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Cryopreservation of Andalusian donkey (*Equus asinus*) spermatozoa: Use of alternative energy sources in the freezing extender affects post-thaw sperm motility patterns but not DNA stability

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ABSTRACT

The aim of this study was to compare the effect of three sugars and Equex paste in a freezing extender for donkey sperm cryopreservation. Ejaculates ($n = 18$) were collected from six Andalusian donkeys of proven fertility were pooled (two ejaculates per pool) and cryopreserved using a freezing extender containing three different sugars (glucose, fructose and sorbitol), with or without the addition of Equex paste. Sperm quality was assessed before and after freezing-thawing for motility, morphology, plasma membrane integrity, acrosome integrity and DNA integrity. The use of sorbitol in the freezing extender improved total and progressive sperm motility ($P < 0.05$) and amplitude of lateral head displacement ($P < 0.01$), but it reduced the values for other sperm motility variables compared with glucose ($P < 0.001$). The use of fructose resulted in a reduction in values for most CASA variables ($P < 0.05$), whereas addition of Equex paste did not have any beneficial effect on values for these variables ($P > 0.05$). Glucose was more effective in maintaining sperm morphology ($P < 0.05$), while there was no beneficial effect with the addition of Equex paste ($P > 0.05$). Supplementation of fructose and Equex paste in the freezing extender decreased plasma membrane integrity ($P < 0.05$) as compared with glucose, but there were no differences between treatments for acrosome and DNA integrity ($P > 0.05$), even after 24 h of incubation. The use of different sugar sources in the extender could affect the *in vitro* post-thaw quality of cryopreserved donkey spermatozoa, with sorbitol being an interesting alternative for improving the sperm quality. Results of the present study indicate the use of Equex paste could negatively affect post-thaw outcomes for sperm viability in this species.

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1. Introduction

The domestic donkey is an important livestock species mainly used for draught purposes around the world (Shai et al., 2016). The Andalusian donkey is an endangered breed (Real Decreto 2129/2008, regulating the National Catalogue of Endangered Species) from the south of Spain, widely used due to its adaptation to the harsh environmental conditions of the area. This breed is characterized by a very small population size and isolated breeding areas as well as by a reduced genetic variability and increased relatedness among individuals within herds (Herrera and Lopez Rodriguez, 2008). Thus, the use of cryopreserved semen from jacks selected from different herds and regions could be an important management strategy to cope with this situation.

Cryopreservation of semen from horses, and particularly from donkeys, is still a challenge (Smits et al., 2012). Sperm freezing capacity is highly variable with large differences in post-thaw quality among individuals (Vidament et al., 1997). In practice, the main strategy to improve the performance of the process was the adaptation of the techniques developed for stallions, considering the apparent similarity between species. Thus, the effect of different variables such as cooling and freezing rates (Demyda-Peyrás et al., 2018), compounds used and amounts included in the extenders (Oliveira et al., 2016; Montoya et al., 2017) as well as the use of permeating (Acha et al., 2016) and non-permeating cryoprotectants (Diaz-Jimenez et al., 2018) have been analyzed to improve the overall outcome. For example, the use of permeating cryoprotectants glycerol and ethylene-glycol improved post-thaw sperm motility rates of donkey sperm (Acha et al., 2016) and pregnancy rates in jennies (Rota et al., 2012), and the use of non-permeating agents such as sugars and polyols in horse extenders has been extensively reported (Squires et al., 2004; Pojprasath et al., 2011; Consuegra et al., 2018). Other than the promising results when conducting a previous study (Diaz-Jimenez et al., 2018), the use of these compounds in donkey semen cryopreservation have been very little.

Monosaccharides are naturally present in the seminal plasma of animals (Størset et al., 1978). It has been proposed that the primary function of these compounds is to regulate osmolarity and function as energy substrates which could be utilized by spermatozoa (Rodriguez-Gil, 2006). In donkeys, glucose (Talluri et al., 2017) and fructose (Trimeche et al., 1997) concentrations are related to sperm motility in fresh but not in frozen-thawed samples. Similarly, the use of sorbitol, a polyol derived from the reduction of glucose largely used in semen extenders (Alvarez and Storey, 1993), has not yet been assessed in donkeys even though there was an improved motility in rams (Wu et al., 2016), stallions (Pojprasath et al., 2011) and boars (Chanapiwat et al., 2012) with use of sorbitol.

Additives are components with an important function in several semen extenders. Among these, Equex paste (sodium lauryl sulfate, EP) is a surfactant molecule currently utilized in freezing extenders of diverse domestic animal spermatozoa (Jimenez, 1987; Nizański and Bielas, 2003; Niasari-Naslaji et al., 2008; Morton et al., 2010; Wu et al., 2013). Its addition, there has been improvement of post-thaw survival rates of spermatozoa, probably by releasing more lipids and lipoproteins from the egg yolk to the freezing extender, thus increasing sperm membrane stability during cooling or thawing (Wu et al., 2013). Even though membrane damage is a major cause of lack of fertility in donkey frozen-thawed sperm (Rota et al., 2010), the use of EP has not yet been tested in this species.

