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RESEARCH ARTICLE

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Effect of warming temperatures on donkey sperm vitrification in 0.5 mL straws in comparison to conventional freezing

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Abstract

Aim of study: There is little information about vitrification of sperm in large volumes (up to 0.5 mL). This study aimed to develop the vitrification technique in 0.5 mL straws in donkey sperm, evaluating the effect of three warming temperatures.

Area of study: Cordoba, Spain.

Material and methods: Ejaculates from five donkeys were divided in four groups: one control subjected to conventional slow freezing (C) and three vitrified in 0.5 mL straws and warmed using different protocols (W1: 37ºC/30s, W2: 43ºC/20s and W3: 70ºC/8s + 37ºC/52s). Sperm motility, kinematic parameters, plasma membrane and acrosome integrity were evaluated. Conventional freezing resulted in significantly higher values for total (42.7 ± 19.6%), and progressive motility (30.3 ± 16.7%), plasma membrane (49.1 ± 10.4%) and acrosome integrity (39.6 ± 14.5%) respect to vitrification method.

Main results: Values after warming ranged between 0.2-2.8% for total motility; 0.2-2.1% for progressive motility; 5.5-20.0% for plasma membrane integrity and 14.5-29.8% for acrosome integrity in all warming protocols after sperm vitrification. However, no differences were found between W3 and C for kinematic parameters; and W3 resulted in significantly higher values for membrane integrity (20.0 ± 11.0%) in comparison to W1 (5.5 ± 3.6%) and W2 (9.3 ± 8.4%).

Research highlights: High warming rates seem to be better for donkey sperm vitrification in large volumes; but this methodology is still not an alternative to conventional sperm freezing.

Additional keywords: equine; cryopreservation; large-volume vitrification; aseptic; thawing.

Abbreviations used: ALH (amplitude of lateral head displacement); AO (acridine orange); BCF (beat cross frequency); CASA (computer-assisted sperm motility analysis); FITC (fluorescein isothiocyanate); LIN (linear coefficient); PI (propidium iodide); PM (progressive motility); PMI (plasma membrane integrity); PNA (peanut agglutinin); STR (straightness coefficient); TM (total motility); VAP (average path velocitiy); VCL (curvilinear velocity); VSL (straight line velocity); WOB (wobble coefficient).

Authors' contributions: MDJ and MH contributed to all sections. JD, IO, EI and VI contributed to the study design, data analysis and interpretation. CC, BP and CV performed the experiment. All authors read and approved the final version of the manuscript.

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Introduction

Since sperm vitrification in straws was first described in human by Isachenko *et al.* (2005), it has been investigated in different animals including ram (Jiménez-Rabadán *et al.*, 2015), goat (Daramola *et al.*, 2016), fish (Zilli *et al.*, 2018) and horse (Consuegra *et al.*, 2018; Restrepo *et al.*, 2019). This technique has also been developed in donkey sperm in absence of permeable cryoprotectants, showing promising

results regarding *in vitro* parameters (Diaz-Jimenez *et al.*, 2017, 2018b). In this sense, vitrification in straws would be an interesting alternative to conventional slow freezing in this species, whose results after artificial insemination in jennies using frozen sperm are very poor, with pregnancy rates ranging from 0 to 36% (Rota *et al.*, 2012; Oliveira *et al.*, 2016). However, the relatively small volume employed (100 µL) seems to be a major disadvantage in comparison to conventional freezing (Slabbert *et al.*, 2015). To solve this limitation, vitrification of large volumes of sperm (up to 0.5 mL) has been developed in human beings, showing similar (Slabbert *et al.*, 2015) and higher (Isachenko *et al.*, 2011) sperm motility parameters after warming when compared to conventional freezing. Nevertheless, to our knowledge, there is little information about its use in animals, and if so, no motile sperm was recovered after warming (Restrepo *et al.*, 2019).

Temperature and time during thawing have shown to affect sperm motility, plasma membrane and acrosome integrity after conventional freezing (Athurupana *et al.*, 2015); and they seem to be also key factors for sperm vitrification success (Mansilla *et al.*, 2016). A few studies comparing warming temperatures after vitrification could be found in the literature: Mansilla *et al.* (2016) reported optimum results in vitrification of human sperm in 0.25 mL straws after warming at 42ºC in comparison to lower temperatures; however, heterogeneous results were obtained after sperm vitrification following the spheres method. In this sense, high warming temperatures (up to 60ºC) seemed to be more adequate for mouflon (Pradiee *et al.*, 2016) and ibex (Pradiee *et al.*, 2015) than for dog (Caturla-Sánchez *et al.*, 2018) sperm, in which slower warming rates (37ºC) showed better sperm quality after vitrification. To the best of our knowledge, there are no previous studies comparing different temperatures and times for warming after vitrification in donkey sperm. Therefore, the aim of this study was to vitrify donkey sperm in 0.5 mL straws using different temperatures and rates for warming in comparison to conventional freezing regarding sperm motility parameters, plasma membrane and acrosome integrity.

