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Horse embryo diameter is influenced by the embryonic age but not by the type of semen used to inseminate donor mares

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ABSTRACT

The diameter of embryos recovered from mares on Day 8 after ovulation varies greatly, from as little as 130 μm to as much as 2500 μm. Several factors have been proposed to affect embryo size at recovery, one of which is the type of semen (frozen vs fresh or cooled-transported) used to inseminate the mare. In addition, it has been shown that smaller embryos (<300 μm) recovered on Day 8 are less likely than larger embryos to result in successful pregnancy after transfer. However, whether the actual age of the embryo (interval from fertilization to flushing) in relation to its size also influences the post-transfer viability is unclear. The aims of this study were: a) To determine the effect of semen type (frozen-thawed vs cooled-transported) on embryo diameter after pre-ovulatory insemination; and b) To establish the relationship between embryonic age, embryo size and likelihood of pregnancy and pregnancy loss following transfer. A total of 179 embryos were recovered from mares inseminated with: frozen semen post-ovulation 8 days previously (G1; n = 35); cooled-transported semen pre-ovulation 8.5 days previously (G2; n = 95); frozen semen pre-ovulation 8.5 days previously (G3; n = 30); and frozen semen post-ovulation 9 days previously (G4; n = 19). The effect of embryonic age, type of semen, donor mare and its age, number of ovulations and embryos per flush on embryo diameter was tested using a general linear model of variance. In addition, the proportions and survivals of small embryos (<300 μm) in each group were compared with those of respective larger embryos by Fisher's exact test. Embryonic age ($P < 0.001$) and age of the donor mare ($P = 0.07$), but no other factor, influenced embryo diameter. The proportion of small embryos was 42.9, 10.5, 10.0 and 10.5% for Groups 1, 2, 3 and 4, respectively. The pregnancy status of recipient mares 35 days post-transfer for small embryos from Group 1 (12/15; 80.0%) was not different ($P > 0.1$) from that of recipients of small embryos from Groups 2 to 4 combined (8/15; 53.3%).

1. Introduction

Embryo donor mares are typically flushed for embryo collection 7–9 days after ovulation, with many veterinarians having a preference for Day 8. It is well accepted that the embryo, once it enters the uterus between approximately 5.5 and 7 days after ovulation (range from 130 h to 168 h) expands quickly, increasing its diameter rapidly as it gets older [1,2]. Mean diameter for Day 6, 7, 8 and 9 embryos have been reported as 191.8 μm, 354.0–404.9 μm, 623.9–660.3 μm, and 912.4 μm, respectively [3,4]. However, within a given day of recovery (e.g. Day 8), embryo diameter can vary greatly, from as little as 130 μm to as much as 2500 μm [4,5]. The diameter of the recovered embryo has been shown to influence the post-transfer likelihood of pregnancy. Indeed, small day 8 embryos (<300 μm) have been reported to be less likely to result in a viable pregnancy [5–7]. However, it is unknown whether the apparent reduced viability of these small embryos is due to intrinsic poor embryo quality (i.e. small-for-age embryos, reflecting preexisting growth retardation) or extrinsic factors, such as greater difficulty in handling small embryos, inadvertent increase in recipient's uterine age-embryo asynchrony, etc.

Several theories and factors have been put forward to explain the origin of the variation in embryo diameter, including exact interval between ovulation and flushing (could vary by 24 h if mares are only checked once a day for ovulation), the age of the donor mare [3], and the use of frozen semen as opposed to fresh or cooled-transported semen [4]. While differences in exact time of ovulation, oocyte quality, oviductal and uterine environment are

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likely to play important roles in the variation in embryo diameter on a given day after ovulation, there is debate about the effect of using frozen semen on embryonic development and size. Some studies [3,4] reported a smaller diameter of embryos flushed from mares bred with frozen semen compared to those from mares inseminated with cooled-transported or fresh semen. Indeed, McCue et al. [4] concluded that the reduced size could be due to a delay in embryonic development following fertilization with frozen semen. On the other hand, mares bred with frozen semen are often inseminated after ovulation has been detected (Day 0), whereas mares bred with fresh or cooled semen are usually inseminated the day before ovulation (Day −1). This may account for differences of up to 24 h in embryonic age. Furthermore, it has been shown that post-ovulatory insemination [8,9] yields smaller embryonic vesicles than does pre-ovulatory insemination, regardless of the type of semen used [8].