The aim of the present study, therefore, was to compare the effect of three different sugars (glucose, fructose, and sorbitol) as source of energy, and the possible interaction with Equex paste in a freezing extender for donkey semen cryopreservation.

2. Materials and methods

2.1. Experimental animals

This study was conducted during the March-May 2013 breeding season at the Equine Center for Assisted Reproduction of the Centro de Selección y Reproducción Animal (CENSYRA), Badajoz, Spain. Ejaculates from six healthy, mature (7–18 years old) Andalusian donkeys of proven fertility were collected twice a week. The animals were housed in individual paddocks and fed daily with hay and grain. Water was available *ad libitum*. All animal procedures were conducted in accordance with the Spanish laws for animal welfare and experimentation (Real Decreto 53/2013).

2.2. Freezing extenders

Glucose monohydrate (454337) and glycerol (453752) were obtained from CARLO ERBA Reagents SRL (Milano, Italy). Lactose monohydrate (LA00601000) and potassium citrate monohydrate (PO01860500) were purchased from Scharlau Chemie SA (Sentmenant, Barcelona, Spain). Raffinose pentahydrate (195670250) was from Acros Organics - Thermo Fisher Scientific (New Jersey, MA, USA). Sodium citrate dihydrate (131655.1210) and apyrogenic ultrapure water (131074) were supplied by Panreac Química SLU (Barcelona, Spain). Penicillin G sodium (P0142) and D-Fructose (F0801) were purchased from Duchefa Biochemie BV (Haarlem, The Netherlands). Gentamycin sulfate (G570) was purchased from PhytoTechnology Laboratories (Lenexa, KS, USA), D-Sorbitol (BP439) was from Fisher Bioreagents - Fisher Scientific (Pittsburgh, Pennsylvania, USA), and HEPES (H3375) was purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Equex paste (13560/0030) was from Minitub GmbH (Tiefenbach, Germany).

The basic extender (BE) used for sperm cryopreservation was a modified INRA 82 (Vidament et al., 2000) composed of 500 mL commercial ultra-heat treated skim milk, 25 g glucose monohydrate, 1.5 g lactose monohydrate, 1.5 g raffinose pentahydrate, 0.41 g potassium citrate monohydrate, 0.25 g sodium citrate dihydrate, 4.76 g HEPES, 50 mg gentamycin sulfate, 50,000 IU Penicillin G sodium, and apyrogenic ultrapure water to make 1000 mL, supplemented with 2.5% (v:v) glycerol and 2% (v:v) centrifuged egg yolk.

The three different sugar sources glucose (GLU), fructose (FRU) and sorbitol (SOR) with or without the addition of EP were tested in six treatments, as follows: 1) BE (GLU, as control); 2) GLU plus 0.5% (v:v) EP (GLU-EP); 3) BE in which GLU was replaced by 25 g/L FRU; 4) FRU plus 0.5% (v:v) EP (FRU-EP); 5) BE in which GLU was replaced by 25 g/L SOR; and 6) SOR plus 0.5% (v:v) EP (SOR-EP). The

Table 1
Composition of the six freezing extenders used in this experiment.

Components	Freezing extender					
	GLU	GLU-EP	FRU	FRU-EP	SOR	SOR-EP
Ultra-heat treated skim milk (ml)	500	500	500	500	500	500
Lactose monohydrate (g/l)	1.5	1.5	1.5	1.5	1.5	1.5
Raffinose pentahydrate (g/l)	1.5	1.5	1.5	1.5	1.5	1.5
Potassium citrate monohydrate (g/l)	0.41	0.41	0.41	0.41	0.41	0.41
Sodium citrate dehydrate (g/l)	0.25	0.25	0.25	0.25	0.25	0.25
HEPES (g/l)	4.76	4.76	4.76	4.76	4.76	4.76
Apyrogenic ultrapure water (ml)	500	500	500	500	500	500
Main sugar						
Glucose monohydrate (g/l)	25	25	–	–	–	–
Fructose (g/l)	–	–	25	25	–	–
Sorbitol (g/l)	–	–	–	–	25	25
Glycerol (%)	2.5	2.5	2.5	2.5	2.5	2.5
Egg yolk (%)	2	2	2	2	2	2
Equex paste (%)	–	0.5	–	0.5	–	0.5
pH	6.65	6.67	6.63	6.64	6.69	6.69
Osmotic pressure (mOsm/kg)	776	742	674	719	748	668

