One-step warming does not affect the *in vitro* viability and cryosurvival of Cryotop-vitrified donkey embryos

Short title: *One-step warming of donkey embryos*

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ABSTRACT

The objective of this study was to compare the effects of two warming protocols (three-step *vs.* one-step dilution) on embryo quality, post-warming embryo survival and embryo cell viability of donkey embryos vitrified by the Cryotop method. Twenty, Day 7-8, grade 1-2 donkey embryos were measured, morphologically evaluated and vitrified using the Cryotop technique. Embryos were then randomly warmed using two different warming procedures: (i) W3 (three-step dilution; n = 11): embryos were warmed in 1 M, 0.5 M and 0 M sucrose, and (ii) W1/0.5 (one-step dilution; n = 9): embryos were warmed directly in 0.5 M sucrose. After 3 and 24 h of warming, the embryos were measured and evaluated for their morphology, developmental stage and viability (Propidium Iodide-Hoechst 33342 dyes). Although both treatments decreased embryo quality after warming ($P < 0.05$), no significant differences ($P > 0.05$) were observed between protocols in terms of post-warming embryo quality, diameter and embryo survival. Greater percentages of dead cells ($P < 0.001$) were observed when embryos were warmed directly in 0.5 M sucrose (one-step dilution) when compared to the three-step protocol. The percentage of ruptured embryos was 27.3% and 0% in W3 and W1/0.5 protocols ($P = 0.0893$), respectively. In conclusion, warming Cryotop-vitrified donkey embryos directly in 0.5 M sucrose had no negative effects on embryo quality and post-warming embryo survival. Moreover, one-step protocol may help to prevent rupture when donkey embryos warmed directly in 0.5 M sucrose. These results observed *in vitro* must be verified by embryo transfer.

Key words: Donkey embryo; Cryotop vitrification; Sucrose dilution; one-step warming; stepwise warming

1. Introduction

Over the last decades, the population of domestic donkeys (*Equus africanus asinus*) has been drastically reduced in Europe [1,2]. The six Spanish donkey breeds are currently at risk of extinction (Real Decreto 2129/2008, regulation of the National Catalogue of Endangered Species) due to their low number of registered individuals (Andaluza = 841; Catalana = 838; Balear = 458; Majorera = 95; Asno de las Encartaciones = 359; Zamorano-Leonés = 1470) [3]. In such critical situation, new strategies for genome resource banking in endangered donkey breeds are advisable.

Successful cryopreservation of equine embryos is crucial for the conservation of genetic resources [4]. Vitrification has been successfully used to cryopreserve embryos from horses [5,6] and donkeys [7,8]. The principles, methodologies and goals for vitrification of equine embryos have been thoroughly described [9-12]. Cryopreservation of embryos by vitrification offers several advantages to the equine industry. The transfer of cryopreserved embryos optimizes the use of suitable donors and minimizes the importance of the immediate availability of a suitable recipient [13]. Moreover, this technique is relatively simple, faster and superior to slow freezing in terms of post-thaw embryo quality and pregnancy rates [14,15].

Warming vitrified embryos requires the removal of cryoprotectants (CPAs) by successive dilution steps in a hypertonic solution before transferring to an isotonic culture medium [16-18]. From a practical point of view, embryo warming becomes problematic when working under field conditions, due to the difficulties associated to the handling of embryos during the stepwise warming procedure. Several techniques have been developed to reduce the need for optical equipment and technical skills during embryo warming, which enable the direct warming (dilution) of embryos that were cryopreserved using different vitrification devices: 0.25 mL straws [19,20], open-pulled straws (OPS)

[21], hand-pulled glass micropipettes (GMP) [22], Cryotops [19] or the VitTrans device [23]. These microscope-free methods have been successfully used to warm vitrified embryos in cows [23-25], sheep [26-28], goats [29], horses [30], pigs [31] and mice [18,32] suitable for the one-step transfer of vitrified-warmed embryos into recipients. In donkeys, no studies have been conducted on this topic.

Given this background, and prior to develop an in-straw dilution method suitable for the one-step transfer of vitrified donkey embryos, the aim of the present study was to compare two different warming protocols (three-step *vs.* one-step sucrose dilution) in terms of their effect on the embryo quality, post-warming embryo survival rate and embryonic cell viability (Hoechst 33342-Propidium Iodide staining) of donkey embryos vitrified by the Cryotop method.

2. Materials and Methods

All animal procedures were approved by the Ethical Committee for Animal Experimentation of the University of Cordoba (no. 31/08/2017/105) and are in accordance with the Spanish laws for animal welfare and experimentation (Real Decreto 53/2013).

