Animal Reproduction Science 204 (2019) 31–38

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Optimization of donkey sperm vitrification: Effect of sucrose, sperm concentration, volume and package (0.25 and 0.5 mL straws)

M. Diaz-Jimenez[a], J. Dorado[a], C. Consuegra[a], I. Ortiz[a], B. Pereira[a], J.J. Carrasco[b], V. Gomez-Arrones[b], A. Domingo[b], M. Hidalgo[a,*]

[a] Veterinary Reproduction Group, University of Cordoba, Cordoba, Spain
[b] Equine Reproduction Center, Centro de Selección y Reproducción Animal, (CENSYRA-Extremadura Government), Badajoz, Spain

ARTICLE INFO

Keywords:
Donkey
Cryopreservation
Vitrification
Sperm
Livestock

ABSTRACT

The aim of this study was to assess the effect of different factors affecting vitrification success of donkey sperm: extender, sperm concentration, volume and storage vessel type. In Experiment 1, sucrose supplementations at 0.25 and 0.1 M were compared using two base extenders (containing or not egg-yolk); in Experiment 2, three sperm concentrations were assessed: 100, 200 or 300 million sperm/mL; and in Experiment 3, three different sperm volumes (100, 160 and 200 µL) and two different storage vessels (0.25 and 0.5 mL straws) were assessed. Sperm motility variables (CASA), plasma membrane and acrosome (evaluated under fluorescence microscopy) and sperm DNA integrity (flow cytometry) were evaluated after warming with comparisons of protocols. There was a greater total (55.7 ± 16.4%) and progressive (44.0 ± 11.5%) motility using the extender with egg-yolk and 0.1 M sucrose. There were no effects of sperm concentrations on vitrification results ($P > 0.05$). The 0.25 mL covered straw showed higher values than the 0.5 mL straw for total (50.0 ± 17.3% vs 2.0 ± 6.7%) and progressive (40.5 ± 14.9% vs 0.9 ± 1.5%) motility, plasma membrane (43.9 ± 14.4% vs 14.0 ± 16.4%) and acrosome integrity (51.5 ± 13.6% vs 28.0 ± 14.7%), respectively. In conclusion, values for donkey sperm quality variables after vitrification were greater using an extender containing egg-yolk and 0.1 M sucrose, at 300 million sperm/mL in 0.25 mL straws with outer covers.

1. Introduction

Vitrification of sperm has been developed in different species as a potential alternative to conventional freezing (Isachenko et al., 2011a; Sánchez et al., 2011; Figueroa et al., 2015; Pradiee et al., 2015). Vitrification has been achieved through an abrupt cooling by dropping the semen samples directly into liquid nitrogen (Isachenko et al., 2003, 2008; Pradiee et al., 2015). This non-aseptic technique requires a small volume of the sample, which is suitable for "*in vitro* fertilization" (IVF) or "intracytoplasmic sperm injection" (ICSI), however, is not practical for intrauterine insemination, as previously described (Isachenko et al., 2011b). To solve these limitations, sperm vitrification has been developed using straws (Isachenko et al., 2011b; Slabbert et al., 2015). This recently-developed method has been used primarily for investigations involving human sperm vitrification (Isachenko et al., 2011b; Sanchez

* Corresponding author at: Department of Animal Medicine and Surgery, Faculty of Veterinary Medicine, University of Cordoba, Campus de Rabanales (Veterinary Teaching Hospital), Ctra. Madrid-Cádiz, km 396, 14071, Córdoba, Spain.
E-mail address: mhidalgo@uco.es (M. Hidalgo).

https://doi.org/10.1016/j.anireprosci.2019.03.002
Received 22 October 2018; Received in revised form 14 January 2019; Accepted 4 March 2019
Available online 05 March 2019
0378-4320/

et al., 2012a; Merino et al., 2015; Slabbert et al., 2015; Mansilla et al., 2016; Schulz et al., 2017) using different combinations of carbohydrates (sucrose or trehalose) and proteins (human serum albumin) instead of permeable cryoprotectants (Sanchez et al., 2012b). There is little information about the use of this technique in animal species and combination of both permeable and non-permeable cryoprotectants were used in previous research (Jiménez-Rabadán et al., 2015; Daramola et al., 2016). Recently, it has been reported that the sperm vitrification technique using 0.25 into 0.5 mL French straws resulted in successful vitrification of donkey semen (Diaz-Jimenez et al., 2017, 2018b); but a fixed concentration of non-permeable agents (sucrose and bovine serum albumin) derived from human studies were used (Sanchez et al., 2012a, b; Slabbert et al., 2015). The concentration of sucrose and proteins to be used for cryopreservation of semen are species-specific (Consuegra et al., 2018a; Diaz-Jimenez et al., 2018a; Hidalgo et al., 2018) and properly designed studies with donkey sperm have not been conducted comparing different concentrations of these compounds. Additionally, results from previous studies indicate usefulness of base extenders containing or not egg-yolk (Sánchez et al., 2011; Pradiee et al., 2015, 2016; Swanson et al., 2017; Bóveda et al., 2018; Consuegra et al., 2018b) for sperm vitrification, but there is little information about comparisons between them (Diaz-Jimenez et al., 2017).

Human sperm have been successfully vitrified at low sperm concentrations, ranging from 1 (Sanchez et al., 2012a) to 15 (Sanchez et al., 2012b) million sperm/mL. This concentration is lower than those usually stored for artificial insemination in animal species, including donkeys, in which sperm are usually frozen in concentrations of 50 (De Oliveira et al., 2017) to 200 million sperm/mL (Acha et al., 2015; Oliveira et al., 2016; Diaz-Jimenez et al., 2018a). Furthermore, the concentration must be increased to compensate for the relatively lesser volumes commonly used for sperm vitrification (30 to 100 µL). But inconsistent results were obtained when there was goat (Daramola et al., 2016) and ram (Jiménez-Rabadán et al., 2015) sperm vitrification at high sperm concentration. This finding indicates that vitrification success in straws could be also related to sperm concentration, and different concentrations for vitrification purposes have not been previously compared in donkeys.