All extenders contained 50,000 IU/l Penicillin G, 50 mg/l Gentamycin sulfate.
GLU: Modified INRA 82-egg yolk-glycerol as basic extender; GLU-EP: Modified INRA 82-egg yolk-glycerol-Equex paste; FRU: Replacing glucose with fructose in the basic extender; FRU-EP: Replacing glucose with fructose in the basic extender plus Equex paste; SOR: Replacing glucose with sorbitol in the basic extender; SOR-EP: Replacing glucose with sorbitol in the basic extender plus Equex paste.

composition of the freezing extenders is detailed in Table 1. All the extenders were prepared before the beginning of the study and kept frozen at -18 °C in single-use aliquots until use. Previously, osmolality and pH were assessed using a Type 6 micro-osmometer (Löser Messtechnik, Berlin, Germany) and a pH meter (HI 2211-02, Hanna Instruments Inc., Woonsocket, RI, USA), respectively (Table 1).

2.3. Semen collection, handling, and cryopreservation

A total of 18 ejaculates (three per jack) were collected using a Missouri-model artificial vagina (Minitüb GmbH, Tiefenbach, Germany) with an in-line gel filter (Minitüb GmbH, Tiefenbach, Germany) to allow the collection of free-gel semen. Immediately after collection, the gel-free fraction of each ejaculate was evaluated to determine volume, sperm concentration, and seminal pH according to our routine methodology (Dorado et al., 2014). At the same time, an aliquot of each ejaculate was also diluted in pre-warmed (37 °C) skim milk base extender (EquiPro, REF. 13570/0201, Minitüb GmbH, Tiefenbach, Germany) to a final concentration of 25×10^6 spermatozoa/mL, which was subsequently used as needed to conduct the appropriate analyses. Only ejaculates with motility, morphology and plasma membrane integrity $\geq$ 70% were included in the study.

Ejaculates from two alternative donkeys were pooled (nine in total) at the time of each collection day to avoid the introduction of uncontrolled male-to-male variation (Dorado et al., 2014). Pooled semen samples were diluted in a 1:1 proportion (semen:extender, v:v) in EquiPro and divided into aliquots (as many as the number of treatments). The aliquots of the diluted semen were centrifuged at 400 x *g* for 7 min at room temperature (20–22 °C) and the sperm pellets were re-suspended in the tested extenders (GLU, GLU-EP, FRU, FRU-EP, SOR, and SOR-EP) to reach a concentration of 200×10^6 spermatozoa/mL. Extended semen was maintained at room temperature for 10 min.

The cryopreservation protocol was based on previously published procedures of Ortiz et al. (2015a). Briefly, vials were slowly cooled in an Equitainer™ I (Hamilton Research, Inc., Danvers, MA, USA) to 5 °C for 120 min. Each cooled sample was packaged in 0.5 mL plastic straws (Minitüb GmbH, Tiefenbach, Germany) at 5 °C and frozen in liquid nitrogen (LN_2) vapor 2.5 cm above the surface for 5 min, after which time they were plunged directly into LN_2. After 1 month of storage, straws were thawed individually in a heater bath (Incudigit horizontal, Instrumentación Científica y Técnica SL, Lardero, Spain) at 37 °C for 30 s, diluted to 25×10^6 spermatozoa/mL with the appropriate extender, and evaluated for sperm quality as subsequently described in this manuscript.

2.4. Semen analysis

Semen assessments were performed after recovery in EquiPro extender and after thawing in the tested extenders. For assessment, diluted semen samples were incubated at 37 °C for 5 (fresh semen) or 10 (frozen-thawed samples) min.

Sperm motility was assessed using the CASA (computer-assisted sperm analyzer) system (Sperm Class Analyzer - SCA®, Microptic SL, Barcelona, Spain), as described by Miró et al. (2005) for donkeys. Briefly, each semen sample was assessed by evaluating three 5-µL drops of the sample using a phase contrast microscope (Eclipse 50i, Nikon, Tokyo, Japan) with a pre-warming stage at 37 °C at 100× magnification. Two microscopic fields per drop were randomly filmed, including a minimum of 200 spermatozoa. Objects incorrectly identified as spermatozoa were minimized using the playback function. With respect to setting parameters of the program, spermatozoa with a mean average path velocity (VAP) < 10 µm/s were considered immotile, spermatozoa with a VAP > 90 µm/s

were considered as rapid, and spermatozoa deviating < 25% from a straight line were designated as linearly motile. The sperm motion variables quantified were total motility (TM; %), progressive motility (PM; %), curvilinear velocity (VCL; μm/s), straight line velocity (VSL; μm/s), average path velocity (VAP; μm/s), linearity (LIN, as VSL/VCL; %), straightness (STR, as VSL/VAP; %), wobble (WOB, as VAP/VCL; %), beat cross frequency (BCF; Hz), and amplitude of lateral head displacement (ALH; μm). Definitions of these descriptors of sperm motility can be found in Dorado et al. (2007).

