Cryopreservation of donkey embryos by the Cryotop method: effect of developmental stage, embryo quality, diameter and age of embryos

Short title: *Vitrification of donkey embryos*

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ABSTRACT

Cryopreservation of embryos has the potential to become a valuable tool for the conservation of endangered donkey breeds. However, there are several factors that can affect cryosurvival of embryos. This study evaluates the effectiveness of the Cryotop method to vitrify donkey embryos and factors affecting the survival of vitrified-warmed embryos. Day 6 to 8 embryos were measured and morphologically evaluated. Embryos were then vitrified-warmed using the Cryotop technique. After 24 h post-warming, the embryos were measured and evaluated for their morphology, development and viability (Propidium Iodide-Hoechst 33342 dyes). A total of 25 embryos were used, of which 17 were classified as Grade 1 (excellent), 5 as Grade 2 (good) and 3 as Grade 3 (fair). Based on their diameter, embryos were grouped as follows: ≤220 µm (n = 10), >220 to 300 µm (n = 8), and >300 µm (n = 7). Post-warming survival of vitrified embryos was similar (P>0.05) to the control fresh embryos, regardless of embryonic diameter, developmental stage, and age of the embryos before vitrification. However, the proportion of embryos that survived vitrification procedures was numerically higher but not significantly different (P>0.05) for Day 7 embryos (84.6%). The ability of Grade 1 (70.6%) and 2 (80%) embryos to survive vitrification procedures was higher (P = 0.0214) than those of Grade 3 (0%). The proportion of dead cells in Grade 3 embryos (56.5%) was higher (P<0.01) than that of Grade 1 (3.2%) and 2 (1.5%) embryos. In conclusion, the Cryotop technique seems to be useful for Grade 1 and 2 donkey embryos. It is likely that donkey embryos show similar survival rates after vitrification in Cryotops irrespective of age, diameter and development stage.

Key words: Andalusian Donkey; Embryo; Vitrification; Cryotop

1. Introduction

For centuries, domestic donkeys (*Equus asinus*) have been used as pack animals or for draught work in agriculture, thereby supporting the economy of rural areas [1]. However, in the last decades, the donkey population has been drastically reduced in several Europe countries [2,3], including Spain [4], mainly due to the intensive mechanization of the countryside [5]. The 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), because their small population size [6]. Considering the importance of biodiversity and of preservation of domestic species resources, the creation of genetic banks for these donkey breeds is advisable.

Cryopreservation of equine embryos and their storage in frozen embryo banks allow the conservation of male and female genetic material, the recovery and management of equine species and the maintenance of their genetic heterozygosis [7]. In addition, the cryopreservation of embryos allows the national and international exchange of genetic resources [7], avoiding the transport of live animals and health risks [8]. Furthermore, embryo transfer optimizes the use of embryo donors and minimizing the number of recipients [8]. In spite of all these advantages, procedures for the cryopreservation of equine embryos have not yet offered consistently satisfactory results [9].

Traditional slow-freezing procedures have been used for the cryopreservation of small equine embryos (<300 μm) with reasonable pregnancy rates (50 to 60%), but lower than those for fresh or cooled embryos [10]. These protocols combine the use of low concentrations of cryoprotectant agents (CPAs) and slow cooling rates to dehydrate the embryos during freezing and to prevent the formation of intracellular ice crystals

[11]. Ice crystal formation is known to be the main responsible of damage in cell membranes and organelles in slow-frozen embryos, decreasing post-warming embryo survival rate [12]. Besides, these procedures require expensive equipments and are laborious and time-consuming [13]. Indeed, in horses, slow freezing has not produced clinically satisfactory results, except for the work of Lascombes and Pashen [14].