Material and methods

Semen collection and processing

Samples were obtained from five healthy, fertile, Andalusian donkeys from 3 to 8 years of age. All the experiments were performed in accordance with the Ethical Committee for Animal Experimentation of the University of Cordoba (project no. 31/08/2017/105) and the Spanish law for animal welfare and experimentation (BOE, 2013). Ejaculates (three per donkey, n=15) were collected using a Missouri artificial vagina and assessed before freezing or vitrification for sperm volume, concentration, motility and plasma membrane integrity as previously described (Diaz-Jimenez *et al.*, 2018a). After that, gel-free semen samples were diluted 1:1 (v/v) with a commercial milk fraction-based extender (INRA-96, IMV Technol., L'Aigle, France), centrifuged at 400 × g for 7 min at 22°C in a corning-adapted centrifuge (Eppendorf, model 5702 RH, Eppendorf AG, Hamburg, Germany) and resuspended to achieve a final concentration of 200 × 10^6 total sperm/mL. A skimmed milk-egg-yolk based extender for equine sperm was used (Gent, Minitüb, Tiefenbach, Germany) (i) containing glycerol (De Oliveira *et al.*, 2017) for conventional slow freezing (control, C); or (ii) without glycerol, but adding instead sucrose (S) to reach a final concentration of 0.1 M (437 mOsm/kg) for sperm vitrification (Diaz-Jimenez *et al.*, 2018b).

Conventional freezing and vitrification procedures

Conventional freezing and thawing were performed following a previous protocol for donkey sperm cryopreservation (Diaz-Jimenez *et al.*, 2018a). Briefly, extended semen was slowly cooled to 5ºC for 2 hours into an Equitainer (Hamilton Research, Inc. Ipswich, MA, USA), and loaded in 0.5 mL plastic French straws. Thereafter, straws were placed in racks 2.5 cm above the surface of liquid nitrogen (LN_2) for 5 min and then stored in tanks. Thawing was performed by immersion in a 37ºC water bath for 30s. For the vitrification technique, sperm extended in S was cooled at 5ºC during 1 hour (Diaz-Jimenez *et al.*, 2017) and loaded into 0.5 mL straws (CBS™, Cryo Bio System, Paris, France). Straws were hermetically sealed at both sides and directly plunged into LN_2 for vitrification (Isachenko *et al.*, 2011; Slabbert *et al.*, 2015). Vitrified samples were stored in LN_2 tank until use. For warming, straws were immersed in a water bath at different temperatures and times according to the following protocols: (W1) 37ºC for 30s, (W2) 43ºC for 20s and (W3) 70ºC for 8s and immediately maintained at 37ºC for 52s. After thawing or warming, all samples were extended with INRA-96 to a final concentration of 25 × 10^6 sperm/mL for sperm evaluation.

Computer-assisted sperm motility analysis (CASA)

Sperm motility was objectively evaluated by the Sperm Class Analyzer (SCA v5.4, Microptic S.L.,

Barcelona, Spain). For each evaluation, two 5 µL drops and three random microscopic fields per drop were evaluated. A minimum of 200 spermatozoa were analysed. The trajectory of each spermatozoa was determined by the software and the following parameters of sperm motion were measured: total (TM, percentage of spermatozoa with a mean average path velocity >10 µm/s) and progressive motility (PM, percentage of motile spermatozoa with >75% of the straightness coefficient); curvilinear (VCL, µm/s), straight line (VSL, µm/s) and average path velocities (VAP, µm/s); linear coefficient (LIN, VSL/VCL×100); straightness coefficient (STR, VSL/VAP×100); wobble coefficient (WOB, VAP/VCL×100); amplitude of lateral head displacement (ALH, µm) and beat cross frequency (BCF, Hz).