Sperm morphology was examined using light microscopy (Olympus BH-2, Olympus Optical Co., LTD, Tokyo, Japan) on smears stained with Diff-Quick® (Medion Diagnostics AG, Düdingen, Switzerland) staining (Brito, 2007). At least 200 spermatozoa per slide were counted to determine the percentage of spermatozoa with abnormal morphology (ASM, %), scoring different types of sperm abnormalities (head, midpiece and tail abnormalities).

Sperm membrane integrity was assessed at 400 X magnification using epi-fluorescence microscopy (Olympus BX40, Tokyo, Japan) with propidium iodide (PI) combined with acridine orange (AO) double staining from the Vital-Test® kit (Halotech SL, Madrid, Spain), as described by Dorado et al. (2014). At least 200 spermatozoa were counted, considering green spermatozoa as membrane-intact spermatozoa (MIS, AO+; %).

To evaluate sperm acrosomes, the PI/peanut agglutinin-fluorescein isothiocyanate (FITC-PNA) double stain (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was used as described by Dorado et al. (2014). At least 200 ethanol permeabilized spermatozoa, stained with PI/FITC-PNA, were evaluated on each slide using 1000 X magnification with epi-fluorescence microscopy. Values were expressed as percentages of acrosome-intact spermatozoa (AIS, PI+/FITC-PNA+; %) and acrosome-reacted spermatozoa (ARS, PI +/FITC-PNA-; %).

Sperm DNA fragmentation (sDF) was evaluated dynamically in each post-thaw semen sample using the Halomax® Kit (Halotech DNA SL, Madrid, Spain), as described by Ortiz et al. (2015b) for donkeys. An aliquot of the original samples was incubated for 24 h at 37 °C and assessed at time (T) 0 (baseline), T6 and T24h. All the slides were stained using a commercial kit for green fluorescence staining (Halotech DNA SL, Madrid, Spain). For each sample, a minimum of 300 spermatozoa were counted using a fluorescence microscope at 400× magnification. The percentage of spermatozoa with fragmented DNA (showing chromatin dispersion halos with a double diameter compared with the core) was calculated and expressed as a percentage of the total sperm count (sDFI, %).

2.5. Statistical analysis

Sperm variables subjected to different semen extenders were evaluated trough general linear mixed model (GLM), considering stallion and treatment as a fixed effect and pool as a random effect. Differences among treatments were analyzed using Bonferroni´s *post-hoc* test. Moreover, a Dunnett's *posthoc* test was used to compare the value of each morphological parameter between all treatments and fresh semen samples. A *t*-test was used to compare differences within the same treatment with or without EP addition. Normality of the data distributions and variance homogeneity were assessed using the Kolmogorov–Smirnov and Cochran-Bartlett tests, respectively. Results are expressed as mean ± SEM. All the analyses were performed using the Statistica V12 statistical package.

3. Results

Data included in Table 2, indicate values for all CASA variables except for STR were differentially affected ($P < 0.01$) when using the freezing extenders selected for evaluation in the present study. In general, the use of SOR with or without 0.5% (v/v) EP (SOR-EP and SOR, respectively) resulted in the most desirable post-thaw motility values (total and progressive motility, $P < 0.05$), but there was a reduction in the values for three sperm velocities (VCL, VSL and VAP), LIN, WOB, and BCF in comparison with the values with use of the control extender (GLU; $P < 0.001$). Furthermore, the addition of SOR to the freezing extender led to increased mean ALH values (1.70 μm; $P < 0.01$). Inconsistent with this finding, supplementation with GLU resulted in intermediate percentages for sperm motility (TM: 49.04%; PM: 39.52%) and the greatest values for the three sperm velocities, LIN, WOB and BCF ($P < 0.05$). The supplementation with FRU resulted in a reduction values for most CASA variables ($P < 0.05$; Table 2). Interestingly, depending on the sugar supplemented, the addition of EP had different effects on values for sperm motility variables. For example, supplementation with EP did not have any beneficial effect on values for CASA variables ($P > 0.05$; *t*-test) when EP was added to both the GLU- and FRU-supplemented extenders (GLU-EP and FRU-EP, respectively). Mean PM values, however, were increased ($P < 0.05$) with supplementation of SOR-EP compared with SOR supplementations to extenders (Table 2).

Sperm morphology was also affected by the type of freezing extender supplemented (Table 3). In general, spermatozoa were affected less ($P < 0.05$) when there was supplementation with GLU compared with the other sugars (FRU and SOR). There was no beneficial effect as a result of supplementation with EP ($P > 0.05$; Table 3).

Addition of both FRU and EP to the freezing extender decreased plasma membrane integrity ($P < 0.05$) in comparison with the control (GLU; Table 3). There were no differences ($P > 0.05$) between treatments for acrosome integrity; however, the percentage of acrosome-intact spermatozoa was numerically greater with use of the GLU-supplemented extender.