Nowadays, vitrification has become a promising alternative to traditional slow-freezing procedures to improve cryotolerance of equine embryos [8]. The vitrification procedure consists of a short incubation time in high concentrations of CPAs followed by rapid temperature drop to avoid ice crystal formation, and to induce a glass-like state [15]. The use of high concentrations of CPAs may be however toxic or results in osmotic stress to the embryo [15,16]. In order to avoid these undesirable effects, new vitrification devices have been developed to minimize the volume of vitrification solution (minimum volume cooling: MVC) and to increase the rate of cooling [17]. The Cryotop method is an advanced version of the MVC procedure that has been successfully used for the cryopreservation of animal [18-20] and human [21] embryos. This device allows the use of a very small volume of fluid (<0.1 µL) and the direct exposure of embryos to liquid nitrogen (LN_2), avoiding ice crystal formation and cell damage and improving embryo survival rates [22].

The size of the equine embryos is the major factor affecting survival rate after cryopreservation [8], and it may be even more critical when vitrification is used as a cryopreservation technique. Previous studies demonstrated that early-stage embryos (<300 µm) produced better survival rates after vitrification and warming than large embryos [23-25]. More recently, Eldridge-Panuska et al. [26] demonstrated that equine morula and early blastocysts can be vitrified and produce pregnancy rates nearly as high as fresh embryos. There are some potential reasons to explain poor freezability of large

equine embryos: (i) the development of the acellular glycoprotein capsule by day 6.5 post-ovulation [27], impedes the penetration of the CPAs [28,29]; (ii) thickness of the capsule has been shown to be correlated to freezability of the embryo [30]; (iii) the large amount of blastocoel fluid induces the formation of ice crystals [31]; and (iv) small surface-area-volume ratio could affect the membrane permeability to water and CPAs [32]. However, to the best of our knowledge, there are no reports in the literature on vitrified donkey embryos at different embyo development stages and ages.

In horses, it seems that vitrification procedures are better than traditional slow-freezing procedures for embryo cryopreservation [33,34]. However, the results obtained so far are highly variable and only a few studies offer data on pregnancy rates after transfer to recipients [26,35]. In donkeys, there are few studies about embryo vitrification [36-39]. Panzani et al. [36] were the first to obtain pregnancies and live foals after transfer of vitrified donkey embryos. However, no studies were found that have used the Cryotop method to vitrify donkey embryos.

The aim of the present study was to determine the effectiveness of the Cryotop method to vitrify donkey embryos and to evaluate the effects of the embryo development stage, embryo quality, embryo diameter and age of embryos on both the ability of the embryos to survive the vitrification-warming procedure and on cell death *in vitro*.

2. Materials and Methods

2.1. Animals

Thirteen healthy Andalusian jennies (3-13 years-old), of known fertility, served as embryo donors and five Andalusian jacks (6 to 9 years-old) known to be fertile were

used to mate the donors. The jennies were housed in paddocks, and the jacks in boxes at Centro Rural Malpica (Palma del Río, Cordoba, Spain).

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

2.2. Oestrus synchronization and mating

Oestrus was induced with one intramuscular injection of 5.25 mg luprostiol (Prosolvin®, Virbac, Barcelona, Spain) during diestrous. Ovarian activity was evaluated daily during oestrus by transrectal ultrasound (Aloka SSD 500, ALOKA Co. Ltd., Tokyo, Japan). Donor jennys received human chorionic gonadotropin (hCG; 1500 IU, intramuscularly; Veterin Corion®, Divasa-Farmavic S.A., Barcelona, Spain) to induce ovulation when a follicle of 35 to 40 mm was detected. Next day, donor jennies were naturally mated and, if necessary, every other day until ovulation. Ovulation was detected via daily transrectal ultrasound (Day 0 = day of ovulation).

2.4. Embryo collection and evaluation

Six to eight days after ovulation, donor jennies were flushed with Lactated Ringer´s solution (B. Braun VetCare S.A., Rubí, Spain), as described by Camillo et al. [40] for donkeys. Embryos (n = 43) were washed ten times in Syngro® holding (Bioniche Animal Health, Washington, USA), as previously described [41].