2.1. Chemicals and media

Human chorionic gonadotropin (hCG) 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). Dimethyl sulfoxide (DMSO; D2650), Ethylene 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 Chemie GmbH (Steinheim, Germany), while Tissue Culture Mediun-199 HEPES (TCM-199-HEPES; M7528), Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham (DMEM/F12; 11330-032), Foetal Calf Serum (FCS; 12483-012) and gentamicin (15750-037) were purchased from Gibco (Life Technologies, Grand Island, New York, USA).

2.2. Embryo recovery and evaluation

Embryos were obtained from six healthy adult fertile Andalusian jennies (3-13 years-old) from the breeding stud of the Centro Rural Malpica (Palma del Río, Cordoba, Spain). During oestrus, ovarian activity was evaluated daily by transrectal ultrasonography (Aloka SSD 500, ALOKA Co. Ltd., Tokyo, Japan), until detection of ovulation (Day 0). Donor jennies received hCG (Veterin Corion®, 1500 IU, intramuscularly) 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 24 h after hCG administration until ovulation was detected.

Seven or eight days 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. [33] for donkeys. Embryos were washed ten times in Syngro® holding, as previously described [34]. The diameter of all embryos (including the zona pellucida) was measured at collection under bright field conditions (SZ51 Olympus optical, Tokyo, Japan) using an ocular micrometer (scale of 1mm/100), as previously described [35]. Embryos were also evaluated for developmental stage and morphology, and were then graded on a scale of 1-4 [36], 1 being excellent, 2 good, 3 fair, and 4 poor, degenerate or dead.

2.3. Vitrification and warming by the Cryotop method

Twenty grade 1 or 2 embryos were vitrified individually using the Cryotop® method (Kitazato BioPharma Co. Ltd., Shizuoka, Japan), as described by Bottrel et al. [7] for donkeys. The holding medium (HM) used to formulate the vitrification-warming solutions was TCM-199-HEPES containing 20% FCS. All steps were performed under a laminar flow hood at room temperature (20-25 °C), using a stereomicroscope to visualize each step.

After washing, each embryo was placed in an equilibration solution (ES) consisting of 7.5% DMSO and 7.5% EG in HM for 10-15 min. After initial shrinkage, each embryo recovered its original volume and was then transferred to the vitrification solution (VS) containing 15% DMSO, 15% EG and 0.5 M sucrose dissolved in HM. After incubating for 30-40 sec, one embryo at a time was loaded onto each Cryotop, the excess of fluid was removed to leave only a thin layer covering the embryo (< 1 µL), and the device was plunged quickly into liquid nitrogen (LN_2). The entire process from the immersion in VS to plunging into LN_2 was completed within 90 sec. The loaded Cryotop was inserted into the cap and stored in LN_2 until warming.

During warming, the protective cap was removed from the Cryotop while still submerged in LN_2. Embryos were then randomly assigned to one of the two different warming protocols: (i) W3 (Cryotop method or three-step dilution protocol with sucrose; n = 11): each embryo was warmed directly in 1 M sucrose dissolved in HM for 1 min, then transferred into 0.5 M sucrose dissolved in HM for 3 min and finally placed in 0 M sucrose in HM for another 6 min; (ii) W1/0.5 (one-step sucrose dilution; n = 9): embryos warmed directly in 0.5 M sucrose dissolved in HM for 3 min. All steps were performed at 38.5 °C. Subsequently, embryos were transferred to DMEM/F12 culture medium supplemented with 10% FCS [37] and 25 µg/mL gentamicin [38], and incubated at 38.5 ºC in a 5% CO_2 humidified atmosphere for 24 h.

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144 *2.4. Evaluation of post-warming viability of embryos*

145 Diameter of embryos, developmental stage and embryo quality were assessed
146 after 3 and 24 h post-warming. Only the vitrified-warmed embryos that progressed to the
147 next developmental stage and/or had an excellent or good morphological quality after
148 culture were considered viable. The post-warming survival rate (%) was defined as the
149 ratio of viable embryos to the total number of cultured embryos.