The success in using vitrification procedures for semen cryopreservation seems to be affected by the procedure used for sperm storage (Rosato and Iaffaldano, 2013), which implies different factors including sperm volume, speed of cooling/warming and storage device. Different sperm volumes and packages have been assessed for human sperm vitrification, ranging from 10 µL in plastic capillaries (Isachenko et al., 2012, 2017b) to 500 µL in plastic straws (Isachenko et al., 2011b); and in animals from 100 µL in 0.25 mL covered straws (Jiménez-Rabadán et al., 2015; Consuegra et al., 2018b) to 200 µL placed directly into 0.5 plastic straws (Daramola et al., 2016). To our knowledge, there are no previous reports about donkey sperm vitrification when there is direct placement in 0.5 plastic straws, nor even a comparison between different storage vessel types for donkey sperm vitrification.

The aims of the present study, therefore, focused on the optimization of donkey sperm vitrification by: (1) assessing the effect of sucrose concentration in extenders containing or not containing egg-yolk; (2) evaluating three different concentrations of sperm and (3) comparing different semen volumes using straws.

2. Materials and methods

All the experiments were performed in accordance with the Ethical Animal Experimentation Committee of Cordoba University (Project No. 31/08/2017/105) according to the Spanish law for animal welfare and experimentation (Decision 2012/707/UE and RD 53/2013).

2.1. Animals

A total of five healthy mature Andalusian donkeys, ranging from 4 to 15 years of age were used as semen donors. Animals were managed in paddocks and fed water *"ad libitum"*, teff hay and oats. Semen was collected twice per week, using a Missouri-model artificial vagina with an in-line gel filter (Minitüb GmbH, Tiefenbach, Germany) in the presence of a jenny in estrus. For each experiment, three different ejaculates from each of the five donkeys were obtained.

2.2. Sperm processing

Immediately after collection, the gel-free semen volume was quantified in the graduated collecting tube and sperm concentration was calculated with a sperm photometer (Sperma-Cue, Minitüb GmbH, Tiefenbach, Germany). Thereafter, semen was extended 1:1 (v:v) with INRA-96 (IMV Technologies, L'Aigle, France). Before vitrification, sperm morphology was determined in diff-quick stained smears and sperm motility, plasma membrane and acrosome integrity were assessed as subsequently described. Extended semen was divided into aliquots and centrifuged (7 min/400 x g/22 °C) in a corning-adapted centrifuge (Eppendorf, model 5702 RH, Eppendorf AG, Hamburg, Germany). The supernatant was removed and the sperm pellets were re-suspended with the corresponding vitrification extender (see *2.3. Experimental design*). Samples were maintained 10 min at room temperature (≈ 22 °C) and then slowly cooled in an Equitainer (Hamilton Research, Inc. Ipswich, Massachusetts, USA) for 1 h as previously described (Diaz-Jimenez et al., 2017). Vitrification and warming processes are subsequently described for each experiment.

2.3. Experimental design

2.3.1. Experiment 1: effect of sucrose and egg-yolk for donkey sperm vitrification in 0.25 mL straws

Sucrose and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Sant Louis, USA). The effect of two sucrose concentrations was evaluated using a procedure that has been described for previous studies: (S1) sucrose powder added directly to

the base extender without egg-yolk (INRA-96) to obtain a final concentration of 0.1 M (Hidalgo et al., 2018); or (S2) sucrose prepared in distilled water and diluted 1:1 with the same base extender to obtain a final concentration of 0.25 M, adding BSA at 1% (Diaz-Jimenez et al., 2018a). A second commercial base extender containing egg-yolk (EY, Gent, Minitüb GmbH, Tiefenbach, Germany) was assessed, and both sucrose concentrations were added using the procedures previously described in this manuscript (S1-EY, S2-EY). Osmolality was measured in each extender using a freezing-point digital micro-osmometer Type 6 (Löser Messtechnik, Berlin, Germany).

Semen was divided in four aliquots. After centrifugation, sperm pellets were extended in each vitrification media to reach a final concentration of 200 million sperm/mL (Diaz-Jimenez et al., 2018a). After the cooling process was conducted, 100 µL of each sample was packaged in 0.25 mL French plastic straws, which were horizontally inserted into 0.5 mL straws (CBS™, Cryo Bio System, Paris, France) (Diaz-Jimenez et al., 2017). Thereafter, both ends of the outer straw were sealed and it was directly plunged into liquid nitrogen for vitrification. For the warming process, 0.5 mL straws were opened with forceps, and each 0.25 mL straw with the vitrified sperm was immersed in vertical position into a tube containing 3 mL of INRA-96 at 43 °C until the vitrified solution became liquid (Diaz-Jimenez et al., 2017). Warmed samples were then centrifuged (7 min/400 x *g*/22 °C) and re-extended with INRA-96 to a final concentration of 25 million sperm/mL for sperm evaluation (as described in *2.4. Post-warming sperm evaluation*).

2.3.2. Experiment 2: effect of sperm concentration on vitrification process in 0.25 mL straws

The treatment with the most desirable results in Experiment 1 (S1-EY) was selected for the subsequent experiments of this study. In Experiment 2, sperm were divided in three aliquots and pellets were extended in S1-EY to reach a final concentration of 100, 200 or 300 million sperm/mL. Vitrification and warming processes were performed using 0.25 mL straws using the procedures described in Experiment 1.

