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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26 **ABSTRACT**

27 The aim of this study was to compare the effect of three sugars and Equex paste in a freezing 28 extender for donkey sperm cryopreservation. Ejaculates (*n* = 18) were collected from six 29 Andalusian donkeys of proven fertility were pooled (two ejaculates per pool) and 30 cryopreserved using a freezing extender containing three different sugars (glucose, fructose 31 and sorbitol), with or without the addition of Equex paste. Sperm quality was assessed before 32 and after freezing-thawing for motility, morphology, plasma membrane integrity, acrosome 33 integrity and DNA integrity. The use of sorbitol in the freezing extender improved total and 34 progressive sperm motility ($P<0.05$) and amplitude of lateral head displacement ($P<0.01$), 35 but it reduced the values for other sperm motility variables compared with glucose ($P<0.001$). 36 The use of fructose resulted in a reduction in values for most CASA variables ($P<0.05$), 37 whereas addition of Equex paste did not have any beneficial effect on values for these 38 variables ($P>0.05$). Glucose was more effective in maintaining sperm morphology ($P<0.05$), 39 while there was no beneficial effect with the addition of Equex paste ($P>0.05$). 40 Supplementation of fructose and Equex paste in the freezing extender decreased plasma 41 membrane integrity ($P<0.05$) as compared with glucose, but there were no differences 42 between treatments for acrosome and DNA integrity ($P>0.05$), even after 24 h of incubation. 43 The use of different sugar sources in the extender could affect the *in vitro* post-thaw quality of 44 cryopreserved donkey spermatozoa, with sorbitol being an interesting alternative for 45 improving the sperm quality. Results of the present study indicate the use of Equex paste 46 could negatively affect post-thaw outcomes for sperm viability in this species.

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48 *Keywords:* Frozen-thawed semen; Donkey; Glucose; Fructose; Sorbitol

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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

75 al., 2018), the use of these compounds in donkey semen cryopreservation have been very
76 little.

77 Monosaccharides are naturally present in the seminal plasma of animals (Størset et al.,
78 1978). It has been proposed that the primary function of these compounds is to regulate
79 osmolarity and function as energy substrates which could be utilized by spermatozoa
80 (Rodriguez-Gil, 2006). In donkeys, glucose (Talluri et al., 2017) and fructose (Trimeche et al.,
81 1997) concentrations are related to sperm motility in fresh but not in frozen-thawed samples.
82 Similarly, the use of sorbitol, a polyol derived from the reduction of glucose largely used in
83 semen extenders (Alvarez and Storey, 1993), has not yet been assessed in donkeys even
84 though there was an improved motility in rams (Wu et al., 2016), stallions (Pojprasath et al.,
85 2011) and boars (Chanapiwat et al., 2012) with use of sorbitol.

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

95 The aim of the present study, therefore, was to compare the effect of three different
96 sugars (glucose, fructose, and sorbitol) as source of energy, and the possible interaction with
97 Equex paste in a freezing extender for donkey semen cryopreservation.
98
99 **2. Materials and methods**

100 *2.1. Experimental animals*

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

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109 *2.2. Freezing extenders*

110 Glucose monohydrate (454337) and glycerol (453752) were obtained from CARLO
111 ERBA Reagents SRL (Milano, Italy). Lactose monohydrate (LA00601000) and potassium
112 citrate monohydrate (PO01860500) were purchased from Scharlau Chemie SA (Sentmenant,
113 Barcelona, Spain). Raffinose pentahydrate (195670250) was from Acros Organics - Thermo
114 Fisher Scientific (New Jersey, MA, USA). Sodium citrate dihydrate (131655.1210) and
115 apyrogenic ultrapure water (131074) were supplied by Panreac Química SLU (Barcelona,
116 Spain). Penicillin G sodium (P0142) and D-Fructose (F0801) were purchased from Duchefa
117 Biochemie BV (Haarlem, The Netherlands). Gentamycin sulfate (G570) was purchased from
118 PhytoTechnology Laboratories (Lenexa, KS, USA), D-Sorbitol (BP439) was from Fisher
119 Bioreagents - Fisher Scientific (Pittsburgh, Pennsylvania, USA), and HEPES (H3375) was
120 purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Equex paste
121 (13560/0030) was from Minitub GmbH (Tiefenbach, Germany).

