiology. InTechOpen, Rijeka, pp. 3–40.

Kopeika, J., Thornhill, A., Khalaf, Y., 2015. The effect of cryopreservation on the genome of gametes and embryos: principles of cryobiology and critical appraisal of the evidence. Hum. Reprod. Update 21, 209–227.

López-Fernández, C., Crespo, F., Arroyo, F., Fernández, J.L., Arana, P., Johnston, S.D., Gosálvez, J., 2007. Dynamics of sperm DNA fragmentation in domestic animals. II. The stallion. Theriogenology 68, 1240–1250.

Macedo, S., Bliebernicht, M., Carvalheira, J., Costa, A., Ribeiro, F., Rocha, A., 2018. Effects of two freezing methods and two cryopreservation media on post-thaw quality of stallion spermatozoa. Reprod. Domest. Anim. 53, 519–524.

Nang, C.F., Osman, K., Budin, S.B., Ismail, M.I., Jaffar, F.H.F., Mohamad, S.F.S., Ibrahim, S.F., 2012. Bovine serum albumin: Survival and osmolarity effect in bovine spermatozoa stored above freezing point. Andrologia 44, 447–453.

Nascimento, J., Raphael, C.F., Andrade, A.F.C., Alonso, M.A., Celeghini, E.C.C., Arruda, R.P., 2008. Effects of Sperm Concentration and Straw Volume on Motion Characteristics and Plasma, Acrosomal, and Mitochondrial Membranes of Equine Cryopreserved Spermatozoa. J. Equine Vet. Sci. 28, 351–358.

Ortiz, I., Dorado, J., Ramirez, L., Morrell, J.M., Acha, D., Urbano, M., Galvez, M.J., Carrasco, J.J., Gomez-Arrones, V., Calero-Carretero, R., Hidalgo, M., 2014. Effect of single layer centrifugation using Androcoll-E-Large on the sperm quality parameters of cooled-stored donkey semen doses. Animal 8, 308–315.

Peña, F.J., Garcia, B.M., Samper, J.C., Aparicio, I.M., Tapia, J.A., Ferrusola, C.O., 2011. Dissecting the molecular damage to stallion spermatozoa: the way to improve current cryopreservation protocols? Theriogenology 76, 1177–1186.

Pérez-Marín, C.C., Requena, F.D., Arando, A., Ortiz-Villalón, S., Requena, F., Agüera, E.I., 2017. Effect of trehalose- and sucrose-based extenders on equine sperm quality after vitrification: Preliminary results. Cryobiology 80, 62–69.

Pradiee, J., Esteso, M.C., Lopez-Sebastián, A., Toledano-Díaz, A., Castaño, C., Carrizosa, J.A., Urrutia, B., Santiago-Moreno, J., 2015. Successful ultrarapid cryopre-servation of wild Iberian ibex (Capra pyrenaica) spermatozoa. Theriogenology 84, 1513–1522.

Restrepo, G., Varela, E., Duque, J.E., Gómez, J.E., Rojas, M., 2019. Freezing, Vitrification, and Freeze-Drying of Equine Spermatozoa: Impact on Mitochondrial Membrane Potential, Lipid Peroxidation, and DNA Integrity. J. Equine Vet. Sci. 72, 8–15.

Sanchez, R., Risopatron, J., Schulz, M., Villegas, J., Isachenko, V., Kreinberg, R., Isachenko, E., 2011. Canine sperm vitrification with sucrose: effect on sperm function. Andrologia 43, 233–241.

Sanchez, R., Isachenko, V., Petrunkina, A.M., Risopatron, J., Schulz, M., Isachenko, E., 2012. Live birth after intrauterine insemination with spermatozoa from an oligoasthenozoospermic patient vitrified without permeable cryoprotectants. J. Androl. 33, 559–562.

Schulz, M., Risopatrón, J., Matus, G., Pineda, E., Rojas, C., Isachenko, V., Isachenko, E., Sánchez, R., 2017. Trehalose sustains a higher post-thaw sperm motility than sucrose in vitrified human sperm. Andrologia 49, e12757.

Sieme, H., Bonk, A., Hamann, H., Klug, E., Katila, T., 2004. Effects of different artificial insemination techniques and sperm doses on fertility of normal mares and mares with abnormal reproductive history. Theriogenology 62, 915–928.

Urbano, M., Dorado, J., Ortiz, I., Morrell, J.M., Demyda-Peyrás, S., Gálvez, M.J., Alcaraz, L., Ramírez, L., Hidalgo, M., 2013. Effect of cryopreservation and single layer centrifugation on canine sperm DNA fragmentation assessed by the sperm chromatin dispersion test. Anim. Reprod. Sci. 143, 118–125.

Wündrich, K., Paasch, U., Leicht, M., Glander, H.J., 2006. Activation of caspases in human spermatozoa during cryopreservation - an immunoblot study. Cell Tissue Bank. 7, 81–90.

Yildiz, C., Law, N., Ottaviani, P., Jarvi, K., McKerlie, C., 2010. Comparison of sperm quality and DNA integrity in mouse sperm exposed to various cooling velocities and osmotic stress. Theriogenology 74, 1420–1430.