2.3.3. Experiment 3: comparison of different sperm volumes and packaging methods (0.25 mL or 0.5 mL straws) for donkey sperm vitrification

The sperm concentration that was determined to be most desirable for vitrification from conducting Experiment 2 (300 million sperm/mL) was used in Experiment 3 using the extender S1-EY. Three sperm volumes and two different packaging processes were compared: 100 and 160 µL of sperm stored in 0.25 mL straws as previously described in this manuscript; and 200 µL of sperm directly loaded in 0.5 mL straws (Isachenko et al., 2011b; Slabbert et al., 2015). Vitrification was performed by plunging straws directly into liquid nitrogen. The 0.25 mL straws were warmed as explained in Experiment 1, and the 0.5 mL straws were immersed in a water bath at 42 °C for 20 s using the procedures described by Slabbert et al. (2015), and then samples were diluted to a final concentration of 25 million sperm/mL with INRA-96 for sperm evaluation.

2.4. Post-warming sperm evaluation

2.4.1. Sperm motility

Sperm motility was objectively evaluated using the Sperm Class Analyzer system (SCA, v.5.4; Microptic S.L., Barcelona, Spain) as previously described by Ortiz et al. (2015). For each sample, two 5 µL drops of diluted sample were evaluated and three microscopic fields were randomly filmed for each drop that was placed on slides. A minimum of 200 spermatozoa were analyzed. The following motility features were recorded: total and progressive motility (TM and PM, %), curvilinear velocity (VCL, µm/s), straight line velocity (VSL, µm/s), average path velocity (VAP, µm/s), linearity (LIN, VSL/VCL, %), straightness (STR, VSL/VAP, %), wobble (WOB, VAP/VCL, %), beat cross frequency (BCF, Hz) and amplitude of lateral head displacement (ALH, µm).

2.4.2. Plasma membrane integrity

Plasma membranes were assessed using the VitalTest stain (Halotech DNA SL, Madrid, Spain) according to the manufacturer instructions (Ortiz et al., 2017). In brief, an aliquot of diluted semen from each sample was mixed with 1 µL of propidium iodide stock solution and 1 µL of acridine orange stock solution. At least 200 spermatozoa per sample were evaluated under fluorescence microscopy (Olympus BX40, Tokyo, Japan), using a U-ND25-2 filter (a 460–490 nm excitation filter). Spermatozoa with an intact plasma membrane stained green and those with a damaged membrane stained red. Results were expressed as plasma membrane integrity percentage (PMI, %).

2.4.3. Acrosome membrane integrity

For sperm acrosome evaluation, the double staining (Sigma-Aldrich, Sant Louis, USA) propidium iodide (PI)/peanutagglutinin–fluorescein isothiocyanate (FITC-PNA) procedure was used as described by Dorado et al. (2014). Briefly, a 10 µL aliquot of each diluted sample was spread on a slide and permeabilized with ethanol for 30 s. A mixture of 10 µL PI and 20 µL FITC-PNA was spread over each smear and the slides were incubated in a dark, moist chamber at 4 °C for 30 min. Slides were then evaluated using an epifluorescence microscope and at least 200 spermatozoa were evaluated. Two staining patterns were used to discern: acrosome-intact spermatozoa (acrosomal region of the sperm displayed green fluorescence; PI+/FICT-PNA+) and acrosome-reacted spermatozoa (equatorial segment displayed green fluorescence or no anterior acrosomal staining; PI+/FICT-PNA–). The percentage of acrosome-intact sperm (AIS, %) was recorded.

2.4.4. Sperm DNA analysis

Sperm DNA integrity was assessed with the Sperm Chromatin Structure Assay (SCSA) using a Cytomics FC500 MPL Flow

Cytometer (Beckman Coulter, Miami, FL) with subsequent data analysis using Summit v4.2 software (DakoCytomation, Fort Collins, CO). Two aliquots of 400 µL at 25 million sperm/mL were saved from each treatment, stored in Eppendorf tubes at -80 °C and thawed at room temperature before evaluation as previously described by Salazar Jr et al. (2011). Sperm DNA integrity was assessed in each sample immediately after warming (T0) and after 4 h of incubation at 37 °C (T4). Approximately 10,000 cells/sample were studied, and the percentage of sperm cells with fragmented DNA was recorded as DNA fragmentation index (DFI, %) (Evenson, 2016).

2.5. Statistical analysis

Statistical analyses of the data were conducted using the Statistical Analysis Software (SAS, v.9.0, SAS Institute Inc., Cary, NC, USA). For each variable, normality of the data distribution and homogeneity of variances were assessed using the Kolmogorov–Smirnov and Levene test, respectively. When values were not normally distributed, results were transformed to a logarithmic scale. Comparisons between treatments were assessed using a general lineal model procedure (PROC GLM) followed by the Duncan test for *post hoc* analysis. Animals and ejaculates were considered as random factors. Values were expressed as mean ± standard deviation. Significant differences were considered when $P < 0.05$.

3. Results

Sperm quality variables from the ejaculates used in this study had the following average values: gel-free volume 65.6 ± 22.7 mL (range: 25.0–117.0 mL), sperm concentration 316.5 ± 99.2 million sperm/mL (range: 147.0–557.0 million sperm/mL), total motility 86.6 ± 7.7% (range: 66.2–97.0%), progressive motility 64.4 ± 13.6% (range: 37.0–88.6%), normal forms 87.9 ± 8.1% (range: 61.0–98.0%), plasma membrane integrity 60.5 ± 12.6% (range: 35.0–82.5%) and acrosome-intact sperm 60.8 ± 19.8% (range: 14.7–88.7%). The extenders used in Experiment 1 had the following osmolality values: S1 = 423 mOsm/kg; S2 = 403 mOsm/kg, S1-EY = 442 mOsm/kg and S2-EY = 433 mOsm/kg.