122 The basic extender (BE) used for sperm cryopreservation was a modified INRA 82
123 (Vidament et al., 2000) composed of 500 mL commercial ultra-heat treated skim milk, 25 g
124 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 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 ºC 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 ºC) skim milk base extender (EquiPro, REF. 13570/0201, Minitüb GmbH, Tiefenbach, Germany) to a final concentration of 25 x 10^6 spermatozoa/mL, which was subsequently used as needed to conduct the appropriate analyses. Only ejaculates with motility, morphology and plasma membrane integrity ≥ 70% were included in the study.

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149 Ejaculates from two alternative donkeys were pooled (nine in total) at the time of each
150 collection day to avoid the introduction of uncontrolled male-to-male variation (Dorado et al.,
151 2014). Pooled semen samples were diluted in a 1:1 proportion (semen:extender, v:v) in
152 EquiPro and divided into aliquots (as many as the number of treatments). The aliquots of the
153 diluted semen were centrifuged at 400 x *g* for 7 min at room temperature (20-22 ºC) and the
154 sperm pellets were re-suspended in the tested extenders (GLU, GLU-EP, FRU, FRU-EP,
155 SOR, and SOR-EP) to reach a concentration of 200 x 10^6 spermatozoa/mL. Extended semen
156 was maintained at room temperature for 10 min.
157 The cryopreservation protocol was based on previously published procedures of Ortiz
158 et al. (2015a). Briefly, vials were slowly cooled in an Equitainer™ I (Hamilton Research,
159 Inc., Danvers, MA, USA) to 5 ºC for 120 min. Each cooled sample was packaged in 0.5 mL
160 plastic straws (Minitüb GmbH, Tiefenbach, Germany) at 5 ºC and frozen in liquid nitrogen
161 (LN_2) vapor 2.5 cm above the surface for 5 min, after which time they were plunged directly
162 into LN_2. After 1 month of storage, straws were thawed individually in a heater bath
163 (Incudigit horizontal, Instrumentación Científica y Técnica SL, Lardero, Spain) at 37 ºC for
164 30 s, diluted to 25 x 10^6 spermatozoa/mL with the appropriate extender, and evaluated for
165 sperm quality as subsequently described in this manuscript.
166
167 *2.4. Semen analysis*
168 Semen assessments were performed after recovery in EquiPro extender and after
169 thawing in the tested extenders. For assessment, diluted semen samples were incubated at 37
170 ºC for 5 (fresh semen) or 10 (frozen-thawed samples) min.
171 Sperm motility was assessed using the CASA (computer-assisted sperm analyzer)
172 system (Sperm Class Analyzer - SCA®, Microptic SL, Barcelona, Spain), as described by
173 Miró et al. (2005) for donkeys. Briefly, each semen sample was assessed by evaluating three

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5-μL drops of the sample using a phase contrast microscope (Eclipse 50i, Nikon, Tokyo, Japan) with a pre-warming stage at 37 ºC at 100 X 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

199 described by Dorado et al. (2014). At least 200 ethanol permeabilized spermatozoa, stained
200 with PI/FITC-PNA, were evaluated on each slide using 1000 X magnification with epi-
201 fluorescence microscopy. Values were expressed as percentages of acrosome-intact
202 spermatozoa (AIS, PI+/FITC-PNA+; %) and acrosome-reacted spermatozoa (ARS,
203 PI+/FITC-PNA-; %).

204 Sperm DNA fragmentation (sDF) was evaluated dynamically in each post-thaw semen
205 sample using the Halomax® Kit (Halotech DNA SL, Madrid, Spain), as described by Ortiz et
206 al. (2015b) for donkeys. An aliquot of the original samples was incubated for 24 h at 37 °C
207 and assessed at time (T) 0 (baseline), T6 and T24 h. All the slides were stained using a
208 commercial kit for green fluorescence staining (Halotech DNA SL, Madrid, Spain). For each
209 sample, a minimum of 300 spermatozoa were counted using a fluorescence microscope at 400
210 X magnification. The percentage of spermatozoa with fragmented DNA (showing chromatin
211 dispersion halos with a double diameter compared with the core) was calculated and
212 expressed as a percentage of the total sperm count (sDFI, %).