The objectives of this study were: a) To determine the effect of semen type (frozen-thawed vs cooled-transported) on embryo diameter after pre-ovulatory inseminations; and b) To establish the relationship between embryonic age (Day 8, 8.5 and 9), embryo size (embryos <300 vs >300 μm) and the likelihood of pregnancy and pregnancy loss. It was hypothesized that 1) embryonic age but not the type of semen used for insemination would influence embryo diameter, and 2) that the proportion of small embryos (<300 μm) and the likelihood of them establishing pregnancy would be higher for mares flushed on Day 8 than on Day 8.5–9.

2. Materials and methods

2.1. Animals and insemination protocols

A total of 179 embryos were recovered from 153 embryo flushes performed in 128 Warmblood mares (Mean age 11.4 ± 0.8 years; range 3–21) over three breeding seasons. All donor mares were inseminated and flushed at the Equine Clinic of Utrecht University's Faculty of Veterinary Medicine, so embryonic age could be calculated more accurately. The authors acknowledge that the obvious time "zero" for embryonic age determination corresponds to the moment of fertilization. However, because this exact time is unknown, the embryonic age at the time of flushing was calculated using the following assumptions. In mares inseminated before ovulation, ovulation and fertilization occurred at the mid-point between the time of insemination and detection of ovulation. In mares inseminated after ovulation, fertilization occurred at the time of insemination. Therefore, the embryonic age is reported nominally (Day 8, Day 8.5 and Day 9) ± the standard deviation originated from variations in the time of flushing within a given day (from 9:00 h to 14:00 h).

On the basis of the type of semen used (frozen-thawed vs cooled-transported), insemination protocol (largely dependent on the number of straws available), and the Day of embryo flushing, the following groups were created:

- G1-Day 8 frozen semen (27 mares and 35 embryos): Mares with obvious endometrial edema and a preovulatory follicle ≥ 35 mm in diameter were administered intravenously 1500 IU hCG at 22:00 on Day −2. Mares were checked for diagnosis of ovulation at 8:00 and 16:00 on Day −1. The minority of mares that had ovulated at 08:00 on Day 0 were inseminated immediately with one straw of frozen semen (n = 13 embryos). The rest were re-examined at 12:00 and similarly inseminated after ovulation had been detected at that time (n = 22 embryos). Mares were flushed 8 days later between 10:00 and 14:00. The mean embryonic age of this group was nominally 8 days ± 0.1 days (range of 7.9–8.2 days).
- G2-Day 8.5 fresh semen (69 mares and 95 embryos): Monitoring was as in Group 1, except that the hCG was administered at 09:00 on Day −2. The next day (Day −1), mares were scanned and inseminated pre-ovulation with 15 mL cooled-transported semen of different stallions (containing > 300 million motile sperm) between 16:00 and 17:00 (approximately 32 h after hCG). Mares that had already ovulated at the time of insemination were removed from the analysis. All mares included in this group had ovulated by 8:00 the next morning (Day 0) and were flushed 8 days later between 09:00 and 14:00. Ovulation was assumed to have occurred at midnight of Day −1 (which is similar to the expected interval from hCG treatment and ovulation of 39.1 ± 0.8 h reported previously [10]). The mean embryonic age of this group was nominally 8.5 days ± 0.1 days (range 8.4–8.6 days).
- G3- Day 8.5 frozen semen (18 mares and 30 embryos): Monitoring was as in Group 1, except that the hCG was administered at 09:00 on Day −2. The next day (Day −1), mares were scanned and inseminated pre-ovulation with half the dose of frozen semen (1–4 straws) between 16:00 and 17:00 in the afternoon (approximately 32 h after hCG). The next morning (Day 0), the second half of the dose (1–4 straws) was inseminated post-ovulation at 08:00. Only mares that had ovulated by 08:00 were included in this group. Mares were flushed on Day 8 between 09:00 and 14:00. As in G2, ovulation was assumed to have occurred at midnight of Day −1. The mean embryonic age was nominally 8.5 ± 0.1 days (range 8.4–8.6 days).
- G4-Day 9 frozen semen (14 mares and 19 embryos): As in Group 1 (post-ovulatory insemination with 1 straw of frozen semen), except that mares were flushed 1 day later (Day 9) between 09:00 and 12:00, and inseminations were all performed at 12:00 on Day 0. The mean embryonic age was nominally 8.9 ± 0.1 days (range 8.9–9.0 days). For the sake of simplicity, the embryonic age of this group will be referred as Day 9.

