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Small day 8 equine embryos cannot be rescued by a less advanced recipient mare uterus

J. Cuervo-Arango*, A.N. Claes, T.A.E. Stout

Department of Equine Sciences, Faculty of Veterinary Medicine, Utrecht University, the Netherlands

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ABSTRACT

Equine embryos tolerate an unusually large degree of negative uterine asynchrony (recipient mare up to 5 days behind the donor mare). By contrast, positive asynchrony of more than 2 days results in a high incidence of early embryonic loss (EEL). Day 8 embryos range in diameter from approximately 130-1300 μm, with embryos smaller than 300 μm reported to suffer an increased incidence of EEL. However, it is not known whether this reduced viability is due to intrinsically poor embryo quality, or to inadvertent recipient uterine stage-embryo (positive) asynchrony. To examine whether small embryos survive better in Day 4–5 recipients than in recipients with a more advanced uterine stage, the likelihood of pregnancy (PR) and EEL for 62 small (<300 μm) and 215 larger Day 8 horse embryos were compared after transfer to recipients at different uterine stages (Days 4–5, 6–7 and 8–9) using logistic regression. Overall, EEL was higher (21.2%; $P < 0.05$) for small than larger embryos (7.1%). However, neither PR nor EEL were influenced by the recipient's uterine stage at the time of transfer ($P > 0.1$). The EEL for small embryos transferred into Day 4–5, 6–7 and 8–9 recipients was 20.8, 18.7 and 25.0%, respectively. We conclude that embryos recovered on Day 8 with a diameter <300 μm are at increased risk of EEL due to reasons other than inadvertent positive asynchrony with the recipient mare's uterus.

1. Introduction

Equine embryos allow an unusually large degree of negative uterine asynchrony (i.e. the recipient mare ovulating after the donor), with no difference in the likelihood of pregnancy apparent after transferring embryos into recipients that ovulated up to 5 days after the donor mare (−5) [1]. On the other hand, a relatively modest positive asynchrony between donor and recipient mares (i.e. >2 days) does negatively affect embryo survival [2]. Indeed, in the latter study a high incidence of embryo loss (75%) was observed when Day 8 embryos (Day 0 = Day of ovulation) were transferred into Day 11 recipient mares (+3 asynchrony). Furthermore, when Day 7 embryos were transferred into Day 11–12 recipient mares (+4 to +5 asynchrony) no pregnancies resulted (0/8) [2].

In practice, embryos are typically collected from donor mares on Day 8 after ovulation. At this stage, embryo diameter has been reported to vary from 130 to 1344 μm, with a mean of 583 ± 272 μm [3]. Moreover, the diameter of the recovered embryo has been shown to influence the likelihood of early embryonic loss (EEL) following initial establishment of pregnancy in recipient mares [4]. In Carnevale and coworkers' survey [4], embryos measuring 100–299 μm in diameter were more likely to be lost between Days 12 and 50 of gestation than larger embryos. However, it is not known whether the apparent reduction in viability of pregnancies resulting from small embryos is due to intrinsically poor embryo quality (i.e. retarded, small-for-age embryos) or extrinsic factors, such as inadvertent recipient uterine age-embryo (positive) asynchrony, resulting in an inappropriately advanced uterine environment [2]. In this respect, embryos measuring <300 m in diameter are more typically recovered from flushes performed on Day 6 after ovulation [5]. Nevertheless, < 300 μm embryos are also recovered, albeit less frequently, from mares flushed 8 days after ovulation [3]. It is possible that these small Day 8 embryos (comparable in size to 'normal' Day 6 embryos) would survive and develop better in recipients with a less advanced uterine stage (i.e. Day 4–7) than in mares with a more advanced uterine stage (i.e. Day 8–9 recipients).

