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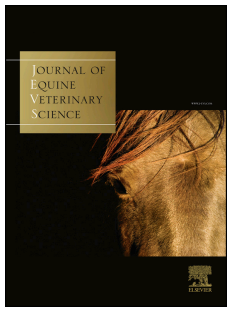

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Update on donkey embryo transfer and cryopreservation

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Abstract:
Donkey species reproductive biotechnology studies had a dramatic increase after the Convention of Biological Diversity that took place in Rio de Janeiro (Brazil) in 1992. The mechanization of agriculture of the 20th century took most of the developed countries donkey breeds close to extinction and, after Rio, the development of effective reproductive programs to save them were encouraged. Moreover, the recent interest in donkey milk, meat and skin products transformed the donkey in a potential productive animal leading to the research and dissemination of desired productive tracts into its population. Amongst reproductive biotechnologies the production and cryopreservation of embryos has a key role due to the possibility of producing and stocking valuable genetics potentially forever. Each species gametes and embryos from each species need specific media and protocols due to their particular characteristics. After more than 10 years of unsatisfactory results, embryo transfer and cryopreservation in the donkey moved from horse protocols to procedures more specifically tailored to the donkey species
Nowadays pregnancy rates of 50% and 36% after the transfer of fresh and vitrified embryos, respectively, is possible in this species.

Keywords: Donkey, Embryo Transfer, Embryo cooling, Vitrification

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1. Introduction:

Embryo transfer (ET) is an assisted reproductive technique that consists in the transfer of one or more embryos from a female, called donor, to one or more females, called recipients [1]. Embryo transfer is a valuable tool able to maximize the reproductive potentialities of a female. Using this reproductive technology, we could be, theoretically, able to give birth to a living being for each ovulation of its mother throughout all of her reproductive life. To be eligible for this technique a female should be able to ovulate, the oocyte to be fertilized and the genital

apparatus to let the embryo reach the uterus and survive until it will be flushed away [2]. The approach to ET in the donkey species initially moved from guidelines derived from the equine consolidated protocols, and its results marked some interesting differences between these two species of equids [3-5]. After the Convention of Biological Diversity [6] [7], in Europe, where most of the donkey local breeds are faced with the risk of extinction [8], the interest on donkey biotechnologies increased, in order to find a way to build a donkey germplasm bank. More recently, the interest in the donkey as a production animal, and the related reproductive technologies, has grown after the evidence of the beneficial effects of donkey milk [9-14], the healthiness of donkey meat [15] and the several applications of donkey-hide gelatin in Chinese traditional medicine [16-18].

2. Brief history of embryo transfer in domestic mammals:

The scientist who produced the first live animals by this technique was Walter Heape in April 1890. Heape surgically transferred two fertilized ova from an Angora doe rabbit into the upper end of the Fallopian tube of a Belgian Hare doe rabbit which had been fertilized three hours before by a Belgian Hare buck rabbit. The Belgian Hare doe gave birth to four Belgian Hare and two Angora breed offspring [19]. During the twentieth century embryo transfer was attempted successfully for the first time in ovine and caprine species in 1932-1933, in bovine and swine species in 1951 and in the horse in 1974 [1].

At the dawn of this technique embryo recovery and transfer were performed by laparotomy in all species; for embryo recovery, uterine tuba washing was carried out after catheterization, while, for the transfer of embryos, a little opening was made into the exteriorized uterine horn ipsilateral to the corpus luteum of the recipient, where the embryos were gently deposed by a sterile pipette [20].

The techniques above described prevented the commercial application of ET, that in the bovine and horse species has not been possible before the introduction of non-surgical embryo recovery and transfer techniques. This transition took more than 20 years to complete [1,21]. The first step on this way was to collect embryos trans-cervically by uterine lavages [21]. The first successful non-surgical ET was obtained in 1964, in the bovine, but before this technique was applied commercially in large scale we had to wait until the ‘70 [21] because “the efforts made to transfer embryos through the cervix were frustrated by problems of infection and egg expulsion” [1]. The development of non-surgical embryo recovery and transfer techniques moved the bovine ET from the labs to the practice, but what made it profitable was superovulation [22]. The event that led to this revolution took place in 1981, when George Seidel Jr. induced the

superovulation and subsequently artificially inseminated a dairy cow; after a week, 10 embryos were non-surgically recovered and immediately transferred trans-cervically to 10 synchronized recipients, and obtaining 10 live calves [23].