3.1. Experiment 1: effect of sucrose and egg-yolk for donkey sperm vitrification in 0.25 mL straws

Sperm values for TM, PM and velocity features (VCL, VSL and VAP) were greater ($P < 0.001$) with use of the extenders containing egg-yolk (S1-EY and S2-EY) in comparison to the extenders without egg-yolk (S1 and S2). The values for other variables that were assessed indicated there were no differences between sucrose concentrations nor extenders ($P > 0.05$). The S1-EY extender was selected for the subsequent experiments because of the tendency for a greater TM, PM, PMI and AIS than S2-EY. Results from Experiment 1 are provided in Table 1.

3.2. Experiment 2: effect of sperm concentration on vitrification process in 0.25 mL straws

Mean values for TM and PM ($P < 0.05$); VCL, VSL and VAP ($P < 0.01$) were greater with 200 and 300 million sperm/mL than 100 million sperm/mL. There were no differences in values assessed for the other sperm variables ($P > 0.05$). The greater

Table 1
Mean values of sperm variables from vitrified-warmed samples ($n = 15$) using different concentrations of sucrose with extenders containing or not egg-yolk.

Sperm variables	Vitrification media				*P*-values
	S1	S2	S1-EY	S2-EY	
TM (%)	34.3 ± 15.2[b]	28.7 ± 12.3[b]	55.7 ± 16.4[a]	48.4 ± 12.7[a]	< 0.001
PM (%)	24.9 ± 15.3[b]	20.5 ± 9.8[b]	44.0 ± 11.5[a]	39.0 ± 11.6[a]	< 0.001
PMI (%)	37.1 ± 9.7	37.7 ± 9.1	43.2 ± 18.1	34.1 ± 7.2	> 0.05
AIS (%)	46.1 ± 15.4	37.4 ± 17.4	43.8 ± 18.0	38.6 ± 18.6	> 0.05
VCL (µm/s)	75.1 ± 21.7[b]	79.2 ± 11.2[b]	94.1 ± 9.9[a]	91.2 ± 12.2[a]	< 0.001
VSL (µm/s)	65.2 ± 22.4[b]	69.7 ± 11.1[b]	82.2 ± 7.6[a]	81.7 ± 9.9[a]	< 0.001
VAP (µm/s)	68.7 ± 22.7[b]	73.2 ± 11.5[b]	87.2 ± 8.6[a]	85.5 ± 10.8[a]	< 0.001
ALH (µm)	1.7 ± 0.1	1.7 ± 0.3	1.8 ± 0.2	1.7 ± 0.3	> 0.05
LIN (%)	83.9 ± 14.7	87.8 ± 4.2	87.6 ± 6.4	89.8 ± 3.8	> 0.05
STR (%)	93.2 ± 1.6	95.2 ± 1.6	94.3 ± 4.8	95.6 ± 1.0	> 0.05
WOB (%)	89.1 ± 11.9	92.0 ± 6.7	92.9 ± 2.9	94.0 ± 3.7	> 0.05
BCF (Hz)	9.2 ± 1.9	8.9 ± 2.0	9.1 ± 1.7	8.9 ± 2.4	> 0.05
DFI T0 (%)	5.8 ± 6.9	5.9 ± 7.2	5.2 ± 6.3	4.6 ± 5.9	> 0.05
DFI T4 (%)	5.0 ± 5.4	5.0 ± 5.4	5.5 ± 6.7	6.0 ± 6.8	> 0.05

Different letters within the same rows indicate significant differences among treatments; TM = total motility; PM = progressive motility; PMI = plasma membrane integrity; AIS = acrosome intact sperm; VCL = curvilinear velocity; VSL = straight line velocity; VAP = average path velocity; ALH = amplitude of lateral head displacement; LIN = linearity; STR = straightness; WOB = wobble; BCF = beat cross frequency; DFI T0 = fragmentation index measured at 0 h; DFI T4 = fragmentation index measured after 4 h of incubation; S1 = sucrose 0.1 M; S2 = sucrose 0.25 M + 1% BSA. EY = egg-yolk; Values are expressed as mean ± SD.

Table 2
Effect of sperm concentration on mean values of sperm variables from vitrified-warmed samples (n = 15).

Sperm variables	Sperm concentration in vitrified 0.25 mL straws [million sperm/mL]			*P*-values
	[100]	[200]	[300]	
TM (%)	39.2 ± 18.4[b]	42.3 ± 16.0[ab]	51.3 ± 14.3[a]	< 0.05
PM (%)	27.8 ± 13.5[b]	33.6 ± 14.1[ab]	39.8 ± 15.8[a]	< 0.05
PMI (%)	32.9 ± 11.4	38.7 ± 15.1	44.2 ± 14.9	> 0.05
AIS (%)	54.1 ± 21.6	55.2 ± 15.4	55.5 ± 12.9	> 0.05
VCL (μm/s)	65.3 ± 27.1[b]	82.6 ± 17.2[a]	89.3 ± 19.9[a]	< 0.01
VSL (μm/s)	57.0 ± 25.3[b]	73.7 ± 17.3[a]	78.7 ± 17.3[a]	< 0.01
VAP (μm/s)	59.9 ± 25.9[b]	76.9 ± 17.7[a]	82.8 ± 18.2[a]	< 0.01
ALH (μm)	1.7 ± 0.5	1.8 ± 0.3	1.9 ± 0.3	> 0.05
LIN (%)	80.6 ± 22.9	88.5 ± 4.7	88.1 ± 4.1	> 0.05
STR (%)	88.3 ± 24.6	95.7 ± 1.0	95.0 ± 2.2	> 0.05
WOB (%)	85.1 ± 23.8	92.4 ± 4.5	92.8 ± 2.9	> 0.05
BCF (Hz)	8.2 ± 2.4	8.9 ± 1.3	9.3 ± 1.7	> 0.05
DFI T0 (%)	3.5 ± 2.8	3.6 ± 2.4	3.0 ± 2.4	> 0.05
DFI T4 (%)	3.7 ± 3.0	3.6 ± 3.0	3.0 ± 2.4	> 0.05

Different letters within the same rows indicate significant differences among treatments; TM = total motility; PM = progressive motility; PMI = plasma membrane integrity; AIS = acrosome intact sperm; VCL = curvilinear velocity; VSL = straight line velocity; VAP = average path velocity; ALH = amplitude of lateral head displacement; LIN = linearity; STR = straightness; WOB = wobble; BCF = beat cross frequency; DFI T0 = fragmentation index measured at 0 h; DFI T4 = fragmentation index measured after 4 h of incubation; S1 = sucrose 0.1 M; S2 = sucrose 0.25 M + 1% BSA; EY = egg-yolk; Values are expressed as mean ± SD.

concentration (300 million sperm/mL) of sperm was selected for conducting Experiment 3 for practical reasons. Results are provided in Table 2.