The objective of this study was to examine the relationship between embryo diameter at ET and the recipient's uterine stage on likelihood of pregnancy and EEL. It was hypothesized that the percentage of small embryos (<300 μm) yielding a pregnancy or

* Corresponding author.
E-mail address: j.cuervo-arangolecina@uu.nl (J. Cuervo-Arango).

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suffering subsequent EEL would not be influenced by the recipient's Day of ovulation (uterine stage).

2. Materials and methods

2.1. Donor mares and embryo collection

A total of 135 donor mares (predominantly Warmblood; mean age 11.1+/-0.9 years; range 3–25) were presented to Utrecht University's Equine Clinic for embryo recovery. Two hundred and thirty-six embryo flushes yielded at least 1 embryo, and a total of 277 embryos (195 single embryos and 41 set of twins) were included in the study. Mares that yielded embryos did so once (n = 75), twice (n = 37), and ≥3 times (n = 23) over three breeding seasons. Insemination of donor mares was performed at the University's Equine Clinic (n = 170 cycles) or at other locations (n = 66 cycles), and all flushes were performed on Day 8: Day 0 = Day of detected ovulation (first ovulation for mares with asynchronous twin ovulations). Donor mares were inseminated with cool-transported or cryopreserved semen. The proportion of mares inseminated with cryopreserved or chilled semen was only known for donors inseminated at the University clinic and was 90 cycles with cool-transported semen and 80 cycles with frozen-thawed semen. However, it has been recently shown that the type of semen (fresh *vs.* frozen/thawed) used to inseminate donor mares did not influence the embryo size at flushing [3].

All embryo flushes were performed using 3 × 1 L of a commercial Lactated Ringer's solution (Baxter Nederland BV, Utrecht, The Netherlands) supplemented with 0.5% v:v fetal calf serum. After recovery into an in-line filter (Emcom: IMV Technologies Netherlands, Leeuwarden, NL), embryos were located and measured (outer diameter) using a dissecting microscope (Olympus SZ60, Olympus Nederland B.V., Leiderdorp, NL) equipped with an eye-piece micrometer. After washing, the embryos were held in holding medium (Syngro; Bioniche Animal Health INC, Athens, GA, USA) at room temperature between 30 min and 2 h before transfer. At the time of embryo flushing, the following data were recorded from donor mares; age and number of embryos recovered per flush (single versus twin).

2.2. Recipient mares and embryo transfer (ET)

All recipient mares included in the study belonged to the University's Equine Clinic and were maintained at the clinic until at least day 40 of gestation. Recipient mares were Warmbloods, aged 3–12 years old (mean of 8.2 ± 0.6 years) and were kept in grass paddocks in groups of 5–20. Detection of ovulation was performed by daily transrectal ultrasonography once the mares were in late estrus. In 28.9% of all estrous cycles (80/277), hCG was used to induce ovulation. The day of ovulation was considered to be Day 0.

For ET, embryos were loaded into sterile 0.5 mL straws (IMV Technologies Netherlands, Leeuwarden, NL)) in the middle of 3 drops of holding medium separated by air. Each straw was loaded into a disposable transfer pipette with openings at both sides of the tip, and covered by a sterile plastic chemise (IMV Technologies). These sheaths were compatible with a stainless-steel "cassou" type rigid transfer stylet for Day 8 embryos (IMV Technologies) which was introduced into the transfer pipette, pushing the straw to the tip. Embryos were transferred transcervically into mares on Day 4 to Day 9 after ovulation (Synchrony of +1 to −4 days). No hormonal treatments were administered to recipient mares before or after transfer, but all mares were sedated with a single intravenous administration of 4 mg detomidine hydrochloride (Domosedan; Vetoquinol BV, 's Hertogenbosch, The Netherlands) immediately prior to ET, to avoid any sudden movement during ET and so facilitate the passage of the cervix with the embryo transfer gun. Pregnancy detection was performed by transrectal ultrasonography (Esaote Mylab gamma, Esaote Euope B.V., Maastricht, The Netherlands) 4 days after ET (Day 12 of pregnancy), and subsequently at two week intervals (approximately Days 26 and 40 of pregnancy).