As previously reported, the first successfully equine ET was performed in Japan in 1974 injecting the embryo into the uterine lumen through the vaginal fornix using a special catheter "the apparatus consisting of three plastic tubes, one inside the other, was guided to the anterior fornix of the vagina where a needle attached to the middle tube was passed through the vaginal wall and on through the uterine wall into the uterine lumen " [24]. The development of this technique followed approximately the same steps of bovine ET. As all the other species, for the first years, both embryo recovery and transfer were performed surgically. In the early studies, to recover embryos, oviducts were catheterized and washed after flank laparotomy, but due to the limited repeatability of this technique on the same donor a trans-cervical uterine lavage was performed with the same success rate [25]. Since the beginning of this century, surgical ET was discontinued [26]. One of the main reasons was because some studies reported a release of PGF2α during cervical manipulation of non-surgical ET in some mares, [27] followed by a decrease of progesterone plasma concentration [27,28]. In addition, this technique was costly and further studies showed, as happened in the bovine species, that the pregnancy rates with the non-surgical technique were similar to those obtained with the surgical method [29]. After the transfer technique moved definitively from the surgical to the non-surgical technique, the critical points of this method in the horse have been classified into factors pertaining to the donor and to the recipients. The age and the reproductive history and health of the donor, the ovulations per cycle and the type of semen employed (fresh, cooled or frozen thawed), all showed to have an influence on embryo recovery rate and embryo diameter and quality [29-32]. The age, the reproductive health of the recipients, the day after ovulation and ovulation synchrony with the donor at the time of ET and the embryo diameter and quality, all have an effect on pregnancy rate after transfer [30,31,33,34].

3. Donkey and Embryo Transfer, a troubled relationship:

The first studies on donkey embryo transfer were reported by Allen and Short in 1982 [3] and 1985 [4]. In these studies, authors surgically transferred donkey embryos into recipient mares and vice versa to study extra-specific pregnancies in the equid species. Embryo recovery in donor mares and jennies was performed by trans-cervical uterine lavage and ETs by mid-ventral laparotomy (n=9 horse-to-donkey and n=95 donkey-to-horse ETs), performed under general anesthesia. Six out of 9 horse embryos transferred into jennies resulted into a pregnancy. Two

out of the six pregnancies were left to continue uninterrupted until the birth of two healthy foals. The development of endometrial cups was studied into the 4 horse-to donkey pregnancies interrupted pharmacologically was physiologic. Pregnancy rates after horse–to-donkey (67%) and donkey-to-horse (63%) ETs at 40 days were analogue. Differently from horse-to-donkey ET pregnancies, in the 70% of donkey-to-horse ET pregnancies, endometrial cup formation and fetus implantation never occurred. All these fetuses were aborted between day 80 and day 100 of pregnancy. In the remaining 30% of donkey-in-horse pregnancies endometrial cups formed at a slower rate than in normal pregnancies and half of the pregnancies gave birth to a normal donkey foal [4]. The other half of these pregnancies ended with an abortion during the last two months of pregnancy [4]. A subsequent study reported as the unique successful treatment supporting the donkey-in-horse pregnancies, the active immunization against genetic relatives' lymphocytes with the birth of 6/9 (67%) live foals [5].

The first trace of donkey-in-donkey pregnancy after ET was reported in France in 1997 [35], showing the first differences between asinine and equine ET came out. The authors of this study obtained 21 embryos between 6 and 8 days of age out of 33 cycles (63.6%) of 6 Poitou jennies. : Of the twenty-one embryos, 8 were transferred surgically, 10 were transferred non-surgically, all into synchronized donkey recipients, and the remaining 2 were frozen and then transferred into a single recipient. Out of 18 ETs, just three (16.7%) resulted in a pregnancy. In this study recipients were considered synchronized if ovulating from the day before till the 3 day after the donor (synchrony from +1 to -3) as for the horse [30].