The diameter of embryos was measured under bright field conditions (SZ51 Olympus optical, Tokyo, Japan) using an ocular micrometer (scale of 1mm/100), as

143 previously described [13]. The morphology was evaluated and the embryos were graded
144 on a 1 to 4 scale [42], 1 being excellent, 2 good, 3 fair, and 4 poor, degenerate or dead.
145
146 *2.5. Embryo vitrification and warming*
147 Embryos (n = 25) were cryopreserved using the Cryotop® method (Kitazato
148 BioPharma Co. Ltd., Shizuoka, Japan) as described by Kuwayama [22]. All steps were
149 performed under a laminar flow hood at room temperature (20 to 25 °C), using a
150 stereomicroscope to visualize each step [43]. The holding medium (HM) consisted of
151 TCM-199 HEPES (M7528, Sigma-Aldrich Chemie GmbH, Steinheim, Germany)
152 supplemented with 20% foetal calf serum (FCS; Gibco, Life Technologies, Grand
153 Island, New York, USA).
154 After washing, embryos were transferred into equilibration solution (ES)
155 consisting of 7.5% dimethylsulfoxide (DMSO; D2650, Sigma-Aldrich Chemie GmbH,
156 Steinheim, Germany) and 7.5% ethylene glycol (EG; 293237, Sigma-Aldrich Chemie
157 GmbH, Steinheim, Germany) in HM for 10 to 15 min. In this first step, after an initial
158 shrinkage, embryos recovered their original volume. Then, embryos were transferred to
159 the vitrification solution (VS) containing 15% DMSO, 15% EG, and 0.5 M sucrose
160 (Suc; S9378, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) dissolved in HM.
161 After incubation of 30 to 40 s, the embryos were loaded onto Cryotop, the excess of
162 fluid was removed to leave only a thin layer covering the embryos (< 1 µL), and device
163 was quickly plunged into LN_2. From the exposure to VS to plunging, the elapsed time
164 did not exceed 90 s. The loaded Cryotops were inserted in the cap and stored in LN_2
165 until warming.
166 All warming steps were performed at 38.5 °C. After removing the protective cap
167 while still in LN_2, the Cryotop was directly immersed into the warming solution (WS)

containing 1 M Suc dissolved in HM. After 1 min, the recovered embryos were transferred to the dilution solution (DS), which contained 0.5 M Suc dissolved in HM. The embryos were incubated for 3 min and CPA diffusion out of the embryo was promoted by gentle pipetting. After that, the embryos were incubated in HM for 5 min. After a final rinse in HM for 1 min, embryos were transferred to a culture dish well containing 950 µL Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham (DMEN/F12; Gibco, Life Technologies, Grand Island, New York, USA), 10% FCS [44], and 25 µg/mL gentamicin (Gibco, Life Technologies, Grand Island, New York, USA) [45], and allowed to recover at 5% CO_2 in air at 38.5 ºC for 24 h. Embryo diameters and quality grades were assessed after 24 h post-warming.

2.6. Analysis of embryo viability

To assess the percentage of dead cells at 24 h culture, embryos were stained with Propidium iodide (PI; P4170, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and Hoechst 33342 (HO; 14533, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) dyes, according to the methods of Lagares et al. [13] and Oberstein et al. [33] with modifications. Briefly, embryos were washed in 100 µL droplet of Dulbecco´s phosphate buffer saline (DPBS; D5773, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and incubated in 100 µL droplet of DPBS supplemented with 1% bovine serum albumin (BSA; A7906, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) 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 on four droplets of a paraffin oil/petroleum jelly mixture. Finally, stained embryos were

examined under a fluorescence microscope (Olympus BX40, Tokyo, Japan), using a narrow band filter (a 360 to 370 nm excitation filter). This resulted in all nuclei fluorescing blue (HO +) and dead cells fluorescing pink (HO+/PI +). Three different and independent estimates of the percent dead cells were estimated and the average of these numbers was taken [33].

2.7. Toxicity test

The embryo toxicity of CPAs was tested by exposing fresh embryos (n = 9) to the vitrification and warming solutions without freezing. The exposed embryos were then cultured under the conditions described above. Embryo diameter, embryo quality and viability at 24 h were recorded and compared with such data obtained from the control group (non-exposed fresh embryos, which were transferred directly to culture medium, as control of culture conditions; n = 9).

