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The cryoprotective effect of Ficoll 70 on the post-warming survival and quality of Cryotop-vitrified donkey embryos

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Short title: *Vitrifying donkey embryos with Ficoll*

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ABSTRACT

Many domestic donkey breeds are at risk of extinction, there is a critical urgency for genome resource banking. In the present study, we examined whether the use of Ficoll 70 added to the vitrification medium containing ethylene glycol (EG), dimethyl sulfoxide (DMSO) and sucrose improves the cryotolerance of donkey *in vivo* derived embryos. Day 7-8, grade 1-2 donkey embryos were measured and morphologically evaluated and then vitrified-warmed using the Cryotop technique. Before vitrification, embryos were randomly distributed into two groups: (i) VS1 (n = 14): vitrified using 15% EG + 15% DMSO + 0.5 M sucrose; and (ii) VS2 (n = 10): vitrified in the same medium supplemented also with 18% of Ficoll 70. After 24 h of warming, the embryos were measured and evaluated for their morphology, development and viability (Propidium Iodide-Hoechst 33342 dyes). Post-warming survival was numerically higher but not significantly different ($P > 0.05$) when embryos were vitrified in VS2 (70%) compared to VS1 (57.1%). Embryo rupture was only observed in the VS1 group (21.4%, 3/14). Higher embryo diameter was observed in all groups after 24 h culture ($P < 0.05$). No significant differences ($P > 0.05$) were observed among treatments in terms of percentages of cell death. These results demonstrate that the addition of Ficoll 70 to the vitrification medium is not a pre-requisite for successful vitrification of donkey embryos. However, its addition seems to enhance some of the post-warming embryo quality characteristics. Since no statistically significant evidence was found, further studies should be conducted in order to confirm our findings.

Key words: Donkey; Embryo; Macromolecule; Vitrification; Viability

1. Introduction

Cryopreservation of embryos and their storage in embryo banks offer several advantages to the equine industry. For instance, it allows the conservation of male and female genetic material and the exchange of genetic resources [1], avoiding the transport of live animals and health risks [2]. In addition, the transfer of cryopreserved embryos optimizes the use of donors and minimizes the number of recipients, and thus decreases the cost of embryo transfer [3]. All these advantages are also important for the preservation and management of equine endangered species and the maintenance of their genetic heterozygosis [1].

Nowadays, the population of domestic donkeys (*Equus asinus*) has been drastically reduced in several Europe countries [4,5], including Spain [6]. All six Spanish donkey breeds (Andaluza, Catalana, Balear, Majorera, Asno de las Encartaciones, and Zamorano-Leonés) are currently at risk of extinction (Real Decreto 2129/2008, regulation of the National Catalogue of Endangered Species). In 2017, the population size of Andalusian donkeys in Spain was estimated to be 841 animals [7]. Considering the importance of preservation of equine biodiversity, strategies for the cryopreservation of embryos for these donkey breeds is advisable.

Vitrification seems to be a promising alternative to traditional slow-freezing procedures to improve cryotolerance of equine embryos [2]. This technique requires high concentrations of permeable cryoprotectant agents (CPAs) and rapid cooling rates to avoid ice crystal formation, and to induce a glass-like state [8]. However, all known permeable CPAs are toxic at the high concentrations required to avoid or minimize ice formation when oocytes or embryos are vitrified-warmed. Despite numerous attempts to decrease these toxic effects by i.e. using shorter exposure times or different equilibration steps, many research efforts have focused on the use of novel, less toxic

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CPAs to improve the efficiency of embryo vitrification. Non-permeable cryoprotective substances such as disaccharides are less toxic than permeable CPAs and can help to lessen the cryodamage induced by permeating CPAs by helping the embryo to stabilize internal solute concentrations under osmotic stress [9]. However, it has been shown that the inclusion of other non-permeable macromolecules, with larger molecular weight than disaccharides and trisaccharides, in the solution also facilitates vitrification [8]. In this study, we hypothesized that the addition of the polymer Ficoll 70 as a non-permeable CPA into the cryopreservation media might protect vitrified-warmed donkey embryos from cryoinjury. Ficoll 70 is a relatively inert polymer characterized by a high solubility with low viscosity, and, when added to the media, reduces mechanical stress, protects the cell membrane and prevents re-crystalization during warming [10]. The cryoprotective action of Ficoll has been proven successfully for cryopreservation of embryos of different domestic animals, including horses [11,12], cattle [13], goats [14], sheep [15], rabbits [16], cats [17] and mice [18,19]. However, to our knowledge, no studies have been previously reported in the literature on the cryoprotective effect of Ficoll 70 during the vitrification of donkey embryos.