These disappointing results were confirmed in an extensive study on ET in the Pantesca breed performed in our lab [36]. In this study, donors were flushed three times for embryo recovery using a total of 2 liters of flushing media kept at 37 °C. The flushings were performed using either DPBS (ZE067; I.M.V., L'Aigle, France) and a one-way tubing system without filter or lactated Ringer (Galenica Senese, Siena, Italy) and a self-built ''Y'' tubing system using an EZ Way filter (PETS, Canton, TX, USA). For both techniques, an Equine Lavage Catether 32 F (AB Technology, Pullman, WA, USA) was used. Sixty-three, 7-to-9 days old embryos, were recovered out of 83 estrous cycles (75.9%) and 98 ovulations (64.3%) of five Pantesca jennies, 2 to 5 years old, naturally mated or artificially inseminated with fresh semen. This result was similar to what was reported by Vendramini (63.6%){Vendramini:1997vo}. Each recovered embryo was evaluated for morphology [30], washed 10 times, placed in a 0.25-mL French straw with a small amount of the washing medium, and transferred transcervically to a recipient treated intravenously with 3.3 mg/100 kg acepromazine (Prequillan; Fatro, Bologna, Italy) using a guarded French gun [11]. Some of the embryos were measured. Washing medium was either

PBS (ZE067, n = 14 embryos), lactated Ringer (n = 33 embryos), or EmCare Holding Solution (Bio98 Srl, Milano, Italy; n = 11 embryos). After the quality evaluation, the embryos were gently aspirated into a 0.25 or 0.5 ml French straw preceded and followed by a small amount of holding solution and a bubble of air. The French straw was then inserted into a French Gun designed for bovine ET (IMV Technologies, Bicef, Piacenza, Italy) and non-surgically transferred into synchronized recipients treated 10 min before transfer with acepromazine (3.3 mg/iv/100 kg; Prequillan, Fatro, Bologna, Italy). Recipients were led to a stock, the rectum evacuated of feces, the tail wrapped and tied up, and the perineum washed three times with a povidone iodine soap (Esoform Jod 75, Esoform S.p.a., Rovigo, Italy), rinsed and dried with clean paper towels. The operator inserted the guarded gun protected by a sanitary sheath through the vagina. The vaginal part of the cervix was grabbed with three fingers and pulled backwards, the tip of the gun was blindly inserted in the cervical os, the sanitary sheath was then broken, and the cervix was manipulated to aid the gun insertion and progression. The embryos were released in the body of the uterus of jennies, without any trans-rectal manipulation [37].

Embryo recovery rate was influenced by number of ovulations per cycle (133% and 63% for double and single ovulations, respectively), by the day of embryo recovery attempt (12%, 83%, and 75% at Days 7, 8, and 9 after ovulation, respectively), and by the repetition of the embryo recovery attempt on successive cycles (60%, 79%, and 100% for cycles 1 to 7, 8 to 14, and 15 to 24, respectively).

All recovered embryos but three were classified as good or excellent. Of 58 non-surgical embryo transfers to Ragusana jenny recipients, 13 (22.4%), 10 (17.2%), and 9 (15.5%) resulted in a pregnancy at Days 14, 25, and 50, respectively. Recipients' pregnancy rate was not influenced by the evaluated parameters: embryo quality and age, days after ovulation of the recipient, or experience of the operator. The only numerical, but not statistical, difference was the effect among the 3 different holding media employed to wash the embryos. While washing embryos after recovery with Ringer Lactate (n = 33 embryos) and EmCare Holding Solution (Bodinco®, The Netherland; n =11 embryos) gave pregnancy rates of 27.2%, the use of DPBS (ZE067, n = 14 embryos) resulted in only the 7% of pregnancies after ET.

Between 14 and 50 d of pregnancy, 4 of 13 (30.7%) embryos were lost, showing an influence of the number of days from ovulation of the recipient: Day 5 and 6 recipients kept all pregnancies (n=7), whereas day 7 and 8 recipients lost 3 of the 4 pregnancies and day 3 recipients lost 1 out of 2 pregnancies."