2.8. Statistical analysis

Data are shown as mean ± standard error of the mean (SEM). The effect of vitrification on diameters and quality scores was analyzed by general linear model (GLM) repeated measures analysis of variance. The percentage of embryos with different quality was analyzed by Chi-square. Analysis of variance (ANOVA) and Duncan´s test was used to evaluate the effect of vitrification on the percentage of dead cells. Pearson correlation analysis was used to determine correlations. All analyses were performed using the statistical package SAS version 9.0. Differences were considered statistically significant when $P < 0.05$.

3. Results

A total of 43 embryos were used, of which 31 (72.1%) were classified as Grade 1 (excellent), 9 (20.9%) as Grade 2 (good) and 3 (7%) as Grade 3 (fair). The most frequent stages of development observed were morula (23.3%, 10/43), early blastocyst (41.9%, 18/43) and expanded blastocyst (34.9%, 15/43; Table 1). Mean diameter of embryos was 192.50 ± 16.69 µm (range: 154.75 to 230.25 µm) for morula, 211.11 ± 9.09 µm (range: 191.94 to 230.29 µm) for early blastocyst, and 820.00 ± 190.26 µm (range: 411.94 to 1228.06 µm) for expanded blastocyst.

From the 43 embryos that were used, 9 embryos were used for toxicity control while 9 embryos were used as fresh non-vitrified control. The remaining 25 embryos were first grouped based on their diameter into three categories: (i) ≤220 µm (n = 10), (ii) >220 to 300 µm (n = 8), and (iii) >300 µm (n = 7); and then vitrified (Table 2). Fresh non-vitrified embryos (Control) showed similar quality (1.22 *vs.* 1.33; $P>0.05$) but increased in diameter (788.89 µm *vs.* 979.68 µm; $P = 0.023$) after 24 h culture. Although there was no significant ($P>0.05$) increase in diameter over time, embryos exposed to CPAs (CPA) and CPA and vitrified embryos (CPA + VIT) had a significant ($P<0.05$) decline in embryo quality after 24 h culture. At 24 h, embryo grade was higher (signifying a lower quality) in CPA + VIT group compared to the control group. After 24 h culture, the mean number of dead cells did not differ ($P>0.05$) between treatments.

In general, vitrification led to a decrease in embryo quality after 24 h culture (on average 1.28; $P<0.05$) when compared to fresh embryos (Table 3). Cryotop-vitrified donkey embryos had an average score of 2.72 after 24 h culture, whereas fresh donkey embryos had an average score of 1.44. No influence ($P>0.05$) of the studied variables (embryonic diameter, developmental stage, age or grade of the embryos before vitrification) on the quality of embryos after 24 h culture was detected (Table 3). Although there was no significant ($P>0.05$) increase in overall embryonic diameter

(Before vitrification: 328.00 ± 49.48 µm; After vitrification: 308.33 ± 32.05 µm), Day 7 embryos had significantly ($P<0.01$) higher diameter after 24 h culture than before vitrification (246.15 ± 29.12 µm *vs.* 307.69 ± 33.42 µm). Whereas early blastocyst stage embryos had significantly ($P<0.05$) higher diameter after 24 h culture (270.45 ± 18.41 µm) compared to initial diameter (220.45 ± 13.85 µm), the opposite was observed in Day-8 embryos (705.00 ± 145.22 µm *vs.* 537.50 ± 68.08 µm).

There was a significant ($P<0.01$) effect of embryo grade on the percentage of dead cells (Table 3). The greatest increase in the percentage of dead cells was observed in Grade 3 embryos (56.5%). However, no differences ($P>0.05$) were observed between the other groups of embryos (day of recovery, development and diameter) in the percentage of dead cells. Repeated measures ANOVA of embryo viability showed a significant ($P<0.05$) interaction of embryo grade with embryo age. The percentage of dead cells and embryo quality grade after 24 h culture were significantly correlated ($r = 0.764$; $P<0.01$), thereby indicating that morphological grading was an acceptable indicator of viability [33].