3.3. Experiment 3: comparison of different sperm volumes and packaging methods (0.25 mL or 0.5 mL straws) for donkey sperm vitrification

Depicts results after warming of samples vitrified in different volumes and with different packaging methods. There were no differences between volumes (100 or 160 μL) when storage was in 0.25 mL plastic straws ($P > 0.05$). Conversely, values for sperm variables were markedly decreased when 200 μL of sperm were directly loaded in 0.5 mL straws ($P < 0.001$); except for DFI, for which there were no differences between volumes or packaging methods ($P > 0.05$).

4. Discussion

In the present study, sperm concentration, volume and packing method were important considerations regarding success of donkey sperm vitrification, as well as the media composition and sucrose concentration, as has been widely reported for other species (Consuegra et al., 2018a; Diaz-Jimenez et al., 2018a; Hidalgo et al., 2018). The effect of these factors were evaluated in the present study and results were used to optimize aseptic vitrification approaches in straws for donkey sperm. Furthermore, sperm quality after vitrification in the present study was similar to that previously reported using conventional slow freezing of donkey sperm (Ortiz et al., 2015).

Experiment 1 of the present study was performed to ascertain the most suitable combination of non-permeable cryoprotectants for donkey sperm vitrification. Two different extenders conventionally used for stallion sperm were assessed in combination with two different concentrations of sucrose by taking into consideration previous studies of sperm vitrification in mammals (Sánchez et al., 2011; Pradiee et al., 2015; Diaz-Jimenez et al., 2017; Swanson et al., 2017; Caturla-Sánchez et al., 2018). From one perspective, a combination of 0.25 M sucrose prepared in distilled water plus 1% BSA has been used to protect sperm from cryodamage during vitrification (Isachenko et al., 2008), and has been successfully used in human (Isachenko et al., 2008; Sanchez et al., 2009, 2012a; Sanchez et al., 2012b), dog (Sánchez et al., 2011) and donkey (Diaz-Jimenez et al., 2017) sperm vitrification. A concentration of 0.1 M sucrose without inclusion of BSA has been used to protect wild goat (Pradiee et al., 2015), wild sheep (Pradiee et al., 2016) and stallion (Hidalgo et al., 2018) sperm after vitrification. Interestingly, there were no differences in vitrification outcomes when there were different sucrose concentrations used in combination with or without BSA in the present study. In this regard, the positive effect of this sugar might depend not only on its final concentration in the extender, but also on the addition procedures to the extender. This would explain the different sperm quality in the present study using water-diluted sucrose in comparison to results from a previous report with stallion sperm vitrification where the same concentration of sucrose (0.25 M) was used but added directly to the extender as powder (Pérez-Marín et al., 2018). There should be consideration that vitrification was performed in spheres in the stallion study; however, when compared with the same vitrification procedure, values of stallion sperm quality variables were greater after reducing the powdered sugar concentration (0.02 M) (Hidalgo et al., 2018). The relatively higher osmolality of the extender after sugar addition could be balanced by a previous dilution in water, as there were similar osmolality values when 0.25 M water diluted sucrose or 0.1 M powdered sucrose were added to the extender. Conversely, different results were obtained when the two different extenders were compared in the present study: use of the skimmed milk-egg-yolk based extender resulted in improvements

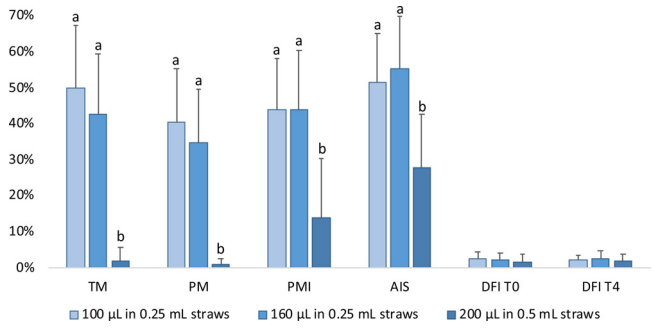

Fig. 1. Sperm variables from vitrified-warmed samples (n = 15) using different sperm volumes and packaging vessels; Samples were diluted in the extender containing egg − yolk and 0.1 M sucrose at 300 million sperm/mL; Different superscripts (a, b) indicate differences ($P < 0.05$); TM = total motility; PM = progressive motility; PMI = plasma membrane integrity; AIS = acrosome-intact sperm; DFI = DNA fragmentation index.

in values for sperm motility and velocity variables in comparison to use of the extender without egg-yolk. Vitrification of human sperm have been conventionally achieved using human tubal fluid enriched with sucrose and human serum albumin (Sanchez et al., 2012a) but in the absence of egg-yolk due to bio-security reasons. More recently, the addition of lipoproteins (from seminal plasma) was found to improve plasma and mitochondrial membrane integrity after human sperm vitrification (Isachenko et al., 2017a). Similarly, in other mammalian species, different cryopreservation extenders containing egg-yolk and skim milk as sources of lipoprotein has been used, which interact with the cell membrane during the freezing-thawing process in a species-specific way, protecting sperm cells from cold shock (Dong and VandeVoort, 2009; Ustuner et al., 2016; Bateman and Swanson, 2017). In donkeys, egg-yolk seems to protect sperm against cold shock during cryopreservation, as described by Zhang et al. (2018). In the present study, the use of the extender containing egg-yolk resulted in greater sperm motility after vitrification but there were no differences for PMI, AIS or DFI percentages. Similar results were previously reported by Dorado et al. (2014) when these extenders were compared for donkey sperm cooling up to 72 h. It appears sperm motility is favourably affected by the use of egg-yolk extenders after cold storage or cryopreservation (Parks and Graham, 1992; White, 1993; Cottorello et al., 2002); nevertheless, acrosome reaction, plasma membrane or DNA integrity were unaffected by the sucrose concentration or medium composition, which is consistent with findings in previous studies (Esteves et al., 1998; Isachenko et al., 2011a) (Fig. 1).