The following recipient mare data was recorded: 1) Day of ovulation at time of ET; 2) outcome of first pregnancy diagnosis; positive (presence of an embryonic vesicle) or negative (absence of an embryonic vesicle: if negative, the recipient mare was rechecked for pregnancy 2–3 days later); 3) the diameter of the embryonic vesicle 4 days after ET (mm); 4) outcome of second pregnancy diagnosis; presence or absence of a heartbeat (anembryonic vesicle) or absence of the embryonic vesicle; and finally, 5) outcome of third pregnancy diagnosis; viable pregnancy (positive heartbeat and normal embryonic membranes) or failed pregnancy (no heartbeat or absence of embryonic vesicle). The incidence of early embryonic loss (EEL%) was calculated as the number of embryo losses between Days 12 and 40 of pregnancy, divided by the total number of pregnant recipients at the first pregnancy diagnosis.

2.3. Statistical analyses

Data were analyzed using Systat13. Two different binary logistic regression models were created to test the effect of different factors on establishment of pregnancy and EEL (dependent variables), respectively. The independent variables investigated were: 1) Age of donor mare (4 levels: 3–5 years, 6–12 years, 13–17 years and 18–25 years old), 2) Day of ovulation of recipient mare at ET (3 levels: Day 4–5, Day 6–7 and Day 8–9), 3) Size of embryo (3 levels: small <300 μm, medium 300–600 μm, and large >600 μm in diameter), 4) Type of embryo (2 levels: single or twin embryo), and 5) interaction between size of embryo and recipient's Day of ovulation. If a significance of $\alpha < 0.05$ was detected for any given variable, Fisher's exact test was used to compare differences between levels. Pearson's correlation was used to determine the strength of association between the size of the embryo at ET and the size of the embryonic vesicle in pregnant recipients 4 days after ET. On 5 occasions, the embryonic vesicle was not detected 4 days after ET but was visible at the recheck (2–3 days later). For analytical purposes (so that all vesicles could be represented in the scatter plot) an "estimated size" of 1.5 mm (just below the resolution capacity of the ultrasound scanner) was assigned to these 5 embryonic vesicles.

3. Results

The overall percentage of recipient mares pregnant 4 days after ET and the incidence of EEL, irrespective of embryo size, were 84.8% and 10.2%, respectively (Table 1). None of the variables investigated (age of donor, Day of recipient's ovulation, embryo size, type of embryo) influenced ($P > 0.05$) the likelihood of establishing pregnancy following ET. Furthermore, the interaction between the recipient mare's Day of ovulation and the size of the embryo at ET also failed to influence the likelihood of pregnancy ($P > 0.05$).

On the other hand, embryo size at ET did influence the likelihood of subsequent EEL ($P = 0.003$): EEL was highest (21.2%) for small embryos (<300 μm in diameter), followed by large (10.8%) and medium sized embryos (4.0%). On the other hand, there was no effect of the interaction between embryo size and the recipient's Day of ovulation ($P > 0.1$), and therefore small embryos were no more likely to survive in Day 4–5 recipients than in Day 6–7 or Day 8–9 recipients (Table 1). The vast majority (90.9%) of small embryos that were lost, never reached the heartbeat stage (Table 2). Instead,

Table 1

Recipient Days after OV	Embryo diameter at ET (μm)						Overall[B]	
	<300		300–600		>600			
	PR (%)	EEL (%)	PR (%)	EEL (%)	PR (%)	EEL (%)	PR (%)	EEL (%)
4–5	24/27	5/24	27/30	1/27	18/24	1/16	69/81	7/69
	88.9	20.8	90.0	3.7	75.0	6.2	85.2	10.1
6–7	16/21	3/16	32/39	1/32	36/41	4/38	84/101	8/84
	76.2	18.7	82.1	3.1	87.8	10.5	83.2	9.5
8–9	12/14	3/12	41/47	2/41	29/34	4/34	82/95	9/82
	85.7	25.0	87.2	4.9	85.3	11.8	86.3	10.9
Overall[A]	52/62	11/52	100/116	4/100	83/99	9/83	235/277	24/235
	83.9	21.2[a]	86.2	4.0[b]	83.8	10.8[c]	84.8	10.2