The sDFI increased ($P < 0.05$) during incubation at 6 and 24 h compared with T0 (baseline), but there were no differences ($P > 0.05$) with use of the different extenders at any time point (T0, T6 and T24; Table 4).

4. Discussion

Cryopreservation of donkey sperm is still problematic due to the differences observed among individuals in response to use of the same protocol. The primary strategy for improvement of the outcomes was the use of different freezing extenders (Montoya et al.,

Table 2
Mean values (± SEM) of CASA motility variables assessed in three ejaculates collected from six Andalusian donkeys (n = 18) and nine pooled semen samples of donkeys (two ejaculates per pool) after freezing-thawing using six different freezing extenders (GLU, GLU-EP, FRU, FRU-EP, SOR, and SOR-EP).

Parameters	Fresh semen	Frozen-thawed samples					
		GLU	GLU-EP	FRU	FRU-EP	SOR	SOR-EP
TM (%)[1]	88.82 ± 0.52	49.04 ± 0.32^{c}	47.00 ± 0.37^{b}	38.41 ± 0.26^{a}	48.29 ± 0.44^{c}	58.36 ± 0.33^{d}	58.71 ± 0.47^{d}
PM (%)[2]	63.72 ± 0.35	39.52 ± 0.34^{d}	34.60 ± 0.27^{c}	28.11 ± 0.24^{a}	32.61 ± 0.35^{b}	40.20 ± 0.29^{d}	41.96 ± 0.37^{e}
VCL (μm/s)	125.14 ± 0.67	88.07 ± 0.48^{d}	75.17 ± 0.34^{b}	78.51 ± 0.41^{c}	72.20 ± 0.33^{a}	78.62 ± 0.36^{c}	72.94 ± 0.34^{a}
VSL (μm/s)	96.77 ± 0.71	74.94 ± 0.47^{e}	63.24 ± 0.40^{c}	65.30 ± 0.44^{d}	58.28 ± 0.32^{a}	63.60 ± 0.39^{c}	60.44 ± 0.37^{b}
VAP (μm/s)	114.30 ± 0.65	82.78 ± 0.51^{e}	69.26 ± 0.40^{c}	72.30 ± 0.44^{d}	65.33 ± 0.34^{a}	72.16 ± 0.40^{d}	66.83 ± 0.37^{b}
LIN (%)	0.77 ± 0.00	0.79 ± 0.00^{c}	0.78 ± 0.00^{c}	0.75 ± 0.00^{b}	0.73 ± 0.00^{a}	0.73 ± 0.00^{a}	0.75 ± 0.00^{b}
STR (%)	0.83 ± 0.00	0.85 ± 0.00^{a}	0.86 ± 0.00^{a}	0.83 ± 0.00^{a}	0.82 ± 0.00^{a}	0.81 ± 0.00^{a}	0.83 ± 0.00^{a}
WOB (%)	0.91 ± 0.00	0.89 ± 0.00^{d}	0.88 ± 0.00^{c}	0.86 ± 0.00^{b}	0.85 ± 0.00^{a}	0.86 ± 0.00^{b}	0.86 ± 0.00^{b}
ALH (μm)	2.45 ± 0.02	1.68 ± 0.01^{b}	1.64 ± 0.01^{a}	1.68 ± 0.01^{b}	1.70 ± 0.01^{c}	1.70 ± 0.00^{c}	1.63 ± 0.00^{a}
BCF (Hz)	7.45 ± 0.03	7.24 ± 0.03^{d}	7.19 ± 0.03^{d}	6.68 ± 0.04^{c}	6.60 ± 0.03^{b}	6.38 ± 0.02^{a}	6.70 ± 0.02^{c}

GLU: Modified INRA 82-egg yolk-glycerol as basic extender; GLU-EP: Modified INRA 82-egg yolk-glycerol-Equex paste; FRU: Replacing glucose with fructose in the basic extender; FRU-EP: Replacing glucose with fructose in the basic extender plus Equex paste; SOR: Replacing glucose with sorbitol in the basic extender; SOR-EP: Replacing glucose with sorbitol in the basic extender plus Equex paste; TM: total motility; PM: progressive motility; VCL: curvilinear velocity; VSL: straight line velocity; VAP: average path velocity; LIN: linearity; STR: straightness; WOB: wobble; ALH: amplitude of lateral head displacement; BCF: beat cross frequency.
Different superscript letters (a–e) in the same row indicate differences between treatments ($P < 0.05$, GLM followed by a Bonferroni´s *post-hoc* test for homogeneous groups).
[1] Total motility is defined as the percentage of spermatozoa with a mean velocity > 10 μm/s.
[2] Progressive motility is defined as the percentage of spermatozoa with a mean velocity > 90 μm/s and straightness > 75%.