Assessment of sperm plasma membrane integrity

Plasma membrane integrity (PMI, %) was assessed using the double stain propidium iodide (PI) with acridine orange (AO) from the VitalTest stain (Halotech DNA SL, Madrid, Spain) as previously described (Diaz-Jimenez *et al.*, 2018a). Briefly, a 10 µL aliquot of diluted semen was mixed with 1 µL PI and 1 µL AO and evaluated under epifluorescence microscopy (Olympus BX40, Tokyo, Japan) using a U-ND25-2 filter (a 460–490 nm excitation filter). At least 200 sperm were counted and those with green emission (viable spermatozoa) were recorded.

Assessment of acrosome integrity

The sperm acrosomes were evaluated using the peanut agglutinin (PNA) conjugated with fluorescein isothiocyanate (FITC) and PI staining, as described by Dorado *et al.* (2014). In brief, 10 µL of diluted sample (25 × 10^6 sperm/mL) was spread on a slide and permeabilized with 70% ethanol for 30 s. A mixture of 10 µL PI and 20 µL FITC-PNA was spread over each smear after 5 min incubation at 38ºC, and the slides were incubated in a dark, moist chamber for 30 min at 4ºC. Slides were then evaluated under epifluorescence microscopy (Olympus BX40, Tokyo, Japan) using the 100x oil immersion objective and the U-ND25-2 filter (a 460–490 nm excitation filter). Sperm acrosomes were classified as intact (spermatozoa displaying intensively bright green fluorescence of the acrosomal cap) and damaged (spermatozoa displaying disrupted fluorescence, fluorescent band at the equatorial segment or no fluorescence, indicating damages to the outer acrosomal membrane). A total of 200 spermatozoa were evaluated, and the percentage of acrosome-intact sperm was recorded (AIS, %).

Statistical analysis

Statistical analysis was performed using the Statistical Analysis Software (SAS, v.9.0, SAS Institute Inc., Cary, NC, USA) and data was presented as mean ± standard deviation (SD). Results were compared between treatments using a general lineal model procedure (PROC GLM) followed by Duncan multiple comparison test as *post-hoc* test. Animals and ejaculates were considered as random factors. Significant differences were considered when $p<0.05$.

Results and discussion

Sperm parameters before cryopreservation had the following average values: gel-free volume = 61.1 ± 25.3 mL, sperm concentration = (354.5 ± 101.2) × 10^6 sperm/mL, TM = 89.5 ± 6.4%, PM = 61.0 ± 15.7% and PMI = 61.8 ± 12.7%. Conventional freezing showed significantly ($p<0.001$) higher results than vitrification for TM = 42.7 ± 19.6%, PM = 30.3 ± 16.7%, PMI = 49.1 ± 10.4% and AIS = 39.6 ± 14.5% despite the warming procedure employed (Fig. 1). No differences ($p>0.05$) were observed among warming temperatures after vitrification for TM (W1: 0.2 ± 0.4%, W2: 2.8 ± 4.3% and W3: 2.4 ± 2.1%) and PM (W1: 0.2 ± 0.4%, W2: 1.2 ± 1.8% and W3: 2.1 ± 1.9%). However, PMI was significantly higher in W3 (20.0 ± 11.0%) than W1 (5.5 ± 3.6%) and W2 (9.3 ± 8.4%) ($p<0.05$). The percentage of AIS resulted also in higher values in W3 (29.8 ± 4.5) than W1 (14.5 ± 3.7) ($p<0.05$).

Parameters regarding velocity features showed no differences ($p>0.05$) between W3 and C for VCL VSL and VAP. Protocol W1 resulted in the lowest values for LIN, STR, WOB, ALH and BCF. Mean values of kinematic sperm parameters assessed are represented in Table 1.

Vitrification of sperm in large volumes has been mostly researched in human showing similar (Slabbert *et al.*, 2015) or higher (Isachenko *et al.*, 2011) sperm quality after warming when compared to conventional freezing. However, in the present study conventional freezing conserved motility, plasma membrane and acrosome integrity better than vitrification in 0.5 mL straws in donkey sperm. A possible explanation for this difference is the higher cryostability of the human sperm due to its structural composition (Isachenko *et al.*, 2003). Cryostability of sperm cells has been negatively correlated with the size, probably due to higher water content and lower internal compaction of the bigger ones (Katkov *et al.*, 2012). Human sperm head size is smaller in comparison to other mammalian species, including equine (Garner, 2006;