These pregnancy rates were lower than those observed after both surgical or non-surgical transfer of horse embryos to horses (50% vs 75%) [31,38] or described after surgical transfer of donkey embryos to mares or of horse embryos to jennies (63% and 67%, respectively) [4].

The reasons for these low pregnancy rates were not explained by the analyzed factors of the study, so the authors decided to investigate if non-surgical transfer techniques or donkey embryo quality could affect donkey recipient pregnancy failure [39]. The impact of transfer technique was investigated by evaluating if cervical catheterization was associated with prostaglandin release and suppression of luteal function and if donkey recipients would become pregnant after nonsurgical transfer of horse embryos more than of donkey embryos. Moreover, donkey embryo viability was investigated by 6-diamidino-2-phenylindole (DAPI) staining of 10 donkey embryos and by the transfer of 6 and 12 donkey embryos to synchronized mare and donkey recipients of known fertility, respectively.

DAPI staining method has been used for the evaluation of embryo damage (described as the percentage of dead per total cells) in horses [40] and other species [41-44] but not yet in donkeys.

The cervix of the jenny is longer, smaller, and tighter than the cervix of the mare [45], and catheterization is more difficult and requires more manipulation [36]. For these reasons, a release of PGF2α , as seen during cervical manipulation in some mares [27,28], followed by a decrease of progesterone plasma concentration [28], was hypothesized for jennies subjected to trans-cervical ET [36]. For this reason, 5 to 8 d after ovulation, 4 jennies were submitted to a sham trans-cervical ET and to evaluation of the PGF2α metabolite 13,14-dihydro-15-keto-PGF2α (PGFM) and progesterone plasma concentrations. Before the sham ET procedure, a cannula was inserted into the jugular vein. Blood samples for PGFM were collected 60, 45, 30, and 15 min before entering the stock, immediately before transfer (Time 0), 2, 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, 180, 240, 300, and 360 min after Time 0, and once daily for 4 days and again at Day 7. Cervical stimulation caused a transient PGF2α release in two of four jennies in the absence of a significant decrease in progesterone plasma concentration.

Embryo recoveries were performed 7 to 9 d after ovulation by a self-made, 2-way tubing system connected to a cuffed 32 French, silicon-made catheter (AB Technology, Pullman, WA, USA) [36]. Jennies' and mares' uteri were flushed 4 to 6 times with a total of 2 to 10 liters of Lactated Ringer's solution (Galenica Senese S.r.l., Siena, Italy) depending on uterine capacity. After the flushing, 3 mg alfaprostol/im (Gabbrostim, CEVA VETEM, SpA, Milano, Italy) was administered to donors to induce luteolysis. Right after recovery, embryos were washed in EmCare Holding Solution (Bio98 Srl, Milano) and stained with DAPI [40]. The number of dead

cells (DAPI stained) was counted and the embryo total number of cells was estimated using the correlation: $n=0.0106d^2$ 2.0542d – 375.28 (n: cells number, d: embryo diameter in μm) calculated for the horse [40]. The estimated proportion of dead cells in DAPI stained embryos was 0.9% (range 0–3.9%) and below what is considered normal (20%) for horse embryos. All the stained embryos were vital.

Five 8 days old horse embryos were non-surgically transferred into synchronized donkey recipients between 5 and 8 d after ovulation. And 8 to 9 days old donkey embryos were transferred into synchronized jennies' (n=12), or mares' (n=6) uteri as described previously by Camillo [36]. All five horse embryos transferred into donkey recipients resulted in pregnancies at 14 and 25 d (5/5)

Three of six and six of 12 of the Donkey-in-Horse and Donkey-in-Donkey ETs, respectively resulted in pregnancies at 14 and 25 d (50%), a higher pregnancy rate than previously reported after Donkey-in-Donkey [36] and comparable to Donkey-in-Horse [4] and horse-in-horse ET [29,31,33,34]. The overall results of this study suggest that the trans-cervical technique for ET and donkey embryo viability are not the reasons for the low pregnancy rates that have previously been described in donkey recipients [35,36], and that nonsurgical ET in donkeys can result in acceptable results. These results were confirmed by Peña-Alfaro and colleagues in the Pega donkey breed in Brazil [46]. These authors transferred fresh 11 9-days-old donkey embryos to as many synchronized recipients obtaining a 45.4% (5/11) of pregnancy rate.