Table 3
Mean values (± SEM) of sperm morphology, acrosome integrity, and membrane integrity in three ejaculates collected from six Andalusian donkeys (n = 18) and nine pooled semen samples of donkeys (two ejaculates per pool) after freezing-thawing using six different freezing extenders (GLU, GLU-EP, FRU, FRU-EP, SOR, and SOR-EP).

Parameters		Fresh semen	Frozen-thawed samples					
			GLU	GLU-EP	FRU	FRU-EP	SOR	SOR-EP
AF		5.33 ± 0.54	9.89 ± 1.58^{a}	11.27 ± 1.92^{a}	13.70 ± 1.94^{b}	16.83 ± 2.80^{b}	13.43 ± 2.05^{b}	13.14 ± 1.86^{b}
	Head abnormalities	0.22 ± 0.13	0.34 ± 0.17	0.69 ± 0.16	0.59 ± 0.28	0.75 ± 0.28	0.79 ± 0.31	0.42 ± 0.16
	Midpiece abnormalities	1.70 ± 0.27*	2.58 ± 0.7^{a*}	3.73 ± 1.08^{ab}	2.73 ± 0.33^{a*}	4.05 ± 1.01^{ab}	3.76 ± 0.79^{ab}	4.60 ± 0.95^{b}
	Tail Abnormalities	3.41 ± 0.47	6.98 ± 1.26^{b*}	6.85 ± 1.30^{b*}	10.38 ± 1.72^{ab}	12.03 ± 2.83^{a}	8.88 ± 1.74^{ab*}	8.12 ± 1.26^{ab*}
MIS		88.00 ± 3.12	46.96 ± 2.82^{b}	42.83 ± 2.77^{ab}	39.67 ± 2.01^{ab}	36.54 ± 2.84^{a}	39.50 ± 4.27^{ab}	38.09 ± 4.33^{ab}
AIS		95.84 ± 0.70*	90.48 ± 2.14*	86.97 ± 2.05	87.35 ± 2.33	86.16 ± 2.73	86.80 ± 2.27	85.62 ± 2.59

GLU: Modified INRA 82-egg yolk-glycerol as basic extender; GLU-EP: Modified INRA 82-egg yolk-glycerol-Equex paste; FRU: Replacing glucose with fructose in the basic extender; FRU-EP: Replacing glucose with fructose in the basic extender plus Equex paste; SOR: Replacing glucose with sorbitol in the basic extender; SOR-EP: Replacing glucose with sorbitol in the basic extender plus Equex paste; ASM: Abnormal sperm morphology; MIS: Membrane-intact spermatozoa; AIS: Acrosome-intact spermatozoa.
Different superscript letters (a–b) in the same row indicate differences between treatments ($P < 0.05$, GLM followed by a Bonferroni´s *post-hoc* test for homogeneous groups).
Asterisks (*) in the same row indicates differences between the treatment and the fresh sperm (GLM followed by a Dunnett´s *post-hoc* test for homogeneous groups).

2017), supplements (Bottrel et al., 2018; Zhang et al., 2018) and freezing conditions (Demyda-Peyrás et al., 2018) developed for stallions. In the present study, this experimental approach was utilized to evaluate the effect of three different sugars (glucose, fructose, and sorbitol) and a surfactant (Equex paste) on post-thaw semen quality of Andalusian donkeys.

It is well known that the use of different sugar sources can markedly affect post-thaw mammalian sperm velocity and motility patterns (Bucci et al., 2011). Results from the present study confirmed the results from this previous study and in addition there was a differential effect based on the type of sugar used (GLU, FRU or SOR). In general, addition of SOR resulted in the greatest mean PM and TM values, although sperm velocities were less compared with supplementation with GLU (control extender) and FRU (only VSL). Similar results have been reported in rams (Wu et al., 2016) when there was inclusion of SOR to the extender with there not only being improvements in post-thaw motility rates, there was also increased sperm velocities (VCL and VAP) as a result of SOR supplementation. Sorbitol, a sugar alcohol (polyol) and natural trace compound in seminal plasma of stallions (Mann, 1975), has been proposed as a substitute energy source for maintenance of baseline motility of frozen-thawed spermatozoa after long periods of incubation, probably by varying the sperm membrane permeability and thereby diminishing the stress on the plasma membrane

Table 4
Mean (± SEM) DNA fragmentation index (sDFI) values associated with the freezing extenders compared with incubation time (immediately after thawing (T0) and after 6 h (T6) and 24 h (T24) of incubation).