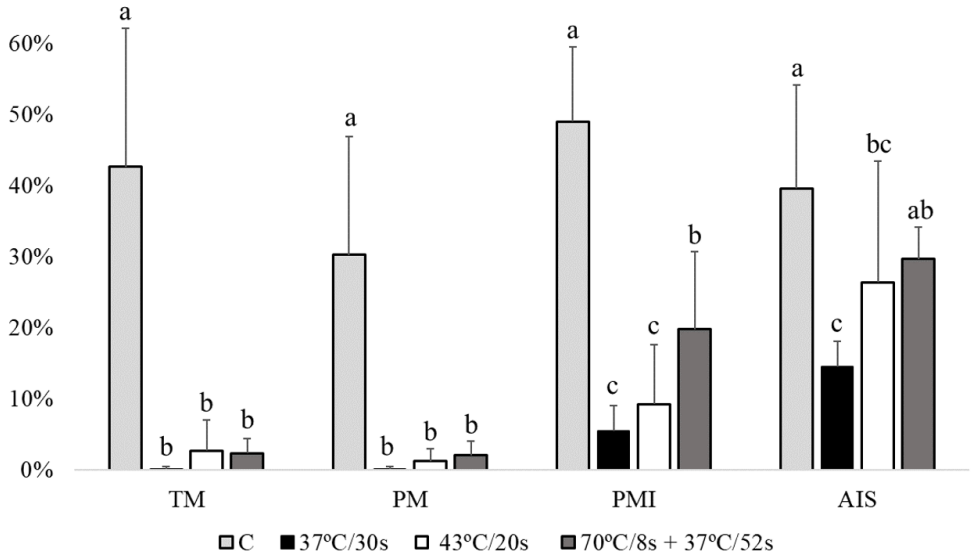

Figure 1. Percentages of total (TM) and progressive (PM) motility, plasma membrane integrity (PMI) and acrosome integrity (AIS) from donkey sperm after conventional slow freezing (C) compared to vitrification in 0.5 mL straws warmed following different protocols. Different superscripts (a, b, c) indicate significant differences between treatments (p<0.05). Values are expressed as mean ± SD.

Jiménez-Rabadán *et al.*, 2015; Arraztoa *et al.*, 2017). This characteristic probably makes human sperm less vulnerable to cryopreservation damage (Arraztoa *et al.*, 2017), which allows successful vitrification in higher volumes. Additionally, differences between these species exist also in plasma membrane composition of the sperm (cholesterol and phospholipid types) and they are also determinants of sperm cryostability. Besides, sucrose concentration employed in the present study was selected from preliminary tests in donkey sperm vitrification in spheres (30 µL), in which 0.1 M sucrose conserved sperm parameters better than the extender commonly used for human sperm vitrification: 0.25 M sucrose prepared in distilled water plus serum albumin (Isachenko *et al.*, 2008; Sanchez *et al.*, 2012 a,b). In this sense, the extender requirements for guaranteeing vitrification of donkey sperm packed in 0.5 mL straws may be different in comparison to spheres method. Therefore, further studies testing higher non-permeable cryoprotectant concentration; other sugars, such as trehalose (El-Badry *et al.*, 2017; Schulz *et al.*, 2017); as well as other substances supplementation, such as

Table 1. Mean values of kinematic parameters from samples subjected to conventional slow freezing (C) and vitrification in 0.5 mL straws warmed using different protocols.

Kinematic parameters	C	Warming procedures after vitrification		
		37ºC/30s	43ºC/20s	70ºC/8s+37ºC/52s
VCL, µm/s	75.3±11.8^{a}	13.6±30.4^{b}	29.5±38.5^{b}	79.3±56.6^{a}
VSL, µm/s	64.0±10.9^{a}	13.1±29.4^{b}	25.2±32.9^{b}	70.2±49.2^{a}
VAP, µm/s	69.4±11.8^{a}	13.3±29.7^{b}	23.4±34.5^{b}	72.6±51.0^{a}
LIN, %	84.9±2.8^{a}	19.3±43.1^{c}	34.1±44.0^{bc}	62.5±43.5^{ab}
STR, %	92.3±1.4^{a}	19.8±44.2^{c}	38.3±49.4^{bc}	67.8±46.8^{ab}
WOB, %	92.0±2.2^{a}	19.5±43.6^{c}	35.6±46.0^{bc}	64.4±44.7^{ab}
ALH, µm	1.8±1.3^{a}	0.2±0.4^{c}	0.9±1.1^{bc}	1.6±1.3^{ab}
BCF, Hz	8.8±0.4^{a}	1.3±2.9^{c}	4.1±5.3^{bc}	7.7±4.7^{ab}

VCL: curvilinear velocity. VSL: straight line velocity. VAP: average path velocity. LIN: linear coefficient. STR: straightness. WOB: wobble. ALH: amplitude of head displacement. BCF: beat cross frequency. Different superscripts $^{(a, b)}$ indicate significant differences between protocols (p<0.05). Values are expressed as mean ± SD.

cyclodextrins (Madison *et al.*, 2013; Partyka *et al.*, 2016; Lone, 2018) would be interesting to improve results after sperm vitrification in 0.5 mL straws in this species.