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The aim of the present study was to assess the ability of donkey *in vivo* derived embryos to survive the vitrification-warming procedure using the Cryotop device when Ficoll was added to the cryoprotective solutions. In addition, the study was designed to determine the effects of the use of Ficoll 70 during vitrificacion on cell death after warming.

2. Materials and Methods

The present study was conducted at Centro Rural Malpica (Palma del Río, Cordoba, Spain). All animal procedures were performed in accordance with the Spanish

laws for animal welfare and experimentation (Real Decreto 53/2013) and approved by the Ethical Committee for Animal Experimentation of the University of Cordoba (no. 31/08/2017/105).

2.1. Chemicals and media

Human chorionic gonadotropin (hCG; Veterin Corion®) was obtained from Divasa-Farmavic S.A. (Barcelona, Spain). Lactated Ringer´s solution was purchased from B. Braun VetCare S.A. (Rubi, Spain) and Syngro® holding from Bioniche Animal Health (Washington, USA). Ficoll 70 (Ficoll; F2878), Dimethyl Sulphoxide (DMSO; D2650), Ethylen Glycol (EG; 293237), Sucrose (S9378), Bovine Serum Albumin (BSA; A7906), Propidium iodide (PI; P4170), Hoechst 33342 (HO; 14533) and Dulbecco´s phosphate buffer saline (DPBS; D5773) were supplied by Sigma-Aldrich (Steinheim, Germany), while Tissue Culture Mediun-199 HEPES (TCM199 HEPES; M7528), Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham (DMEN/F12; 11330-032), Foetal Calf Serum (FCS; 12483-012) and gentamicin (15750-037) were purchased from Gibco (Life Technologies, New York, USA).

2.2. Embryo recovery and evaluation

Ten healthy adult fertile Andalusian jennies (3-13 years-old) were used as embryo donors. Reproductive tracts of donors were evaluated daily by transrectal ultrasonography (Aloka SSD 500, ALOKA Co. Ltd., Tokyo, Japan) to track follicular activity and confirm ovulation. Donor jennies received hCG (1500 IU, IM) to induce ovulation when a follicle of 35-40 mm was detected. All jennies were mated naturally with a fertile jack every other day, beginning from 24 h after hCG administration until ovulation was detected (Day 0).

At day seven or eight after ovulation, embryos were recovered by transcervical flushing of the uterus using 3 x 1 L of Lactated Ringer´s solution, as described by Camillo et al. [20] for donkeys. Embryos were washed ten times in Syngro® holding, as previously described [21]. The diameter of all embryos was measured at collection under bright field conditions (SZ51 Olympus optical, Tokyo, Japan) using an ocular micrometer (scale of 1mm/100), as previously described [11]. Embryos were also evaluated for developmental stage (morula, early blastocyst, blastocyst or expanded blastocyst) and morphological quality, and graded on a modified four-point scale (Grade 1: excellent; Grade 2: good; Grade 3: fair; Grade 4: poor, degenerate or dead) [22].

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2.3. Cryopreservation of embryos

Only grade 1-2 donkey embryos were selected (n = 33). A total of 24 embryos were vitrified in a Ficoll-free vitrification medium (VS1, n = 14) or in Ficoll-containing medium (VS2, n = 10), while the remaining 9 embryos were used as fresh (n = 5) and CPA toxicity (n = 4) controls.

The embryos in groups VS1 and VS2 were vitrified using the Cryotop device and vitrification and warming solutions described by Bottrel et al. [23]. The holding medium (HM) used to formulate the vitrification-warming solutions was TCM-199 HEPES supplemented with 20% FCS. All steps were performed under a laminar flow hood at room temperature (20-25 °C), using a stereomicroscope to visualize each step.

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After washing, embryos were transferred into equilibration solution (ES) consisting of 7.5% DMSO and 7.5% EG in HM for 10-15 min. After an initial shrinkage, embryos regained their original volume; then, they were randomly transferred to: VS1 (HM supplemented with 15% EG, 15% DMSO and 0.5 M sucrose)