Freezing extender	sDFI		
	T0	T6	T24
GLU	18.12 ± 1.85	40.35 ± 2.53	51.36 ± 2.80
GLU-EP	18.53 ± 2.49	38.91 ± 2.50	47.12 ± 2.07
FRU	19.18 ± 1.70	36.59 ± 2.43	49.59 ± 3.48
FRU-EP	19.06 ± 2.61	39.33 ± 1.31	48.83 ± 2.89
SOR	19.05 ± 1.27	35.88 ± 1.90	46.98 ± 1.54
SOR-EP	19.18 ± 1.64	43.32 ± 5.04	52.02 ± 3.70

GLU: Modified INRA 82-egg yolk-glycerol as basic extender; GLU-EP: Modified INRA 82-egg yolk-glycerol-Equex paste; FRU: Replacing glucose with fructose in the basic extender; FRU-EP: Replacing glucose with fructose in the basic extender plus Equex paste; SOR: Replacing glucose with sorbitol in the basic extender; SOR-EP: Replacing glucose with sorbitol in the basic extender plus Equex paste. Different superscript letters (a–b) in the same row indicate differences between treatments ($P < 0.05$, GLM followed by a Bonferroni´s *post-hoc* test for homogeneous groups).

during the freezing process (Alvarez and Storey, 1993). Although the SOR-protective mechanism against cryoinjury is not clearly understood, its effect could be similar to that of glycerol due to the structural similarity, producing a synergistic effect (Wu et al., 2016). The structure of the SOR molecule, however, may hinder its penetration into the cell with this penetration only being possible as a result of the presence of a SOR dehydrogenase (SORD) located near the plasma membrane. Even at relatively lesser concentrations, SOR is an efficient cryoprotectant of mice sperm and has functions through SORD (Cao et al., 2009). The SOR compound, however, was not detected in boar sperm (Chanapiwat et al. (2012), where there was the use of SOR in semen extenders, there was a negative effect of this supplementation. Even though there is the presence of SORD in donkey semen, it has not yet been determined in donkeys whether there is this negative effect similar to that detected in stallion spermatozoa (Pojprasath et al., 2011), suggesting that this pathway could be responsible for the results observed in the current study. The metabolism of FRU to ATP, however, is relatively little in spermatozoa of several domestic species, thus, there is relatively lesser motility in anaerobic conditions (Storey, 2008). In donkeys, semen contains very little FRU (Mann et al., 1963). Trimeche et al. (1997) reported that addition of FRU to the INRA 82 extender did not improve sperm motility or velocities, which is consistent with the results in the present study where with FRU supplementation there was the least values for sperm velocity and motility rates. The effect of FRU could be mediated by increased production of oxygen radicals generated during FRU metabolism as a result of functions in the glycolytic pathway, compared to other sugars such as GLU (Goodson et al., 2012; Visconti, 2012). This thought is also supported by the increased percentages of motility and the more vigorous motility patterns observed in the sperm when there was supplementation with GLU. In donkeys, a positive correlation between GLU supplementation and motility has been previously described (Talluri et al., 2017). This monosaccharide is also associated with the induction of capacitation in human (Williams and Ford, 2001), mice (Goodson et al., 2012) and domestic species (Bucci et al., 2010) spermatozoa, probably inducing an increase in the hypermotility of the viable sperm. The localization and relative abundance of GLUT transporters, however, may vary between species (Bucci et al., 2010); therefore, more study is necessary to elucidate what the situation is in this regard in donkey semen.

Equex paste has been widely used as an additional protective compound in the cryopreservation of sperm from dogs (Rota et al., 1997), cats (Axnér et al., 2004), bulls (Chaveiro et al., 2006), rams (Šterbenc et al., 2014), boars (Buranaamnuay et al., 2009), and wildlife species (de Paz et al., 2012; Favoretto et al., 2012), with there being different outcomes. To our knowledge, its effect has not yet been evaluated in donkey jack sperm. In the present study, this additive had inconsistent effects based on the sugar source supplemented in the extender. As an example, while supplementation with SOR-EP and GLU-EP resulted in improvements, supplementation with GLU-EP decreased progressive motility. Even though the differences were about 10% of the variation in motility in all the cases, these were statistically significant. It has been proposed that the EP effect is mediated by its capacity to induce the release of low-density lipoproteins from egg-yolk and by functioning as a surfactant to stabilize and protect cell membranes against cold shock and freezing injury (Anel et al., 2010). The interaction between EP and constituents in the freezing extender used for supplementations was also evident, probably as a result of the different chemical composition and glycerol concentrations (Schembri et al., 2003; Wu et al., 2013). In the present study, because only sugar sources differed between treatments (with or without EP), it is hypothesized that the interaction observed was caused by the sugars used for supplementations. Nevertheless, the addition of EP to the freezing extender led to reduced sperm velocities (VCL, VSL, and VAP) compared with EP-free extenders. There were similar results with dog sperm, in which supplementation with Equex® STAMP (sodium dodecyl sulfate) improved sperm motility but decreased the velocity of spermatozoa (Bencharif et al., 2012). Even though there is a lack of previous research on this topic in donkeys, a possible variation in the density of the extender, mediated by the increase of the free lipoproteins available or a direct detrimental effect on spermatozoa, could account for results in the present study.