213

214 *2.5. Statistical analysis*

215 Sperm variables subjected to different semen extenders were evaluated trough general
216 linear mixed model (GLM), considering stallion and treatment as a fixed effect and pool as a
217 random effect. Differences among treatments were analyzed using Bonferroni´s *post-hoc* test.
218 Moreover, a Dunnett´s *posthoc* test was used to compare the value of each morphological
219 parameter between all treatments and fresh semen samples. A t-test was used to compare
220 differences within the same treatment with or without EP addition. Normality of the data
221 distributions and variance homogeneity were assessed using the Kolmogorov–Smirnov and
222 Cochran-Bartlett tests, respectively. Results are expressed as mean ± SEM. All the analyses
223 were performed using the Statistica V12 statistical package.

224

225 **3. Results**

226 Data included in Table 2, indicate values for all CASA variables except for STR were
227 differentially affected (*P*<0.01) when using the freezing extenders selected for evaluation in
228 the present study. In general, the use of SOR with or without 0.5% (v/v) EP (SOR-EP and
229 SOR, respectively) resulted in the most desirable post-thaw motility values (total and
230 progressive motility, *P*<0.05), but there was a reduction in the values for three sperm
231 velocities (VCL, VSL and VAP), LIN, WOB, and BCF in comparison with the values with
232 use of the control extender (GLU; *P*<0.001). Furthermore, the addition of SOR to the freezing
233 extender led to increased mean ALH values (1.70 µm; *P*<0.01). Inconsistent with this
234 finding, supplementation with GLU resulted in intermediate percentages for sperm motility
235 (TM: 49.04%; PM: 39.52%) and the greatest values for the three sperm velocities, LIN, WOB
236 and BCF (*P*<0.05). The supplementation with FRU resulted in a reduction values for most
237 CASA variables (*P*<0.05; Table 2). Interestingly, depending on the sugar supplemented, the
238 addition of EP had different effects on values for sperm motility variables. For example,
239 supplementation with EP did not have any beneficial effect on values for CASA variables
240 (*P*>0.05; t-test) when EP was added to both the GLU- and FRU-supplemented extenders
241 (GLU-EP and FRU-EP, respectively). Mean PM values, however, were increased (*P*<0.05)
242 with supplementation of SOR-EP compared with SOR supplementations to extenders (Table
243 2).

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

248 Addition of both FRU and EP to the freezing extender decreased plasma membrane
249 integrity ($P<0.05$) in comparison with the control (GLU; Table 3). There were no differences
250 ($P>0.05$) between treatments for acrosome integrity; however, the percentage of acrosome-
251 intact spermatozoa was numerically greater with use of the GLU-supplemented extender.
252 The sDFI increased ($P<0.05$) during incubation at 6 and 24 h compared with T0
253 (baseline), but there were no differences ($P>0.05$) with use of the different extenders at any
254 time point (T0, T6 and T24).
255
256 **4. Discussion**
257 Cryopreservation of donkey sperm is still problematic due to the differences observed
258 among individuals in response to use of the same protocol. The primary strategy for
259 improvement of the outcomes was the use of different freezing extenders (Montoya et al.,
260 2017), supplements (Bottrel et al., 2018; Zhang et al., 2018) and freezing conditions
261 (Demyda-Peyrás et al., 2018) developed for stallions. In the present study, this experimental
262 approach was utilized to evaluate the effect of three different sugars (glucose, fructose, and
263 sorbitol) and a surfactant (Equex paste) on post-thaw semen quality of Andalusian donkeys.
264 It is well known that the use of different sugar sources can markedly affect post-thaw
265 mammalian sperm velocity and motility patterns (Bucci et al., 2011). Results from the present
266 study confirmed the results from this previous study and in addition there was a differential
267 effect based on the type of sugar used (GLU, FRU or SOR). In general, addition of SOR
268 resulted in the greatest mean PM and TM values, although sperm velocities were less
269 compared with supplementation with GLU (control extender) and FRU (only VSL). Similar
270 results have been reported in rams (Wu et al., 2016) when there was inclusion of SOR to the
271 extender with there not only being improvements in post-thaw motility rates, there was also
272 increased sperm velocities (VCL and VAP) as a result of SOR supplementation. Sorbitol, a