All mares included in the study had single or twin ovulations (in the latter case, both ovulations were first detected during the same examination: either at 08:00 or 12:00). All donor mares were flushed with 3 L of a commercial Lactated Ringer's solution (Baxter Nederland BV, Utrecht, The Netherlands) supplemented with 0.5% v:v fetal calf serum. Embryos were searched for and measured once (outer diameter) using a dissecting microscope (Olympus SZ60, Olympus Nederland B.V., Leiderdorp, NL) equipped with an eyepiece micrometer and, after washing, were held in holding medium (Syngro; Bioniche Animal Health INC, Athens, GA, USA) at room temperature for between 30 min and 2 h before being transferred transcervically into recipient mares that had ovulated between 1 day before and 4 days after the donor mare. No hormonal treatment was administered to recipient mares before or after transfer, but all mares were sedated with a single intravenous administration 3–4 mg detomidine hydrochloride (Domosedan, Vetoquinol BV, 's Hertogenbosch, The Netherlands) immediately before embryo transfer. Pregnancy diagnoses were performed at 4–6 days and 35–38 days following transfer.

2.2. Statistical analyses

Data were analyzed using Systat13. A general linear model of variance was created to test the effect of the embryonic age, donor mare and their age (young: 3 to 5, middle aged: 6 to 12, old: 13–21 years), number of ovulations (single *vs.* twin), number of embryos recovered per flush (single *vs.* twin) and type of semen used for insemination (fresh cooled-transported *vs.* frozen-thawed) on embryo diameter (independent variable). If a significance of $\alpha \leq 0.1$ was obtained for any dependent variable, a Post-hoc multiple

comparisons Tukey-test was used to detect any difference amongst levels. Fisher's exact test was used to test the difference in the proportion of small embryos (<300 μm), post-ET pregnancy status and early pregnancy loss amongst the embryonic age groups (Days 8, 8.5 and 9).

3. Results

None of the following variables: number of ovulations, donor mare, number of embryos recovered per flush, or the type of semen used for insemination influenced the diameter of recovered embryos ($P > 0.1$). The age of the donor mare tended ($P = 0.07$) to influence embryo diameter. A separate analysis for embryos recovered on Day 8.5 showed that young donor mares (3–5 years old) produced larger embryos (710.8 ± 40 μm; $P = 0.04$; $n = 27$) than middle-aged mares (6–12 years old; 605.3 ± 37 μm; $n = 30$) or old mares (13–21 years old; 576.5 ± 44 μm; $n = 66$). Embryo diameter did not differ ($P > 0.1$) between middle-aged and old mares.

The embryonic age had the strongest influence on embryo diameter at flushing ($P < 0.001$), with older embryos being larger (Fig. 1). The proportion of small embryos (<300 μm) was higher ($P < 0.01$) in mares flushed on Day 8 (42.9%) than after longer intervals (Table 1). However, the proportion of small embryos did not differ ($P > 0.1$) between mares flushed on Day 8.5 (10.4%) and Day 9 (10.5%; Table 1). The post-transfer viability of small embryos according to their age is shown in Table 2.

4. Discussion

The first hypothesis, i.e. that embryo diameter would be influenced by embryonic age but not by the type of semen used, was supported by the results of the current retrospective study. These results are in agreement with a previous study which showed similar embryonic vesicle diameters at the first pregnancy diagnosis for mares inseminated with fresh or frozen semen, provided