Sperm vitrification in straws has been performed in a wide range of sperm concentrations: from 1 million sperm/mL in humans (Sanchez et al., 2012a), to 1148 million sperm/mL in goats (Daramola et al., 2016), obtaining good sperm quality after warming in both studies. Nevertheless, in the study of Jiménez-Rabadán et al. (2015) there was poor sperm quality after ram sperm vitrification at concentrations of 20 and 50 million sperm/mL. Appropriate sperm concentrations for vitrification in straws is apparently different for animal species, which could be related to the sperm cell characteristics of each species, as previously described with use of slow freezing procedures (Alvarez et al., 2012). Human sperm heads are smaller in size in comparison with other mammalian species, therefore, human sperm have maximal cryostability (Gao et al., 1997). In addition, assisted reproductive techniques such as IVF or ICSI allow human sperm vitrification in low concentration ranges: from 1 (Sanchez et al., 2012a) to 15 million sperm/mL (Sanchez et al., 2012b); however, vitrification of donkey sperm requires greater concentrations for its use in artificial insemination of jennies (Acha et al., 2015; Oliveira et al., 2016; De Oliveira et al., 2017). In addition, it has been hypothesized that increasing the number of sperm per dose, combined with the use of the deep-horn insemination technique, could improve pregnancy rates in jennies (Miró and Papas, 2018). In the present study, donkey sperm could be vitrified with desirable outcomes at a very high concentration of 300 million sperm/mL. Similarly, in other species such as fish, dogs and sheep, there was a desirable sperm quality after cryopreservation at high sperm concentrations (Alvarez et al., 2012). Although there were no differences in the sperm quality after warming of sperm vitrified at 200 or 300 million sperm/mL in the present study, the greatest sperm concentration was selected to conduct Experiment 3 because of practical considerations and general convenience.

Experiment 3 was also conducted to assess the use of a similar insemination dose that is conventionally used in donkeys, through increasing the vitrified sperm volume. Two storage approaches and different sperm volumes were evaluated: with 0.25 into 0.5 mL straws being filled with A) 100 µL of semen, as previously described in humans (Sanchez et al., 2012a, b; Sanchez et al., 2013; Merino et al., 2015; Mansilla et al., 2016; Schulz et al., 2017), rams (Jiménez-Rabadán et al., 2015), stallions (Consuegra et al., 2018b) and donkeys (Diaz-Jimenez et al., 2017) sperm; and B) 160 µL of semen, as the maximum volume that could be placed in the inner straw. With the second approach, 200 µL of sperm were directly loaded in 0.5 mL French straws (Isachenko et al., 2011b; Slabbert et al., 2015). Unexpectedly, use of the 0.5 mL straw method resulted in almost no motile sperm after warming, in contrast to successful large volume vitrification of human sperm using this procedure (Isachenko et al., 2011b; Slabbert et al., 2015). Inconsistent with this finding, the use of the two-straw packaging method resulted in the greatest values for all the sperm variables assessed, as previously described for humans (Sanchez et al., 2012a, 2013; Merino et al., 2015; Mansilla et al., 2016; Schulz et al., 2017). These inconsistent results between species using the 0.5 mL straw technique might be a consequence of the greater cryostability of human sperm (Gao et al., 1997), and poor repeatability of the technique, as described by Katkov et al. (2012). The greater distribution of the sperm sample along the thinner straw, allow for cooling by the liquid nitrogen at more rapid rates using the two-straw method, resulting in the most suitable technique for donkey sperm vitrification. In this direction, sperm vitrification success is probably not completely related to the volume of semen stored because there were no differences with the use of 100 and 160 µL, but rather with the

distribution of the liquid nitrogen on the straw surface. Similar results were reported by Diaz-Jimenez et al. (2018b) where there were greater values for the sperm quality variables with use of the two-straw method when compared with the use of spheres for donkey sperm vitrification. Furthermore, the main inconvenience with use of the sphere method is the risk of cross-contamination during cryopreservation in liquid nitrogen tanks (bacteria transfer from infected semen pellets to sterile pellets), as was previously described by Isachenko et al. (2017a). The two-straw method of sperm vitrification not only allows for rapid cooling and for greater vitrification volumes, but also isolation of the sperm from liquid nitrogen.

In conclusion, the process of donkey sperm vitrification is affected by the factors analyzed in the present study: extender, sperm concentration, volume and storage vessel. Donkey sperm could be vitrified using an extender containing egg-yolk and 0.1 M sucrose, at 300 million sperm/mL in 0.25 mL straws with outer covers.

Author declaration

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

We are grateful to the “Centro de Selección y Reproducción Animal” (CENSYRA, Badajoz, Spain) for providing animals and facilities. This project was supported by grant AGL-2013-42726-R from MINECO (“Ministerio de Economia Industria y Competitividad”). M. Diaz-Jimenez is supported by an FPU fellowship from Spanish MECD (“Ministerio de Educación, Cultura y Deporte”, Spain). The authors thank Dr. A. Álvarez-Barrientos and Dr. JM. Lopez Martin for their help in the acquisition, analysis and interpretation of data concerning the flow cytometric assays.