Interaction between diameter of horse Day 8 embryos and recipient mare's uterine stage (Days after ovulation) on likelihood of pregnancy (PR) and early embryonic loss (EEL).
A: Overall PR and EEL irrespective of Day of ovulation (Day 4–9) for small, medium and large embryos. Different superscripts (a,b,c) indicate a significant difference ($P < 0.05$) in EEL.
B: Overall PR and EEL irrespective of embryo diameter following ET into Day 4–5, 6–7, and 8–9 recipients.

45.4% of these small embryos developed as anembryonic vesicles, compared to only 15.4% of embryos larger than 300 μm on day 8 (Table 2). Twin embryos tended ($P = 0.09$) to have a higher incidence of EEL (14.5%) than singleton embryos (8.2%), although the likelihood of pregnancy at Day 40 was similar (P > 0.1) for singleton (75.1%) and twin embryos (78.7%). Neither the recipient's Day of ovulation at ET nor the age of the donor mare affected the incidence of EEL ($P > 0.1$).

There was a strong association ($r = 0.81$; $P < 0.001$) between embryo diameter at ET and the diameter of the embryonic vesicle as detected by ultrasound 4 days later (Fig. 1). Five embryonic vesicles could not be identified at the first pregnancy diagnosis but were detected 2–3 days later (6–7 days after ET). Three of these 5 embryonic vesicles subsequently suffered EEL (Fig. 1).

For mares that yielded an embryo on 3 or more occasions ($n = 23$), 13 mares yielded at least 1 small embryo in one of their positive flushes. From those 13 mares, two (15.4%) always produced (in 100% of positive flushes) small embryos (<300 μm); 5 and 3 embryos from 4 to 3 positive flushes, respectively. The pregnancy and EEL statistics for these two mares were 60.0% (3/5) and 66.6% (2/3) for mare 1, and 100.0 (3/3) and 33.3% (1/3) for mare 2, respectively. The ages of mares one and two at the beginning of the study were 20 and 15 years, respectively.

4. Discussion

The hypothesis that the likelihood of pregnancy or EEL for small day 8 equine embryos would not be influenced by the recipient's uterine stage (Day of ovulation) is substantiated by this retrospective study. We therefore conclude that embryos recovered on Day 8 with a diameter less than 300 μm are at an increased risk of undergoing early embryonic loss for reasons other than inadvertent positive asynchrony with the recipient mare. The small embryos were equally likely to result in a pregnancy 4 days after ET as larger embryos, but more likely to be lost subsequently, as reported previously [4]. This higher incidence of EEL suggests pre-existing compromise or poor quality of some of these small-for-age embryos, presumably reflecting deficiencies intrinsic to the oocyte or acquired during fertilization and/or the early stages of embryo development.

The association between increased EEL and 'small for age' size of Day 8 embryos resembles that reported for embryonic vesicles that were small for their gestational age when detected ultrasonographically in normal pregnant mares 13–15 days after ovulation [6,7]. These small for gestational age vesicles have been reported to be first detectable on Days 13–14 after ovulation, or sometimes even later, when they measured only 3–4 mm instead of the expected 10–15 mm [7]. One study reported that 78% of embryonic vesicles that were more than one standard deviation smaller than the mean had undergone EEL by the next examination [8]. In the current study, there was a strong association between the size of an

Table 2
Characteristics of horse embryo transfer (ET) pregnancies lost between days 12 and 40 of gestation in relation to embryo diameter at ET.