Table 4 shows that average embryo survival rates at 24 h post-warming was 64% (16/25). After warming, the proportion of ruptured embryos was 24% (6/25). No significant ($P<0.05$) differences were observed between average survival rates at 24 h post-warming among the development stages (72.7% for early blastocysts *vs.* 57.1% for morulas and expanded blastocysts). The proportion of embryos that survived vitrification procedures was numerically higher, although not significant, for Day 7 embryos (84.6%; $P>0.05$). At Day 6, none of the early blastocysts survived to the vitrification-warming process, whereas the survival rate of early Day 7 blastocysts was numerically higher (80%; $P>0.05$). Similar results (100%) were observed for Day 7 morulas and expanded blastocysts, although there were no significant differences

between these groups (P>0.05; Table 4). Lower rates were observed when expanded Day 8 blastocysts were vitrified-warmed (40%, 2/5), although the differences did not reach statistical significance (P>0.05).

On the other hand, significant (P<0.05) differences were observed for embryo grading (Table 4). No differences (P>0.05) were observed between Grade 1 and 2 embryos (70.6% *vs.* 80%), but none of the Grade 3 embryos survived to the vitrification-warming process. Moreover, a significant (P<0.05) increase in survival rate was noted when morulas of Grade 1 were vitrified-warmed (100%, 4/4). No significant (P>0.05) differences were observed for early and expanded blastocysts, but there was a tendency for higher survival rates in Grade 1 and 2 embryos.

Post-warming survival rates were similar (P>0.05) for embryonic diameter (Table 4), although the post-warming survival rate was slightly higher in small embryos (≤220 µm; 70%) than in the other groups (>220 to 300 µm: 62.5%; >300 µm: 57.1%). When survival rates were compared, considering the developmental stage, this same tendency was observed for morula and expanded blastocyst stages (P>0.05; Table 4).

4. Discussion

Embryo vitrification has been studied in several species, including horses, but only few studies are available on the use of vitrification to cryopreserve donkey embryos. To the best of our knowledge, this is the first report about the use of the Cryotop method to vitrify donkey embryos.

Several studies have reported differences in survival rates or viability after vitrification and warming of equine embryos, emphasising that the length of embryo culture affects embryo cryotolerance. An incubation time of 24 h was chosen because of previous studies on horses demonstrated that the culture of embryos for 20-72 h did not

affect embryo survival rates [31,33,44,46]. The *in vitro* culture conditions used in this study were found to be optimal for culture of donkey embryos, because of no deterioration in the quality of the control embryos was observed after 24 h culture. In addition, substantial increase in embryo diameter (on average 190.79 μm) was found together with a low percentage of dead cells at 24 h culture (2.7%), which evidences the quality of the culture system. When the toxicity of CPAs was tested, embryo grade and the percentage of dead cells of embryos exposed to vitrification and warming solutions without freezing were similar to those of the culture control group, thereby confirming the safety of these solutions.

The use of the Cryotop method as a cryopreservation technique resulted in a decline in embryo quality after 24 h culture. Cryotop-vitrified donkey embryos had an average score of 2.72, which is consistent with previous studies in horses [13,33] and donkeys [37]. Previous studies observed 57% of embryo development after culture, when equine embryos were exposed to solutions containing EG, Ficoll and Sucrose for 60 s at 20 ºC [23,35]. In our study conditions, when donkey embryos were exposed to CPAs (15% DMSO + 15% EG) for 60-70 s at room temperature (22 to 25 ºC), we obtained average embryo survival rates at 24 h post-warming of 64%. Our findings were similar to those involving equine embryos [47], but less than the 44% and 50% reported for small equine embryos subjected to open pulled straw (OPS) vitrification and slow-cooling procedure respectively [34]. It is interesting to note that all these previous works vitrified only Grade 1 or 2 embryos. In line with this approach, Cryotop vitrification of 17 Grade 1 donkey embryos resulted in 70.6% of embryos surviving after warming. Similarly, the proportion of Grade 2 embryos surviving vitrification and warming was 80% (4/5), which was comparable to those of previous works [31,32]. Our

data suggest therefore that the use of the Cryotop method seems to offer acceptable donkey embryo survival rates.