In addition, human sperm vitrification has been traditionally performed after sperm selection of motile and viable cells; whereas sperm selection techniques prior to freezing in stallions has shown no improvements on sperm motility after thawing (Hidalgo *et al.*, 2017). In this regard, it is also important to point out that vitrification in 0.5 mL straws has obtained despair results regarding species, even among studies in human sperm, as previously described by Katkov *et al.* (2012). Conventional freezing has shown better results than sperm vitrification in other domestic animals as dog (Caturla-Sánchez *et al.*, 2018), and ram (Pradiee *et al.*, 2016); but the spheres method was used in both.

In order to maximize the possibilities of sperm vitrification success in large volumes in donkeys, different warming protocols were compared. Unexpectedly, no differences in motility percentages between warming rates were found; although higher temperatures during shorter periods of time (W3, 70ºC/8s + 37ºC/52s), showed significantly higher membrane integrity and velocity features than lower temperatures during longer periods of warming (W1 and W2). Moreover, W3 warming protocol outcomes similar sperm velocity parameters (VSL, VCL and VAP) after vitrification than those obtained after conventional freezing. In this regard, hyperactivation of sperm cells might be suspected; however, it has been generally defined as a change in the pattern of sperm motility (Hinrichs & Loux, 2012), which has been set in stallion sperm as (i) VCL>180 µm/s and mean ALH>12 µm (Rathi *et al.*, 2001) or (ii) VCL between 225 and 350 µm/s and ALH between 8 and 13 µm (Hinrichs & Loux, 2012). In this sense, none of the kinematic sperm parameters assessed in this study had those characteristics, so sperm hyperactivation after vitrification could be dismissed. On the other hand, sperm velocity parameters ascertained by CASA assessments have been previously correlated with in vivo fertility in mammals (Gomendio & Roldan, 2008). Unfortunately, fertility has not been assessed in this study.

Additionally, the better sperm quality found in the present study using high warming rates (W3), is in agreement to those results previously reported by Pradiee *et al.* (2016), in which rapid warming prevented damage after vitrification in spheres in mouflon sperm (60ºC better than 37ºC). Similarly, Athurupana *et al.* (2015) found higher motility and membrane integrity sperm values using 70ºC/8s + 39ºC/52s in comparison to 39ºC/60s in conventional freezing of boar sperm. On the contrary, a recent work comparing warming procedures after dog sperm vitrification in spheres (Caturla-Sánchez *et al.*, 2018), showed better sperm quality after warming at 37ºC rather than 65ºC. In the present study, almost no motile sperm were recovered using the conventional thawing protocol for 0.5 mL French straws (W1, 37ºC/30s), which may be explained because the warming rate for sperm vitrification in large volumes must be raised in comparison to conventional freezing. In this sense, W2 (43ºC/20s) was expected to outcome good sperm quality after vitrification because similar warming protocols were successfully employed in donkey (Diaz-Jimenez *et al.*, 2017, 2018b) and human (Mansilla *et al.*, 2016) sperm vitrification using 0.25 mL straws. Nevertheless, volume and package were modified in this report in comparison to the previous ones, so the vitrification procedure was completely different. The ratio between volume and surface occupied by the semen samples in 0.5 mL straws was different in comparison to 0.25 mL straws, reducing thereby the cooling rates achieved in this study. According to this, the ultra-rapid cooling rates required for successful 'kinetic vitrification' of equine sperm may not occur (Hidalgo *et al.*, 2018), which could explain the poor sperm quality obtained after warming.

Sperm motility after vitrification yielded substantially lower percentages in comparison to conventional freezing, being therefore deficient for its use in routine artificial insemination practices. However, percentage of sperm viability using the W3 warming protocol was higher than other warming rates tested, reaching values around 20%. In this regard, selection of sperm cells after warming might be performed (Ortiz *et al.*, 2015) and possibly be used for advanced reproduction techniques such as "intracytoplasmic sperm injection" (ICSI) (Gonzalez-Castro & Carnevale, 2019).

In conclusion, high warming rates seem to be better for donkey sperm vitrification in large volumes; but this methodology is still not an alternative to conventional sperm freezing.

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