150 The percentage of dead cells was assessed after 24 h of culture, as described by
151 Lagares et al. [35] and Oberstein et al. [15] with slight modifications. Briefly, embryos
152 were washed in a 100 µL microdroplet of DPBS and then placed in a 100 µL microdroplet
153 of DPBS containing 1% BSA and 125 µg/mL PI, and incubated for 5 min at 38.5 ºC in
154 the dark. Thereafter, embryos were loaded in a 100 µL microdroplet of 90%
155 glycerol/DPBS with 100 µg/mL HO during 5 min at 38.5 ºC in the dark. After staining,
156 embryos were washed twice in DPBS and mounted on a glass slide in an 11 µL
157 microdroplet of DPBS, which was covered with a cover slip mounted with droplets of a
158 paraffin oil/petroleum jelly mixture on the corners. Finally, stained embryos were
159 examined under a fluorescence microscope (Olympus BX40, Tokyo, Japan), using a
160 narrowband filter (360-370 nm excitation filter). This resulted in all nuclei fluorescing
161 blue (HO +) and dead cells fluorescing pink (HO +/PI +). To calculate the percentage of
162 dead cells, three different and independent estimates of the percent dead cells were
163 estimated and the average of these numbers was taken [15].

164

165 *2.5. Statistical analysis*

166 Data are shown as mean ± standard error of the mean (SEM). ANOVA (PROC
167 GLM) was used to evaluate the effect of the warming protocol (three-step or one-step

sucrose dilution) on embryo diameter and grade before and post-vitrification and percentage of dead cells post-warming. Percentage data were subjected to arcsine transformation prior to analysis. The statistical model included fixed effect for treatment (W3 or W1/0.5), random effect for donor (six jennies), embryo diameter (≤ 300 µm or > 300 µm) and embryo developmental stage (morula, early blastocyst or expanded blastocyst) at collection, and their interaction. Differences in studied variables within treatments over time were analyzed by GLM repeated measures analysis. Mean values were compared by Duncan´s test. Post-warming survival rates of vitrified-warmed embryos were compared between groups using the Chi-square test. All analyses were performed using the statistical package SAS version 9.0. The significance level was set at $P < 0.05$.

3. Results

A total of 20 embryos collected on day 7 or 8 post-ovulation were used in this study, all of which were classified as good (30%, 6/20) or excellent (70%, 14/20; Table 1). As shown in Table 1, the mean diameter of the embryos collected was 262.50 ± 29.83 µm (ranged from 150 to 550 µm in diameter). The most frequently observed stage of development at collection was early blastocyst (65%, 13/20).

No differences in embryo survival rate were observed between warming treatments, neither at 3 nor at 24 h post-warming ($P > 0.05$; Table 2). When the survival rate of donkey embryos warming by W1/0.5 or W3 protocols were compared according to their initial developmental stages, no significant differences emerged ($P > 0.05$). However, the number of dead cells after 24 h culture was significantly higher ($P < 0.001$) for the W1/0.5 protocol than for W3. This difference between warming protocols was observed in all embryo developmental stages at collection ($P < 0.05$; Table 2).

193 Furthermore, the percentage of ruptured embryos tended to be higher ($P = 0.0893$) in the
194 W3 protocol (27.3%; 3/11) than in the W1/0.5 protocol (0%; 0/9).
195 As shown in Table 3, no significant differences ($P > 0.05$) were observed between
196 the warming protocols in terms of embryo quality after 3 and 24 h post-warming.
197 However, vitrified-warmed embryos had a significant ($P < 0.05$) decrease in embryo
198 quality after 3 and 24 h culture, regardless of the warming protocol. Warming did not
199 affect ($P > 0.05$) embryo diameter (Table 3).
200 The GLM revealed that only cell death rate was influenced ($P < 0.05$) by the
201 treatment (W3 and W1/0.5). The dependent variables were not influenced ($P > 0.05$) by
202 donor, embryo diameter and embryo developmental stage at collection, and no interaction
203 effect was observed ($P > 0.05$).
204
205 **4. Discussion**
206 In the last two decades, several one-step warming in-straw cryoprotectant dilution
207 techniques have been developed to allow the direct transfer of vitrified embryos of
208 domestic animals [18,24,31]. However, to the best of our knowledge, there are no studies
209 in donkeys that support its use either under field or laboratory conditions. The present
210 study compares for the first time the effects of two warming protocols (three-step *vs.* one-
211 step sucrose dilution) on embryo quality, post-warming embryo survival rate and
212 embryonic cell viability of donkey embryos vitrified by the Cryotop method.
213 The conventional methods of vitrification used to preserve embryos require the
214 use of a high concentration mixture of CPAs to achieve sufficient intracellular
215 concentrations during brief exposure times [39] and to avoid ice crystal formation [40].
216 However, the exposure to such high levels brings other injuries such as chemical embryo
217 toxicity and osmotic shock during removal of permeating CPAs [30,41]. During embryo

warming, there is a danger of over-swelling [42] as the influx of water can be faster than the efflux of the CPAs [43,44]. The warming protocol for Cryotop-vitrifed embryos includes the exposure of embryos to decreasing concentrations of a non-permeable solute (mainly sucrose) until isosmotic conditions are restored [45], which is vital to avoid sudden osmolarity changes during warming and to remove the high concentration of CPAs used during vitrification [46].