Sperm morphology was affected by the sugar used for supplementations but not by the use of EP. The greatest protective effect resulted with use of GLU-supplemented extender, which increased the percentage of morphologically normal spermatozoa. Glucose metabolism involves the hexokinase family of enzymes, which can bind to mitochondria, exerting tissue protection against cell death (Sun et al., 2008). Although this mechanism has not been previously associated with the use of FRU or SOR supplementations to extenders, a protective effect on the tail morphology was also observed with use of the SOR-supplemented extender, without there

being any differences with fresh semen. The SOR compound can osmo-stabilize the sperm plasma membrane by protecting the phospholipid components against fusion and cell content leakage (Hincha and Hagemann, 2004), with results having already been reported for this in horses (Pojprasath et al., 2011), but not donkeys. The FRU supplementation increased the number of sperm tail abnormalities when compared with fresh sperm. This monosaccharide is present in seminal fluid of Poitou jacks (Trimeche et al., 1997) and in stallion sperm, although at a lesser concentration (Gamboa et al., 2011). In the present study, however, the use of FRU as extender additive did not result in production of any changes in the post-thaw quality of jack sperm. To our knowledge, this is the first study where there was assessment of the effect of this sugar when there was supplementation to donkey semen extender, suggesting that addition of FRU as the main energetic substrate to the extender should be avoided. Further studies, however, are necessary to confirm whether this is a problem when FRU is used for extender supplementations.

A similar pattern was observed in the percentage of spermatozoa having intact plasma membranes post-thawing, where GLU had the greatest protective effect. On the contrary, there was the least protective effect with use of the FRU-EP extender. Regarding acrosome integrity, there were no differences between treatments, even though with only supplementation with GLU were there similar results to those observed for fresh semen (Dunnett´s test). In stallions, FRU-based extenders were less effective in protecting stallion spermatozoa against the destabilization of the acrosomal membrane with GLU and SOR being more effective options (Pojprasath et al., 2011). There, however, are no data available for the domestic donkey. The greater protective effect of GLU in donkey semen cryopreservation, therefore, could be attributed to differences in sperm metabolism between species. The effect of thawing rate and post-thaw temperature on sperm membranes should be taken into account, as has been previously described for stallion semen (Pugliesi et al., 2014). Determination of the existence of an interaction among sugars, Equex paste, thawing rate and post-thaw temperature, therefore, may be an interesting topic for further studies.

It was noteworthy that addition of EP to the freezing extenders in the present study did not improve sperm membrane integrity, although it was proposed that this addition may serve for additional protection in several mammal species such as dogs (Rota et al., 1997), goats (Anakkul et al., 2010) and sheep (Šterbenc et al., 2014). Although in previous studies there has not been assessment of the effects of EP on frozen-thawed donkey spermatozoa, results of the present study are consistent with those reported by Jimenez (1987), where there were no differences in the post-thaw quality of stallion spermatozoa, with or without the use of EP. Accordingly, it is suggested based on results in the present and previous studies that the beneficial effect of this molecule as a supplement to semen extender could be species-specific.

The DNA fragmentation is a determining factor in the fertilizing capacity of spermatozoa (Karoui et al., 2012). Cryopreservation results in stress to spermatozoa, leading to a decreased membrane stability, oxidative stress and reduced DNA integrity (Kopeika et al., 2015). In horses and donkeys, sperm DNA fragmentation increases as the post-thaw time progresses, without being affected by the cryopreservation procedure (Lopez-Fernandez et al., 2007; Cortes-Gutierrez et al., 2008). Findings in the present study are consistent with these previous results because DNA fragmentation was greater after 6 and 24 h of incubation with all the treatments assessed, without there being differences between these at similar time points. Although a small protective effect of DNA integrity in spermatozoa stored in a diluent has been reported for other species (Perez-Llano et al., 2006; Bottrel et al., 2018), results of the present study indicate supplementation with different sugars does not affect DNA quality.

5. Conclusions

Considering results of the present study, supplementation of the freezing extender with different sugar sources could affect the *in vitro* post-thaw quality of cryopreserved donkey spermatozoa. Furthermore, addition of sorbitol improved the overall sperm motility pattern variables, while fructose supplementation was the least beneficial supplement when using the experimental conditions of the present study. Furthermore, addition of glucose to the freezing extender resulted in an enhanced sperm morphology than fructose and sorbitol, without affecting plasma membrane and acrosome integrity. In addition, Equex paste supplementation to the freezing extender affected negatively the post-thaw quality of the Andalusian donkey spermatozoa.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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