144 or VS2 (HM supplemented with 15% DMSO, 15% EG, 0.5 M sucrose and 18% Ficoll
145 70). After incubation of 30-40 sec, one embryo was loaded onto a Cryotop (Kitazato
146 BioPharma Co. Ltd., Shizuoka, Japan), the excess of fluid was removed to leave only a
147 thin layer covering the embryos (< 1 µL), and the open device was quickly plunged into
148 LN_2. From the exposure to VS1 or VS2 to plunging, the elapsed time did not exceed 90
149 sec. The loaded Cryotops were inserted in the cap and stored in LN_2 until warming.
150
151 *2.4. Embryo warming and culture*
152 All warming steps were performed at 38.5 °C. After removing the protective cap
153 while still in LN_2, the Cryotop was directly immersed into the warming solution
154 containing 1 M sucrose dissolved in HM. After 1 min, the recovered embryos were
155 transferred to the dilution solution, which contained 0.5 M sucrose dissolved in HM.
156 Then, embryos were incubated for 3 min in the dilution solution to promote CPA
157 diffusion out of the embryo by gentle pipetting. After that, embryos were incubated in
158 HM for 5 min. After a final rinse in HM for 1 min, embryos were transferred to 950 µL
159 of DMEN/F12 supplemented with 10% FCS [24], and 25 µg/mL gentamicin [25], and
160 incubated at 38.5 ºC in a 5% CO_2 humidified atmosphere.

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161 Embryos exposed to the vitrification (VS2) and warming solutions were used as
162 a control for CPA cytotoxicity (n = 4). The exposed embryos were then cultured under
163 the conditions described above. Fresh, non-vitrified embryos directly transferred to the
164 culture medium served as the non-vitrified fresh control group (n = 5).
165 Embryo diameter, quality grade and developmental stage for all the embryos in
166 each of the experimental groups were assessed at 24 h post-warming as previously
167 described. The vitrified-warmed embryos that progressed to the next developmental
168 stage and/or that had an excellent or good appearance during the culture were

considered viable. Post-warming embryo survival rates were calculated by the number of surviving embryos 24 h after warming divided by the number of vitrified embryos. The capacity of every embryo to develop from one developmental stage to the next during 24 h culture was analysed according to its initial stage before vitrification and classified as: 0: no change; + 1: one positive change; + 2: two positive changes. Differences on embryo quality were analysed according to their initial quality before vitrification compared to their quality 24 h post-warming as described by Poitras et al. [26]: - 3: three negative changes; - 2: two negative changes; - 1: one negative change; 0: no change; + 1: one positive change; + 2: two positive changes. Morphological grading of embryos with a damaged zona pellucida (ZP) was reduced by one point and all ruptured embryos were classified as Grade 4 (poor, degenerated or dead).

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2.5. Analysis of embryo viability

To assess the percentage of dead cells, each embryo was stained 24 h post-warming with PI and HO, according to the methods of Lagares et al. [11] and Oberstein et al. [12] with slight modifications. Briefly, embryos were washed in a 100 µL droplet of DPBS and incubated in a 100 µL droplet of DPBS supplemented with 1% BSA and 125 µg/mL PI at 38.5 ºC for 5 min in the dark. Thereafter, embryos were loaded in a 100 µL droplet of 90% glycerol/DPBS with 100 µg/mL HO during 5 min at 38.5 ºC in the dark. After staining, embryos were washed twice in DPBS and mounted on a glass slide in an 11 µL drop of DPBS, which was covered with a cover slip mounted with droplets of a paraffin oil/petroleum jelly mixture on the corners. Finally, stained embryos were blindly examined in a fluorescence microscope (Olympus BX40, Tokyo, Japan) using a narrowband filter (a 360 – 370 nm excitation filter). This resulted in all nuclei fluorescing blue (HO +) and dead cells fluorescing pink (HO +/ PI +). To assess

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194 the percentage of dead cells, three different and independent estimates of the percent
195 dead cells were calculated and the average of these numbers was taken [12].
196
197 *2.6. Statistical analysis*
198 Data are shown as mean ± standard error of the mean (SEM). The GLM
199 procedure was used to evaluate the effect of treatment on the percentage of dead cells.
200 The statistical models included treatment (as fixed effect), embryo diameter (as random
201 effect) and their interaction. Differences in diameters and quality scores within
202 treatments were also analyzed by paired-samples t-test. Post-warming survival rate and
203 the percentage of embryos with different quality and developmental stage were
204 analyzed by Chi-square. All analyses were performed using the statistical package SAS
205 version 9.0. The significance level was set at $P < 0.05$.
206
207 **3. Results**
208 A total of 33 embryos collected on day 7 or 8 post-ovulation were used in this
209 study. Developmental stages as well as mean diameters and morphological grades of
210 donkey embryos at collection are reported in Table 1. All embryos collected from
211 jennies were classified as good (27.3%, 9/33) or excellent (72.7%, 24/33) quality. Of
212 the 33 recovered embryos, 51.5% (17/33) were early blastocysts, 39.4% (13/33) were
213 expanded blastocysts, 6.1% (2/33) were blastocysts, and only 3.3% (1/33) were morula.
214 The mean diameter of the embryos collected 7-8 days after ovulation was 330.30 ±
215 29.47 µm (ranged from 150 to 800 µm in diameter).
216 Treatment was found to have significant ($P < 0.05$) influences on dependent
217 variables. The diameter of embryos (< 300 µm and > 300 µm) had no significant ($P >$

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0.05) influence on dependent variables as well as there was no interaction ($P > 0.05$) between treatment and embryo diameter (data not shown).