	Embryo diameter at ET (μm)	
	<300	>300
Number of embryos	11	13
Number of mares	9	13
Twin embryos (%)	45.4	46.1
Number of sets of twins lost	2 (4 embryos)	0
Mean diameter (μm)	230.8 ± 46.4[a]	714.7 ± 206.6[b]
Mean donor age (years)	11.1 ± 6.1	11.2 ± ;5.5
% Losses before heartbeat	90.9[a]	46.1[b]
% Anembryonic vesicles	45.4	15.4

Within row, different letters (a,b) indicate a significant difference ($P < 0.05$).

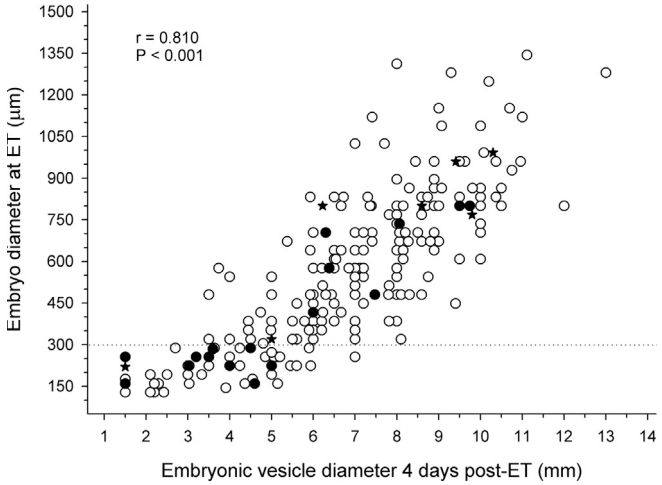

Fig. 1. Scatter plot of day 8 horse embryo diameters (μm) at time of embryo transfer and diameter (mm) of the resulting embryonic vesicle detected in the recipient mare by ultrasound 4 days later. Pearson's correlation between the two diameters was significant ($P < 0.001$) and positive ($r = 0.81$). Open circles represent viable pregnancies at day 40. Closed circles represent pregnancies lost between days 12 and 26. Stars represent pregnancies lost between days 26 and 40 days (after detection of a heartbeat). The broken line delineates pregnancies resulting from embryos <300 μm in diameter. Five embryonic vesicles could not be detected by ultrasound 4 days after transfer, but were visible 2–3 days later. An "estimated diameter" of 1.5 mm was used to represent these five pregnancies in the graph.

embryo at transfer and the diameter of the embryonic vesicle in the recipient mare 4 days later (i.e. at day 12 of embryonic age), with the smallest embryos resulting in the smallest embryonic vesicles, or not being detected 4 days after ET. In short, the small-for-age status of Day 8 embryos is carried over to initial intra-uterine development, regardless of the recipient mare's uterine stage.

Clearly, any attempt to explain why the affected embryos were small and more prone to EEL is speculative. Although advanced donor mare age is a well-accepted risk factor for EEL [9], there is considerable variation between mares and advanced mare age has not been shown to be consistently associated with small-for-age embryos or pregnancies, in this or in previous studies [3,6]. Delayed embryo descent into the uterus from a compromised oviduct could also theoretically result in small embryos, given that the embryo does not begin to expand until reaching the uterus [10]. Anecdotal observations from practitioners suggest that no embryos are recovered from some individual (often older) mares unless embryo flushing is delayed until Day 9 or 10 post-ovulation.

The current study included multiple embryos recovered from individual mares during 3–5 different flushes within or during consecutive breeding seasons. A small percentage of these mares (15%) always produced embryos <300 μm in diameter, and also exhibited above average incidences of EEL. It therefore appears that some individual mares are more likely to produce small-for-age embryos, a phenomenon that could arise from a high proportion of abnormal oocytes, a persistently compromised oviductal environment, or a suboptimal uterine environment. However, the majority (85%) of mares flushed multiple times and that yielded a small embryo in one flush, also delivered larger embryos in previous or subsequent cycles. A final possible explanation for a small embryo on Day 8 is fertilization of a later asynchronous ovulation or development of an embryo from a post-ovulatory insemination, both of which would result in a younger than calculated embryo [3].