4. Cooled and frozen Embryo Transfer in the donkey, first steps:

The selection and propagation of a specific genome could lead to the transmission of desirable phenotypic tracts for donkey productions and the cold preservation of embryos allows, as for the semen, to move valuable genetic materials through space and time [47,48]. In the bovine industry, in 1999, around 75000 frozen embryos were transferred worldwide allowing the best genetics to be spread all over the world [1]. Nowadays there are 3 ways to preserve the embryos: cooling embryos at 4°C and transfer them after maximum 24 hours, or *sine die* in liquid nitrogen after conventional freezing or vitrification [47].

Cooling the embryos after recovery allows the donor to be flushed on the breeding farm and permits transportation of the embryos to a centralized laboratory where the recipient herd resides. Hence, there is no need to transport the donors nor to buy and maintain recipients at the donor's breeding farm [40,47]. Usually pregnancy rates are better when ET is performed by experienced personnel in a dedicated ET facility, so, especially in the horse species, this technique is widely used [47].

According to McCue et al, the cooling technique consists in placing the recovered and washed embryo into a 5mL plastic or glass tube almost full of the warmed holding medium; fill the tube with holding medium until it's full; fix the cap securely in place; seal the tube with Parafilm sealing film; fill a 50 mL centrifuge tube with the saved flush medium; place the 5mL tube containing the embryo into the 50mL centrifuge tube; attach the cap of the centrifuge tube securely in place and seal with Parafilm; load the 50 mL tube containing the embryo into a passive cooling device [47].

Conventional embryo freezing is widely used in the bovine industry, where superovulation provides a large number of embryos recovered per flushing. Bovine embryos have a good vitality after freezing and ET of frozen-thawed embryos have a commercially acceptable success rate [48,49].

Conventional freezing is a procedure that consists in several passages and needs trained personnel and expensive equipment [48]. To avoid cellular damages, most of the embryonic intracellular water needs to be substituted by cryoprotectants. This could be done moving the embryos in media containing increasing concentration of cryoprotectants, step by step. At this point the embryo is loaded in a French straw and the straw is placed in the device and temperature descends at a rate of approximately 3 ºC/minute from room temperature to -6-7 ºC. After seeding, the temperature decreases at a rate of 0.3-0.5 ºC/minute to approximately -35 ºC and finally plunged and stored in liquid nitrogen [48].

For vitrification, instead, the embryo is moved subsequently into drops of vitrification media containing increasing concentration of cryoprotectants, loaded in a french straw, kept for a minute over the liquid nitrogen vapors and finally plunged and stored into liquid nitrogen. The final concentration of cryoprotectants is higher than conventional freezing and the cooling rate is really fast, approximately 2500◦C/min. Both conventional freezing and vitrification resulted in pregnancy rates comparable to the fresh embryos if their diameter was < 300 µm (64-80%) [48,50-54]. Scarce results were reported after transfer of horse frozen-thawed > 300 µm embryos (8-29%) [50,55,56]. A possible explanation for these results, in contrast to embryos from other species, could be that large equine embryos contain a capsule that may negatively limit the cryoprotectants permeability and affect embryo survival after freezing and thawing.

Donkey embryo cooling has been tested in vitro, in our lab, in a study comparing the viability of fresh, cooled or vitrified donkey embryos [57] by DAPI staining method. Eighteen 7 to 8 days old, quality I donkey embryos, recovered by uterine lavages with Ringer Lactate (RL), were divided in 3 groups: 1) Fresh (n=6, 7-days old; FR); 2) Cooled for 24 hours in a 5 ml test tube

