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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