The incidence of ruptured embryos immediately after warming (24%) was similar to those of previous studies for equine embryos vitrified by different methods [13,23,32-34]. Since the rupture of embryos upon warming is however barely recorded in the literature [44], it is plausible that the membranes were ruptured by mechanical pressures of the ice during freezing [32]. Nevertheless, the results of a recent study support the hypothesis that capsule fracture may be reversed *in vivo* [48]. In fact, two of six ruptured embryos appeared viable by Hoechst 33342-Propidium Iodide staining. However, further studies are needed to confirm this assumption in donkeys.

The results of the present study did not demonstrate any influence of the diameter and age of embryos on post-warming survival rate of the embryos. This lack of effect could be partially due to the low number of embryos assessed in each group and consequently resulting in low power for statistical comparison. Regardless of the embryonic developmental stage, numerically higher survival rates of Day 7 embryos (84.6%) were recorded compared to Days 6 and 8 embryos (42.9 and 40% respectively). Similar results have been reported for bovine embryos [43], suggesting that Day 7 embryos had greater chance of surviving the vitrification process after warming. In agreement with previous findings [23,26], the largest embryos (>300 µm) also had a numerically lower post-warming survival rate (57.1%) than smaller ones (>200-300 µm: 62.5%; ≤220 µm: 70%). Eldridge-Panuska [26] suggested that the embryonic capsule, appearing at these later stages, may impair the diffusion of CPAs. Our results emphasize the importance of collecting Day 6 to 7 donkey embryos, smaller than 300 µm in diameter and with high morphological score, for vitrification. However, most

authors agree that the embryo recovery rate is lower when uterine flush is performed at Day 6 [7].

Previous studies conducted in different species, such as the mare [32], the cow [43], the goat [18], the sheep [49] and the sow [50], have shown that the developmental stage of embryos before vitrification affects their post-warming survival. In our study, when we examined the effect of development stage, we observed that morula and expanded blastocyst stages exhibit numerically lower survival rates after vitrification compared to early blastocysts (57.1% *vs.* 72.7%). This finding has been related to greater mechanical damage suffered by expanded than early blastocysts due to greater ice crystal formation in the blastocoele [51]. It is also speculated that the membrane permeability to water and CPAs may increase or decrease depending on the developmental stage of equine embryos [32]. In bovine, Hasler et al., [52] reported, however, that within the different age and grade categories, embryo stage failed to modify pregnancy rates. Embryo transfer experiments are needed to confirm the *in vitro* results shown in this study.

Having established the survival of the Cryotop-vitrified embryos we assessed the percentage of dead cells of individual embryos (Hoechst 33342-Propidium Iodide). The percentage of dead cells observed in this experiment (8.1%) was similar or lower than those reported by other authors when horse (11.1 to 73.3%) [13,33,34,53] and donkey (9%) [39] embryos were vitrified using other vitrification solutions, protocols or devices. These results could be partially explained by species differences in particular characteristics of embryos, which would render different CPAs penetration rate or toxicity [13]. Another possibility could be the lower solution effects (embryo damage caused by prolonged exposure to high concentration of CPAs) induced by the Cryotop device compared to other vitrification carrier systems applied in the previous studies.

In contrast, our results were slightly higher than the rate (4.6%) reported by Pérez-Marín et al. [37], in which donkey embryos were frozen using different cryopreservation protocols (slow freezing and vitrification) combined with different devices (Fibreplugs and 0.25 mL straws) and CPAs (EG, glycerol and galactose). However, this study only used Day 6.5-7.5 donkey embryos (four per treatment), with good morphological quality (Grade 1 or 2), which could explain, at least in part, this difference. In fact, under our experimental conditions, the percentage of dead cells observed in Grade 1 (3.2%) and 2 (1.5%) donkey embryos were significantly lower than that in Grade 3 embryos (56.5%). Indeed, no differences were observed between vitrified-warmed embryos and fresh control embryos for the proportion of dead cells. In any case, in our 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 [34].

5. Conclusions

The Cryotop technique seems to be effective for the cryopreservation of Grade 1 and 2 donkey embryos. It is likely that donkey embryos show similar survival rates after vitrification in Cryotops irrespective of age, diameter and development stage, although small Day 7 embryos at the early blastocyst stage seem to be more prone to vitrification. Further studies should be conducted to address the effects of new CPAs and fertility.

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