273 sugar alcohol (polyol) and natural trace compound in seminal plasma of stallions (Mann,
274 1975), has been proposed as a substitute energy source for maintenance of baseline motility of
275 frozen-thawed spermatozoa after long periods of incubation, probably by varying the sperm
276 membrane permeability and thereby diminishing the stress on the plasma membrane during
277 the freezing process (Alvarez and Storey, 1993). Although the SOR-protective mechanism
278 against cryoinjury is not clearly understood, its effect could be similar to that of glycerol due
279 to the structural similarity, producing a synergistic effect (Wu et al., 2016). The structure of
280 the SOR molecule, however, may hinder its penetration into the cell with this penetration only
281 being possible as a result of the presence of a SOR dehydrogenase (SORD) located near the
282 plasma membrane. Even at relatively lesser concentrations, SOR is an efficient cryoprotectant
283 of mice sperm and has functions through SORD (Cao et al., 2009). The SOR compound,
284 however, was not detected in boar sperm (Chanapiwat et al. (2012), where there was the use
285 of SOR in semen extenders, there was a negative effect of this supplementation. Even though
286 there is the presence of SORD in donkey semen, it has not yet been determined in donkeys
287 whether there is this negative effect similar to that detected in stallion spermatozoa
288 (Pojprasath et al., 2011), suggesting that this pathway could be responsible for the results
289 observed in the current study. The metabolism of FRU to ATP, however, is relatively little in
290 spermatozoa of several domestic species, thus, there is relatively lesser motility in anaerobic
291 conditions (Storey, 2008). In donkeys, semen contains very little FRU (Mann et al., 1963).
292 Trimeche et al. (1997) reported that addition of FRU to the INRA 82 extender did not
293 improve sperm motility or velocities, which is consistent with the results in the present study
294 where with FRU supplementation there was the least values for sperm velocity and motility
295 rates. The effect of FRU could be mediated by increased production of oxygen radicals
296 generated during FRU metabolism as a result of functions in the glycolytic pathway,
297 compared to other sugars such as GLU (Goodson et al., 2012; Visconti, 2012). This thought is

298 also supported by the increased percentages of motility and the more vigorous motility
299 patterns observed in the sperm when there was supplementation with GLU. In donkeys, a
300 positive correlation between GLU supplementation and motility has been previously
301 described (Talluri et al., 2017). This monosaccharide is also associated with the induction of
302 capacitation in human (Williams and Ford, 2001), mice (Goodson et al., 2012) and domestic
303 species (Bucci et al., 2010) spermatozoa, probably inducing an increase in the hypermotility
304 of the viable sperm. The localization and relative abundance of GLUT transporters, however,
305 may vary between species (Bucci et al., 2010); therefore, more study is necessary to elucidate
306 what the situation is in this regard in donkey semen.

307 Equex paste has been widely used as an additional protective compound in the
308 cryopreservation of sperm from dogs (Rota et al., 1997), cats (Axnér et al., 2004), bulls
309 (Chaveiro et al., 2006), rams (Šterbenc et al., 2014), boars (Buranaamnuay et al., 2009), and
310 wildlife species (de Paz et al., 2012; Favoretto et al., 2012), with there being different
311 outcomes. To our knowledge, its effect has not yet been evaluated in donkey jack sperm. In
312 the present study, this additive had inconsistent effects based on the sugar source
313 supplemented in the extender. As an example, while supplementation with SOR-EP and
314 GLU-EP resulted in improvements, supplementation with GLU-EP decreased progressive
315 motility. Even though the differences were about 10% of the variation in motility in all the
316 cases, these were statistically significant. It has been proposed that the EP effect is mediated
317 by its capacity to induce the release of low-density lipoproteins from egg-yolk and by
318 functioning as a surfactant to stabilize and protect cell membranes against cold shock and
319 freezing injury (Anel et al., 2010). The interaction between EP and constituents in the
320 freezing extender used for supplementations was also evident, probably as a result of the
321 different chemical composition and glycerol concentrations (Schembri et al., 2003; Wu et al.,
322 2013). In the present study, because only sugar sources differed between treatments (with or