One additional interesting observation is that the majority (91%) of small embryos that succumbed to EEL did so before or without developing an embryo proper with a visible heartbeat, either by remaining as an anembryonic vesicle or by simply disappearing between the first and second pregnancy examinations at days 12 and 26 of pregnancy, respectively; by comparison only 46% of larger embryos that underwent EEL did so without developing a visible embryo with a beating heart. Pregnancy loss before development of a visible embryo proper has also been reported as the main presentation for EEL in mares inseminated (too) long after ovulation (12–24 h post-ovulation) [11]. In the latter study, 50% (7/14) of pregnant mares inseminated >12 h after ovulation underwent EEL, and 86% of the affected embryos were lost before a heartbeat was detected (between days 12 and 20 of pregnancy) [11]. Mammalian oocytes subjected to post-ovulatory aging have been shown to exhibit ultrastructural and molecular differences to freshly ovulated oocytes. In mice and pigs, these changes include fragmentation of the female pronucleus [12], displacement of chromosomes from the metaphase plate [13], and meiotic spindle abnormalities [14–16], all of which can predispose to aneuploidy and the production of compromised zygote, which in turn increase the risk of EEL and later fetal death [17]. Whether similar molecular alterations occur in equine oocytes fertilized long after ovulation has not been specifically examined but is likely. Although delayed post-ovulatory insemination cannot be ruled out as a contributory factor to some of the small embryos that underwent EEL in the current study (i.e. from mares inseminated at remote locations; n = 2), the majority of cases for which relative timing of ovulation and insemination were known, involved insemination pre-ovulation (n = 4) or within 6 h after ovulation (n = 2). Therefore, factors other than post-ovulation oocyte aging are likely to have resulted in the development of small-for-age embryos.

Embryo diameter at ET was a good predictor of the size of the embryonic vesicle at the first pregnancy examination 4 days later (Fig. 1). The scatter plot can therefore be used as a guide to estimate the best time to perform the first post-ET pregnancy diagnosis, based on the diameter of a recovered embryo. Although most ultrasound scanners can easily detect a vesicle of 2.5–3 mm in diameter, the first examination of a recipient mare could be delayed or advanced according to the size of the transferred embryo and the operator specific circumstances (e.g. experience, quality of ultrasound scanner), bearing in mind that the Day 11 to Day 15 embryonic vesicle grows at between 2.9 and 4.6 mm a day [18].

The inability to detect an embryonic vesicle 4 days after ET was a strong indicator that successful pregnancy was unlikely to result, since in the 5 cases that an embryo was detected at a follow-up scan 2–3 days later the majority subsequently underwent EEL (60%; 3/5). All 5 of the vesicles that were not detected at day 12 originated from small embryos. Although the other 2 embryonic vesicles had developed a fetus with a heartbeat by 40 d of pregnancy, it is not known whether they were carried to term and resulted in a healthy foal. In a previous study, all small for age embryonic vesicles with a size suggesting >2 days delayed growth at days 13–15 pregnancy failed before Day 50, with the exception of three that were carried to term: of these 3, one died at birth from unknown causes, a second failed to thrive and the third, which remained small and weak, was ultimately euthanized; a post-mortem examination revealed a single heart ventricle (Tetralogy of Fallot) following [7].

In conclusion, while horse embryos recovered on Day 8 with a diameter <300 μm exhibit a normal ability to develop into pregnancies, they are more likely to undergo subsequent early embryonic loss (21.2%) than larger embryos, irrespective of the uterine stage of the recipient mare (Day of ovulation) to which they are transferred. The majority of EEL for small day 8 embryos occurs between days 12 and 26 of pregnancy, before the development of an embryo proper.

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