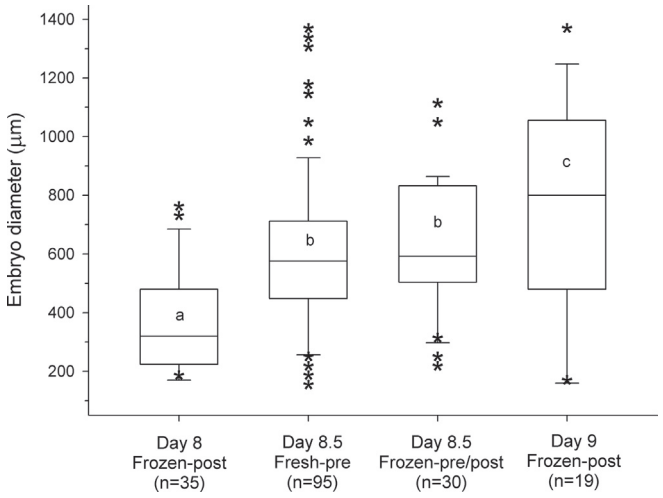

Fig. 1. Box plot distribution of embryo diameters recovered from mares from Groups G1 (inseminated with frozen semen post-ovulation and flushed 8 days later), G2 (inseminated with fresh semen pre-ovulation and flushed 8.5 d after ovulation), G3 (inseminated with frozen semen pre and post-ovulation and flushed 8.5 d after ovulation) and G4 (inseminated with frozen semen post-ovulation and flushed 9 d later). Different letters (a,b,c) indicate a significant difference ($P < 0.05$) in embryo diameter between groups. The upper limit of the box indicates the third quartile, the middle line the median, the lower line the first quartile and the whiskers the maximum and minimum embryo diameters. The asterisks represent outliers (unusually low-frequency observations that lie outside the 10th and 90th percentiles).

that the interval between ovulation and pregnancy diagnosis was similar [8]. The discrepancy with other studies which reported smaller embryo diameter in mares inseminated with frozen semen compared to those inseminated with fresh semen [3,4], appears to originate from differences in the timing of insemination relative to ovulation, and in the moment of ovulation on Day 0. In this respect, mares bred with frozen semen are commonly inseminated post-ovulation on Day 0 (morning to afternoon) whereas fresh and cooled-transported semen are generally inseminated pre-ovulation on Day −1, with ovulation occurring at some time between the afternoon of Day −1 and the morning of Day 0. In addition to the longer ovulation-embryo flush interval in the fresh semen group, it is expected that there will be a further delay to fertilization of the oocyte following post-ovulatory insemination in the frozen semen group [8], because the sperm are thought to require 2.5–4 h to capacitate and acquire the ability to fertilize [11,12]. In contrast to previous studies, the current study included a group (G3) in which frozen semen was inseminated before and after ovulation. Since the mean embryo diameter in this group was similar to that with a single pre-ovulatory insemination with fresh semen (G2) it can be assumed that most embryos were conceived from the first (pre-ovulatory) insemination. If not, the embryos would have been smaller - closer to those of G1. In addition, the proportions of small embryos in G2 and G3 were similar (10.5 and 10% respectively), further supporting the view that most fertilizations in G3 resulted from the pre-ovulatory insemination.

Hypothesis two, that the proportion of small embryos (<300 μm) and the likelihood of them establishing pregnancy would be higher in mares flushed on Day 8 than on Day 8.5–9, was only partially supported by the results of the study. There was clearly a higher proportion of small embryos (42.5%) in mares flushed 8 days after a post-ovulatory insemination (embryos aged 7.8–7.9 days, allowing time for sperm transport and capacitation) than in mares flushed after longer intervals. This finding was expected, since the embryo expands rapidly very soon after entering the uterus. Therefore, the longer the embryo is present in the uterus, the less likely it is to remain as an early blastocyst (i.e. < 300 μm in diameter). On the other hand, the proportion of small embryos recovered after Day 8 appeared to plateau at around 10% of all embryos flushed on Day 8.5–9. The absence of a further reduction in the proportion of small embryos between Day 8.5–9, may indicate a group of embryos suffering from a pre-existing delay in development and growth either due to intrinsic poor oocyte or embryo quality, late descent from a compromised oviduct or a failure to expand in a suboptimal uterine environment.

Unfortunately, the total number of small embryos in the different embryo age groups was small, so meaningful comparison of the post-transfer viability of Day 8 *vs* Day 8.5–9 small embryos could not be made; that is why hypothesis 2 cannot be accepted completely. Nevertheless, previous larger field studies have shown an association between a small-for-age embryo diameter (i.e. < 300–400 μm for Day 8–9 embryos) and an increased likelihood of failure to establish or maintain pregnancy following transfer [5–7]. Further studies with a larger sample size should be carried out to elucidate whether small embryos from Day 7 or early Day 8 flushes are more likely to result in a viable pregnancy than are small embryos from Day 8.5 or Day 9 flushes.