filled with the same RL recovered at the first uterine lavage (n=6, 8-days old; RL24); 3) Vitrified using the Equine Vitrification Kit (Bioniche Animal Health, USA) and the technique described by Eldridge-Panuska (Eldridge-Panuska et al. 2005) (n=6, 7-days old; VIT). After 24 hours of refrigeration in Equitainer® (RL24), after thawing (VIT) or directly after being recovered (FR), embryos were washed three times in EmCare Holding Solution, measured, placed for 5 min into EmCare Holding Solution containing 1µg/mL DAPI and washed three more times in EmCare Holding Solution. Embryo dead cells (DAPI +) were directly counted under a fluorescence microscope, while the total number of cells was estimated as described previously [40,58]. The proportion of DAPI stained cells was compared using One-Way ANOVA and LSD post-hoc test. Embryo diameter, estimated number of total and of DAPI stained cells, and percentage of DAPI stained cells (±sd) are described in Table 1. Embryo dead cell number, for all the 3 groups, was lower than what is reported to be the maximum limit for viability of horse embryos. The results observed in fresh embryos were similar to what has been previously reported in donkeys (07±0.3 vs 0.9±1.17). However, the proportion of dead cells differed between groups (P<0.05): vitrified embryos resulted significantly more damaged compared to fresh or refrigerated embryos (P<0.05).

Table 1: Diameter, total number of cells, number of DAPI stained cells and percentage of fresh (FR), cooled (RL24) or vitrified (VIT) donkey embryos

Treatment	**Ø (µm)**	**Total number of cells**	**Number of DAPI stained cells**	**Percentage of DAPI stained cells**
FR (Day 7)	193±48	439±325	3±2	0.7±0.3[a]
RL24 (Day 8)	738±368	8110±8496	13±17	0.2±0.3[a]
VIT (Day 7)	225±50	650±357	56±58	9.0±9.3[b]

[a,b]: within column, values with different superscripts differ significatively (P<0.05)

The results of this study make the cooling of donkey embryos an interesting opportunity, even though it has to be confirmed by the transfer of cooled donkey embryos into synchronized recipients as already done with vitrified donkey embryos. In fact, in a previous study, we tried to evaluate if it was possible to obtain pregnancies and live foals after transfer of vitrified donkey embryos and to compare day 14 and 25 pregnancy rates following transfer of donkey and horse vitrified embryos. Eleven day-7 donkey embryos and 10 day-6 horse embryos were recovered as previously described [39]. Embryos were washed 10 times in EmCare Holding Solution (Bio98 Srl, Milano, Italy), measured, and morphologically evaluated [30] before being vitrified using

the Equine Vitrification Kit (Bioniche Animal Health, USA) and the technique described by Eldridge-Panuska et al. [53]. During the next breeding season the embryos were thawed and transferred using the non-surgical direct-transfer procedure [53] into selected recipients of the same species at day 5 after ovulation. Pregnancy rates at 14 and 25 days in mares and in jennies were compared by the Fisher's Exact Test. All donkey and horse embryos were below 300 μm in diameter and graded as excellent or good [30]. Pregnancy rates after ET were 4/11 and 4/10 at day 14 for jennies and mare recipients, respectively. One pregnancy was lost in both jennies and mares before. Both One of the 25th day of pregnancy and 1/3 donkey pregnancies resulted in the delivery of a non-viable foetus at 321 days of gestation. The remaining two jennies produced two healthy fillies at term. Pregnancy rate in jennies was slightly lower compared to the latest results after transfer of fresh donkey embryos [39,46].

The development of assisted reproductive techniques for donkeys remains a challenge, mostly due to the little research funding available. Many aspects of donkey reproductive physiology appear similar and others different to the horse. On the other hand, interest in donkey reproduction has had a linear growth since the beginning of the century, and results of applying reproductive biotechnologies to donkey production and gamete preservation are likely to achieve similar results to those in the horse.

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Update on donkey embryo transfer and cryopreservation: a review

Highlights

Most of the European donkey species are at risk of extinction

Donkey is becoming a production animal: milk, meat and skin

Programs for the enhancement of the endangered donkey breeds and for the production of donkeys with desired productive tracts are financed

Embryo transfer and cryopreservation, after two decades of research reached acceptable results

Ethical statement:

The authors of the paper entitled: "**Update on donkey embryo transfer and cryopreservation: a review**" declare that no ethical issues are present for the publication of this review.

Conflict of interests:

The authors of the paper entitled: "**Update on donkey embryo transfer and cryopreservation: a review**" declare that no conflict of interest is present for the publication.