References

Acha, D., Hidalgo, M., Ortiz, I., Gálvez, M.J., Carrasco, J.J., Gómez-Arrones, V., Dorado, J., 2015. Freezability of Andalusian donkey (Equus asinus) spermatozoa: effect of extenders and permeating cryoprotectants. Reprod. Fertil. Dev. 28, 1990–1998.

Alvarez, M., Tamayo-Canul, J., Anel, E., Boixo, J.C., Mata-Campuzano, M., Martinez-Pastor, F., Anel, L., de Paz, P., 2012. Sperm concentration at freezing affects post-thaw quality and fertility of ram semen. Theriogenology 77, 1111–1118.

Bateman, H.L., Swanson, W.F., 2017. Interaction of extender composition and freezing method for effective semen cryopreservation in the North American river otter (Lontra canadensis). Theriogenology 1, 26–34.

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.

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.

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. 2, 59–61.

Cottorello, A., Amancio, R., Henry, M., Borges, I., 2002. Effect of storage temperature and extenders on “in vitro” activity of donkey spermatozoa. Theriogenology 58, 325–328.

Daramola, J.O., Adekunle, E.O., Iyasere, O.S., Oke, O.E., Sorongbe, T.A., Iyanda, O.A., Kehinde, A.R., Aluko, S.P., Olaoye, I.O., Gbadebo, O.E., Falolu, L.I., Olukayode, E.O., Ajayi, R.A., Enikannaye, O.J., Osunjaiye, E.D., 2016. Effects of coconut milk alone or supplementation with pyridoxine in tris-extenders on viability of buck spermatozoa during vitrification. Small Rumin. Res. 136, 208–213.

De Oliveira, R.A., Teixeira, A.B.S., Pignataro, T.A., Freitas, M.L., Teixeira, H.C.A., De Oliveira Carvalho, J., Dode, M.A.N., Pivato, I., Budik, S., 2017. Evaluation of hypo-osmotic swelling test and supra vital staining technique as indicators for donkey semen freezability. Pferdeheilkunde 33, 159–164.

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. 4, 55–57.

Diaz-Jimenez, M., Dorado, J., Ortiz, I., Consuegra, C., Pereira, B., Gonzalez-De Cara, C.A., Aguilera, R., Mari, G., Mislei, B., Love, C.C., Hidalgo, M., 2018a. Cryopreservation of donkey sperm using non-permeable cryoprotectants. Anim. Reprod. Sci. 189, 103–109.

Diaz-Jimenez, M., Dorado, J., Pereira, B., Ortiz, I., Consuegra, C., Bottrel, M., Ortiz, I., Hidalgo, M., 2018b. Vitrification in straws conserves motility features better than spheres in donkey sperm. Reprod. Domest. Anim. 53, 1–3.

Dong, Q., VandeVoort, C.A., 2009. Effect of egg yolk on cryopreservation of rhesus monkey ejaculated and epididymal sperm. J. Androl. 30, 309–316.

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

Esteves, S.C., R.K, S., Thomas Jr., A.J., Agarwal, A., 1998. Effect of in vitro incubation on spontaneous acrosome reaction in fresh and cryopreserved human spermatozoa. Int. J. Womens Health 43, 235–242.

Evenson, D.P., 2016. The Sperm Chromatin Structure Assay (SCSA®) and other sperm DNA fragmentation tests for evaluation of sperm nuclear DNA integrity as related to fertility. Anim. Reprod. Sci. 169, 56–75.

Figueroa, E., Merino, O., Risopatrón, J., Isachenko, V., Sánchez, R., Effer, B., Isachenko, E., Farias, J.G., Valdebenito, I., 2015. Effect of seminal plasma on Atlantic salmon (Salmo salar) sperm vitrification. Theriogenology 83, 238–245.

Gao, D., Mazur, P., Critser, J.K., 1997. Fundamental cryobiology of mammalian spermatozoa. Reproductive Tissue Banking. Academic Press, San Diego, pp. 263–328.

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.