Beyond the clear and direct effect of embryo age on diameter, the great variation of diameters within age groups is striking (e.g. 144–1344 μm in G4). It follows that factors other than embryonic age *per* se must play a crucial role in the rate of embryo expansion and development. In that regard, a recent study [13] showed a delay in the development and growth of Day 8 embryos from donor mares with a suboptimal uterine environment induced by repeated PGF-treatment following ovulation (to prevent a systemic

Table 1
Effect of embryonic age and type of semen on horse embryo diameter and post-transfer viability.

	Groups for insemination protocol/type of semen			
	G1 Frozen post-8d	G2 Fresh Pre-8.5d	G3 Frozen Pre/Post	G4 Frozen post-9d
Embryos (n)	35	95	30	19
Embryonic age (d)	8.0 ± 0.1	8.5 ± 0.1	8.5 ± 0.1	8.9 ± 0.1
Type of semen	Frozen/thawed	Fresh-cooled	Frozen/thawed	Frozen/thawed
Timing of AI relative to OV	Post-OV	Pre-OV	Pre/Post-OV	Post-OV
Embryo diameter (μm)	375.7 ± 29.6[a]	605.7 ± 27.8[b]	639.3 ± 41.8[b]	778.9 ± 80.2[c]
Min-Max diameter (μm)	160–736	128–1344	192–1120	144–1344
Embryos <300 μm (%)	42.9[a]	10.5[b]	10.0[b]	10.5[b]
Post-ET viability (%)	31/35 (88.6)	73/95 (76.8)	24/30 (80.0)	14/19 (73.7)
Pregnancy loss by 45 d (%)	3/31 (9.7)	3/73 (4.1)	2/24 (8.3)	3/14 (21.4)

Embryonic age was calculated as the time from ovulation/insemination to embryo flush, for pre-ovulatory and post-ovulatory inseminations, respectively. In Groups 2 and 3, ovulation was assumed to have occurred at 39 h after hCG, at the mid-point (midnight) of the interval between the pre-ovulation and post-ovulation scan. Post-ET viability: pregnancy status of recipient mares 4–6 days after embryo transfer. Within a row, different superscripts (a,b,c) indicate a significant difference ($P < 0.05$).

Table 2
Post-transfer viability of small horse embryos (<300 μm) recovered on Day 8–9.

	Embryonic age (days)	
	8	8.5 to 9
Embryos (n)	15	15
Post-ET viability (%)	13/15 (86.7)	11/15 (73.3)
Pregnancy loss by 45 d (%)	1/13 (7.7)	3/11 (27.3)

Day 8 embryos were from Group 1, whereas Day 8.5–9 embryos were pooled from Groups 2, 3 and 4. The Post-ET viability (pregnancy status of recipient mares 4–6 days after embryo transfer) and pregnancy loss by 45 days of gestation in recipient mares did not differ between embryo age groups ($P > 0.1$).

progesterone rise). The embryos produced in these 'aluteal' cycles were smaller and of poorer quality than embryos from control cycles (171 *vs* 756 μm in diameter, respectively), underlining the necessity of an adequate progestogenic environment for normal early embryonic development. In this respect, the smaller embryos most frequently found in old mares [3] might reflect a suboptimal oviductal and uterine environment. However, the results of this study showed only a tendency of recovering larger embryos in mares aged 3–5 years old and the mean embryo diameter did not differ between other age groups. Further research should be carried out to establish differences in the timing of oviductal embryo descent and uterine environment between young and old mares to elucidate the exact effect of mare age on embryo diameter and development.

Other factors might play a role, to a lesser extent, in the variation in the embryonic diameter. It has been hypothesized that the sex of the embryo could account for differences in embryonic development. Although this could not be confirmed in the horse [14], bovine male embryos are known to develop and grow faster than female embryos under *in vitro* production and culture conditions [15]. The temporary contraction of mammalian blastocysts has been demonstrated in various species, which could potentially account for variations in diameter of up to 20–30% of the original size [16]. However, this has not been confirmed in the horse. The effect of season was not taken into account in the analysis of the current study, however, it is unlikely to have influenced the embryo diameter, as has been shown by Aurich and Budik [17].

In conclusion, embryonic age but not the type of semen used for insemination influenced embryo diameter at flushing. Young mares tended to produce larger embryos than did older mares but the difference was not great. Finally, approximately 10% of recovered embryos were small (<300 μm) even when flushing was delayed until Day 8.5–9, indicating that some embryos have a delayed oviductal descent, are incapable of expanding rapidly and/or are inhibited by a suboptimal maternal environment.

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