Embryo grades, diameters before vitrification (at collection) and 24 h after warming together with cell death rates after vitrification-warming are given in Table 2. Donkey embryos exposed to CPAs or vitrified showed lower embryo quality than control fresh embryos after 24 h culture, although this difference was only significant ($P < 0.01$) for vitrified groups. Embryos from all groups reached a significantly ($P < 0.05$) higher embryo diameter after 24 h culture, this increase was more significant ($P < 0.01$) when embryos were vitrified in presence of Ficoll 70. No significant differences ($P > 0.05$) were observed among treatments in terms of percentages of cell death (Table 2). As shown in Table 3, survival rates at 24 h post-warming were numerically higher but not significantly different ($P > 0.05$) for those embryos vitrified in VS2 supplemented with Ficoll 70 (70%), when compared to embryos vitrified in VS1 (57.1%).

No significant differences ($P > 0.05$) in both quality and developmental changes were observed after 24 h post-warming when donkey embryos were vitrified using solutions VS1 and VS2 (Figure 1). However, embryo quality after warming was maintained or improved in 50% of the embryos vitrified in VS2. Meanwhile, only 28.6% of the embryos vitrified with VS1 kept their embryo quality. There was no increase in embryo quality in the VS1 group (Figure 1A). After warming, a total of 12.5% (3/24) ruptured embryos were observed; however, all of them (21.4%, 3/14) were observed in the VS1 group (Ficoll-free medium). These three (Day 7) embryos were early blastocysts and their diameters were 200, 225 and 250 µm. Of a total of 24 vitrified-warmed embryos, 13 (54.2%) increased their developmental stage after 24 h of culture, regardless of the vitrification medium used (Figure 1B).

4. Discussion

Embryo cryopreservation allows long-term preservation of genetic material and is an essential tool for worldwide proliferation of endangered species. Interest in donkey reproduction has had a linear growth since the beginning of the century. However, the development of assisted reproductive techniques for donkeys remains a challenge, especially when working with endangered species, in which the number of animals and so the embryos available, is very restricted. Until now, very few studies have been reported on the donkey embryo cryopreservation [23,27]. Previous studies conducted in our group have already demonstrated that vitrification using the Cryotop technique seems to be effective for the cryopreservation of Grade 1 and 2 donkey embryos, with similar post-warming survival regardless of age, diameter and development stage of the embryos [23]. Because of the beneficial effects of the use of Ficoll 70 on post-warming embryo survival in other species, we assessed whether the addition of Ficoll 70 to the vitrification medium could improve the cryotolerance of vitrified-warmed donkey embryos.

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Although no significant differences were detected between the two different cryopreservation protocols, a numerically higher percentage of survival rate (70%) was observed when donkey embryos were vitrified in medium supplemented with Ficoll 70, lending support to the suggestion that Ficoll 70 may have a cryoprotective role during the vitrification process. Embryo survival in this study was higher to that reported by Hochi et al. [28,29] for equine embryos (57%) also vitrified in the presence of EG, Ficoll 70 and sucrose. Vitrification of donkey embryos in VS1 (Ficoll-free medium) resulted in 57.1% of embryos surviving after warming, which was comparable to the embryo survival reported in previous works [23]. It is noteworthy that embryo diameter

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did not had an impact on post-warming survival rate, which corroborate our previous findings [23].

The overall incidence of ruptured embryos immediately after warming (12.5%) was similar to those of previous studies (13% - 25%) for equine embryos vitrified in the presence of 40% EG, 18% Ficoll 70 and 0.3 M sucrose [11,29], but lower to that reported by our group [23]. It is noteworthy that this sudden rupture was only observed in embryos vitrified with VS1 (Ficoll-free medium) while all of the embryos vitrified in VS2 (Ficoll-containing medium) were intact after warming.