Sucrose solutions have been widely used as an osmotic buffer to regulate water permeation and prevent severe swelling of embryos during warming [19,47], reducing membrane damage and increasing the embryo survival [48]. Another advantage of sucrose is the preservation of structural and functional integrity of membranes at low water activities [49]. In sucrose solutions, embryos gradually shrink as permeating CPAs passively diffuse out of the embryo. Finally, after CPA elimination, the embryos are rehydrated in a sucrose-free isotonic medium and regain their normal volume [50].

In our study, after 3 and 24 h of culture, there were no significant differences between the control three-step procedure (W3) and the one-step warming protocol tested (W1/0.5) in terms of embryo diameter, embryo grade or embryo survival. These results suggest that one-step dilution is efficient enough to protect Cryotop-vitrified donkey embryos from osmotic shock during warming. Consistent with our findings, Morató and Mogas [23] found that the CPA dilution system (one-step or three-step) had no effect on *in vitro* development when Cryotop-vitrified *in vitro* produced (IVP) bovine blastocysts were warmed directly in 0.5 M sucrose. Embryo vitrification by the Cryotop method requires a short time of exposure to high concentrations of cryoprotectants. It can be assumed that the intracellular concentration of cryoprotectant is relatively low and therefore the osmotic difference between the embryo and medium during warming is not high enough to cause serious cellular damage [18,39,51].

Previous studies also reported the effectiveness of the one-step dilution of vitrified bovine [19,23,24], ovine [26,52] and porcine [31] embryos using a solution of sucrose compare to a stepwise warming protocol. Moreover, the percentage of embryos that survived the vitrification and warming process in this study (80% and 70% after 3 h and 24 h of culture, respectively) was higher than embryo survival reported in a previous study by Bottrel et al. [7] after 24 h of culture (64%). These results suggest that the combination of vitrification using the Cryotop device and one-step warming in a sucrose dilution has the potential to be successfully applied under field conditions to techniques for direct transfer of cryopreserved donkey embryos.

During warming, equilibration with CPA concentrations is usually associated with large osmotic gradients driving water fluxes that can cause cell volumes to exceed biophysical limits, provoking osmotic cell damage. Typically, damage of this nature has been avoided using stepwise warming procedures that reduce concentration changes so that osmotic changes between steps are not damaging. However, no differences were observed in the total cell number, cell death rate or apoptotic index when bovine IVP embryos were warmed by one-step procedure in comparison to two- or three-step procedures [23,24].

In our study, cell death rate was significantly higher in embryos warmed directly in 0.5 M sucrose (W1/0.5) compared to the three-step dilution protocol (W3), probably caused by the osmotic shock induced by the one-step procedure. However, the overall percentage of dead cells observed in this experiment (8.5%) is similar to that previously reported in donkeys (4.6-9%) [7,8,53], and lower than 20%, which has been previously proposed as the upper limit for viable horse embryos [14]. Further studies are warranted to investigate the effects of different sucrose concentrations or exposure times on post-

warming survival of donkey embryos cryopreserved by the Cryotop method and warmed by the one-step dilution method.

It is noteworthy that the overall incidence of ruptured embryos immediately after warming (15%) was lower than data previously reported by our group [7]. Moreover, only those embryos warmed using the three-step dilution protocol ruptured (27.3%) while embryos warmed in 0.5 M sucrose remained intact. This observation suggests once more that one-step warming in 0.5 M sucrose could be beneficial for Cryotop-vitrified donkey embryos, although these findings require further verification by embryo transfer.

5. Conclusions

One-step warming of Cryotop-vitrified donkey embryos in 0.5 M sucrose showed no negative effects on embryo quality and post-warming embryo survival when compared to the standard three-step dilution method. Although an increase in cell death rate could be observed, one-step warming procedure seemed to prevent embryo rupture at warming. Further experiments are needed to develop a system for the direct transfer of Cryotop-vitrified donkey embryos that could be used under field conditions.

Acknowledgments

We are extremely grateful to the Centro Rural Malpica (Palma del Río, Cordoba, Spain) for providing the animals.

Funding

This work has been supported by Grant AGL2013-42726-R (State Secretary for Research, Development and Innovation, Ministry of Economy and Competitiveness - MINECO, Spain). I. Ortiz was supported by a Ph.D. fellowship from the ceiA3 (Andalusia, Spain)

with funding provided by Banco Santander through its Global Division, Santander Universidades. M. Díaz-Jiménez and C. Consuegra were supported by a FPU fellowship from Spanish Ministry of Education, Culture, and Sports.

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