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.M.I., Risopatrón, M.J., Sánchez, R., 2008. Acrosomal status and mitochondrial activity of human spermatozoa vitrified with sucrose. Reproduction 136, 167–173.
Isachenko, E., Isachenko, V., Sanchez, R., Katkov, I.I., Kreienberg, R., 2011a. Cryopreservation of spermatozoa: Old routine and new perspectives. Principles and Practice of Fertility Preservation. Cambridge University Press, pp. 176–198.
Isachenko, V., Maettner, R., Petrunkina, A.M., Mallmann, P., Rahimi, G., Sterzik, K., Sanchez, R., Risopatron, J., Damjanoski, I., Isachenko, E., 2011b. Cryoprotectant-free vitrification of human spermatozoa in large (up to 0.5 mL) volume: a novel technology. Clin. Lab. 57, 643–650.
Isachenko, V., Isachenko, E., Petrunkina, A.M., Sanchez, R., 2012. Human spermatozoa vitrified in the absence of permeable cryoprotectants: birth of two healthy babies. Reprod. Fertil. Dev. 24, 323–326.
Isachenko, V., Rahimi, G., Mallmann, P., Sanchez, R., Isachenko, E., 2017a. Technologies of cryoprotectant-free vitrification of human spermatozoa: asepticity as criterion of effectiveness. Andrology 5, 1055–1063.
Isachenko, V., Sanchez, R., Mallmann, P., Rahimi, G., Isachenko, E., 2017b. Technology of aseptic cryoprotectant-free vitrification of human ICSI spermatozoa. Chapter 6. Methods Mol. Biol. 79–84.
Jiménez-Rabadán, P., García-Álvarez, O., Vidal, A., Maroto-Morales, A., Iniesta-Cuerda, M., Ramón, M., del Olmo, E., Fernández-Santos, R., Garde, J.J., Soler, A.J., 2015. Effects of vitrification on ram spermatozoa using free-egg yolk extenders. Cryobiology 71, 85–90.
Katkov, I., Bolyukh, V., Chernetsov, O., Dudin, P., Grigoriev, A., Isachenko, V., Isachenko, E., Lulat, A., Moskovtsev, S., Petrushko, M., Pinyaev, V., Sokol, K., Sokol, Y., Sushko, A., 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.
Mansilla, M.A., Merino, O., Risopatrón, J., Isachenko, V., Isachenko, E., Sánchez, R., 2016. High temperature is essential for preserved human sperm function during the devitrification process. Andrologia 48, 111–113.
Merino, O., Aguagüiña, W.E., Esponda, P., Risopatrón, J., Isachenko, E., Isachenko, V., Sánchez, R., 2015. Protective effect of butylated hydroxytoluene on sperm function in human spermatozoa cryopreserved by vitrification technique. Andrologia 47, 186–193.
Miró, J., Papas, M., 2018. Post–Artificial insemination endometrial inflammation and its control in donkeys. J. Equine Vet. Sci. 65, 38–43.
Oliveira, J.V.d, Oliveira, P.Vd.L.F., Melo e Oña, C.M., Guasti, P.N., Monteiro, G.A., Sancler da Silva, Y.F.R., Papa, Pd.M., Alvarenga, M.A., Dell'Aqua Jr, J.A., Papa, F.O., 2016. Strategies to improve the fertility of fresh and frozen donkey semen. Theriogenology 85, 1267–1273.
Ortiz, I., Dorado, J., Morrell, J.M., Crespo, F., Gosálvez, J., Gálvez, M.J., Acha, D., Hidalgo, M., 2015. Effect of single-layer centrifugation or washing on frozen–thawed donkey semen quality: Do they have the same effect regardless of the quality of the sample? Theriogenology 84, 294–300.
Ortiz, I., Dorado, J., Morrell, J., Gosalvez, J., Crespo, F., Jimenez, J.M., Hidalgo, M., 2017. New approach to assess sperm DNA fragmentation dynamics: fine-tuning mathematical models. J. Anim. Sci. Technol. 7, 8–23.
Parks, J.E., Graham, J.K., 1992. Effects of cryopreservation procedures on sperm membranes. Theriogenology 38, 209–222.
Pérez-Marín, C.C., Requena, F.D., Arando, A., Ortiz-Villalón, S., Requena, F., Agüera, E.I., 2018. 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 cryopreservation of wild Iberian ibex (Capra pyrenaica) spermatozoa. Theriogenology 84, 1513–1522.
Pradiee, J., Esteso, M.C., Castaño, C., Toledano-Díaz, A., Lopez-Sebastián, A., Guerra, R., Santiago-Moreno, J., 2016. Conventional slow freezing cryopreserves mouflon spermatozoa better than vitrification. Andrologia 49, 4.
Rosato, M.P., Iaffaldano, N., 2013. Cryopreservation of rabbit semen: comparing the effects of different cryoprotectants, cryoprotectant-free vitrification, and the use of albumin plus osmoprotectants on sperm survival and fertility after standard vapor freezing and vitrification. Theriogenology 79, 508–516.
Salazar Jr, J.L., Teague, S.R., Love, C.C., Brinsko, S.P., Blanchard, T.L., Varner, D.D., 2011. Effect of cryopreservation protocol on postthaw characteristics of stallion sperm. Theriogenology 76, 409–418.
Sanchez, R., Gomez, I., Samper, J.C., 2009. Chapter 15 - artificial insemination with frozen semen. In: Saunders, W.B. (Ed.), Equine Breeding Management and Artificial Insemination, second edition. pp. 175–183 Saint Louis.
Sánchez, R., Risopatrón, 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., Risopatron, J., Schulz, M., Isachenko, E., 2012a. Live birth after intrauterine insemination with spermatozoa from an oligoasthenozoospermic patient vitrified without permeable cryoprotectants. J. Androl. 33, 559–562.
Sanchez, R., Risopatrón, J., Schulz, M., Villegas, J.V., Isachenko, V., Isachenko, E., 2012b. Vitrified sperm banks: the new aseptic technique for human spermatozoa allows cryopreservation at -86 °C. Andrologia 44, 433–435.
Sanchez, R., Schulz, M., Risopatrón, J., Isachenko, V., Isachenko, E., 2013. Vitrificación de espermatozoides: una alternativa a la inyección intracitoplasmática de espermatozoides en paciente con oligoastenozoospermia severa. Rev. Int. Andrologãa 11, 36–39.
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, 1–3.
Slabbert, M., du Plessis, S., Huyser, C., 2015. Large volume cryoprotectant-free vitrification: an alternative to conventional cryopreservation for human spermatozoa. Andrologia 47, 594–599.
Swanson, W.F., Bateman, H.L., Vansandt, L.M., 2017. Urethral catheterization and sperm vitrification for simplified semen banking in felids. Reprod. Domest. Anim. 52, 255–260.
Ustuner, B., Alcay, S., Toker, M.B., Nur, Z., Gokce, E., Sonat, F.A., Gul, Z., Duman, M., Ceniz, C., Uslu, A., Sagirkaya, H., Soylu, M.K., 2016. Effect of rainbow trout (Oncorhynchus mykiss) seminal plasma on the post-thaw quality of ram semen cryopreserved in a soybean lecithin-based or egg yolk-based extender. Anim. Reprod. Sci. 164, 97–104.
White, I., 1993. Lipids and calcium uptake of sperm in relation to cold shock and preservation: a review. Reprod. Fertil. Dev. 5, 639–658.
Zhang, H., Ye, H., Shao, Y., Wu, S., Yu, J., Ji, C., Wang, S., Zeng, S., 2018. The effects of egg yolk concentration and particle size on donkey semen preservation. J. Equine Vet. Sci. 65, 19–24.