Results showed that addition of Ficoll 70 to the vitrification medium did not result in significant differences when embryo grade and percentage of dead cells where analysed 24 h post-warming. Although embryo diameter significantly increased in all groups of embryos after 24 h culture, this increase was more significant for those embryos vitrified in medium supplemented with Ficoll 70. When equine morula and early blastocysts were vitrified using 0.25 mL straws, Lagares et al. [11] observed a significant increase of embryo quality and viable cells associated to the addition of the Ficoll 70 to the vitrification media. However, embryos vitrified using Ficoll 70 showed similar embryo diameters to the embryos vitrified only with EG. Therefore, a positive effect of the addition of Ficoll 70 to the vitrification media could be observed in both studies. Differences observed in the parameters studied may be because equine embryos were evaluated 10 min after warming while donkey embryos were evaluated 24 h post-warming, which may have allowed the embryos to increase their diameter and decrease the percentage of cell death.

The assessment of embryo quality revealed no significant differences between initial and post-warming values of fresh, non-vitrified embryos and embryos exposed to CPAs. However, vitrification of donkey embryos resulted in a decline in embryo quality

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after 24 h culture, but no differences were observed between both vitrification solutions, VS1 and VS2. In agreement with previous findings in horses [11,12] and donkeys [23,27], Cryotop-vitrified donkey embryos had an average score of 2.5. The overall percentage of dead cells observed in this experiment (4.2%) was comparable to or even lower than those reported in donkeys (4.6-9%) [23,27,30], in which donkey embryos were frozen using different cryopreservation protocols (slow freezing and vitrification), devices (Cryotop, Fibreplugs and 0.25 mL straws) and CPAs (DMSO, EG, glycerol, sucrose and galactose). It is known that cell death may be attributed to exposure of embryos to toxic levels of CPAs before freezing [31]. Moreover, different exposure times or temperatures of exposure of equine embryos to the vitrification solutions may also increase toxicity for the embryo [11,12]. In this study, Cryotop-vitrified donkey embryos had a percentage of dead cells lower than 20%, which has been previously proposed as the upper limit for viable horse embryos [32], regardless of the vitrification solution used (VS1 or VS2). In fact, the proportions of dead cells in vitrified-warmed embryos (3.09% and 5.24% for VS1 and VS2, respectively) were comparable, and not significantly different to those observe for fresh control embryos (2.31%) and toxicity control embryos (5.86%). This low percentages of dead cells observed in this study could be due to that only high grade (1 and 2) quality donkey embryos have been vitrified [33] or to the lower toxicity of Ficoll 70 [18,27]. When assessing fresh donkey embryos for cell death, Panzani et al. [21] observed higher proportion of dead cells in lower quality embryos (Grade 3), while Grade 1 and Grade 2 embryos had a low number of dead cells that was not correlated with embryo size. Referring to Ficoll cytotoxicity, Kuleshova et al. [18] studied the replacement of penetrating CPAs by polymers in mouse embryo cryopreservation and they concluded that Ficoll 70 had the lowest toxicity under the conditions tested.

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Regarding to embryo quality, 50% of the embryos vitrified in VS2 kept or increased their quality at 24 h post-warming, whereas 71.4% vitrified in VS1 decreased their quality after 24 h culture. Moreover, higher number of embryos progressed to the next development stage 24 h post-warming when Ficoll 70 was added to the vitrification media, while none of the embryos vitrified in EG alone progress to the next embryo stage. Although they are not significant differences, these data suggest that the use of Ficoll 70 on Cryotop vitrification of donkey embryos could improve survival rates, which is consistent with previous studies in equine [10,11] and ovine [15] embryos. Generally, the mechanism of protection by the large polymers is unclear. It has been suggested that extracellular disaccharides and macromolecules, such as sucrose and Ficoll 70 added to vitrification solutions prevent the osmotic injury caused by the rapid shrinkage of the blastocoele, together with the ability to coat the cells and protect the cell membrane from denaturation [18]. Moreover, the use of these macromolecules means that the concentration of CPA can be decreased reducing so their toxicity associated [9,34]. It has also been reported that during warming, Ficoll 70 acts as a soft capsule for additional dehydration of embryo, such a procedure assists in preventing ice formation inside the embryo by increasing the total concentration of solute and cell contents inside the cells [10], all of which may increase post-warming embryo viability [29].

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5. Conclusions

Our study demonstrates that the addition of Ficoll 70 to the vitrification medium is not a pre-requisite for successful vitrification of donkey *in vivo* derived embryos. However, a tendency to prevent rupture was observed in donkey embryos vitrified with Ficoll-containing medium. Similarly, the use of Ficoll 70 on Cryotop vitrification of

342 donkey embryos seems to improve post-warming survival rates, but no statistically
343 significant evidence was found. In this way, Ficoll 70 is probably a valuable tool for the
344 successful cryopreservation of donkey embryos, but further studies should be conducted
345 in order to confirm our findings.
346
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