323 without EP), it is hypothesized that the interaction observed was caused by the sugars used for
324 supplementations. Nevertheless, the addition of EP to the freezing extender led to reduced
325 sperm velocities (VCL, VSL, and VAP) compared with EP-free extenders. There were similar
326 results with dog sperm, in which supplementation with Equex® STAMP (sodium dodecyl
327 sulfate) improved sperm motility but decreased the velocity of spermatozoa (Bencharif et al.,
328 2012). Even though there is a lack of previous research on this topic in donkeys, a possible
329 variation in the density of the extender, mediated by the increase of the free lipoproteins
330 available or a direct detrimental effect on spermatozoa, could account for results in the
331 present study.

332 Sperm morphology was affected by the sugar used for supplementations but not by the
333 use of EP. The greatest protective effect resulted with use of GLU-supplemented extender,
334 which increased the percentage of morphologically normal spermatozoa. Glucose metabolism
335 involves the hexokinase family of enzymes, which can bind to mitochondria, exerting tissue
336 protection against cell death (Sun et al., 2008). Although this mechanism has not been
337 previously associated with the use of FRU or SOR supplementations to extenders, a
338 protective effect on the tail morphology was also observed with use of the SOR-supplemented
339 extender, without there being any differences with fresh semen. The SOR compound can
340 osmo-stabilize the sperm plasma membrane by protecting the phospholipid components
341 against fusion and cell content leakage (Hincha and Hagemann, 2004), with results having
342 already been reported for this in horses (Pojprasath et al., 2011), but not donkeys. The FRU
343 supplementation increased the number of sperm tail abnormalities when compared with fresh
344 sperm. This monosaccharide is present in seminal fluid of Poitou jacks (Trimeche et al., 1997)
345 and in stallion sperm, although at a lesser concentration (Gamboa et al., 2011). In the present
346 study, however, the use of FRU as extender additive did not result in production of any
347 changes in the post-thaw quality of jack sperm. To our knowledge, this is the first study

348 where there was assessment of the effect of this sugar when there was supplementation to
349 donkey semen extender, suggesting that addition of FRU as the main energetic substrate to
350 the extender should be avoided. Further studies, however, are necessary to confirm whether
351 this is a problem when FRU is used for extender supplementations.

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

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

373 Accordingly, it is suggested based on results in the present and previous studies that the
374 beneficial effect of this molecule as a supplement to semen extender could be species-
375 specific.

376 The DNA fragmentation is a determining factor in the fertilizing capacity of
377 spermatozoa (Karoui et al., 2012). Cryopreservation results in stress to spermatozoa, leading
378 to a decreased membrane stability, oxidative stress and reduced DNA integrity (Kopeika et
379 al., 2015). In horses and donkeys, sperm DNA fragmentation increases as the post-thaw time
380 progresses, without being affected by the cryopreservation procedure (Lopez-Fernandez et al.,
381 2007; Cortes-Gutierrez et al., 2008). Findings in the present study are consistent with these
382 previous results because DNA fragmentation was greater after 6 and 24 h of incubation with
383 all the treatments assessed, without there being differences between these at similar time
384 points. Although a small protective effect of DNA integrity in spermatozoa stored in a diluent
385 has been reported for other species (Perez-Llano et al., 2006; Bottrel et al., 2018), results of
386 the present study indicate supplementation with different sugars does not affect DNA quality.
387

388 **5. Conclusions**

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

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399 **Conflict of interest statement**

400 The authors declare that there is no conflict of interest.

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