

Survival of Day-4 embryos from young, normal mares and aged, subfertile mares after transfer to normal recipient mares

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Summary. The estimated embryonic loss rate between Days 4 and 14 after ovulation for young, normal mares (9%) was significantly lower ($P < 0.01$) than the estimated embryonic loss rate for aged subfertile mares (62%). Fertilization rates, which were based on the recovery of embryos at Day 4 after ovulation, were 96% and 81% ($P < 0.1$) for normal and subfertile mares, respectively. Day-4 embryos were collected from the oviducts of normal and subfertile donor mares. These embryos were transferred to the uteri of synchronized, normal recipient mares to test the hypothesis that the high incidence of embryonic loss in subfertile mares was related to embryonic defects. The hypothesis was supported because embryo survival rates were significantly higher ($P < 0.05$) for Day-4 embryos from normal compared to subfertile mares. These defects may have been intrinsic to the embryo or might have arisen due to the influence of the oviducal environment before Day 4 after ovulation.

Keywords: horse; embryonic loss; embryo transfer; embryonic defects; oviduct

Introduction

Several studies have indicated that embryonic loss is a major cause of reduced pregnancy rates in subfertile or 'repeat breeding' mares. Subfertile mares had a higher incidence of embryonic loss between Days 15 and 50 after ovulation than did other mares (39% versus 17%) (Villahoz *et al.*, 1985). Pregnancy rates were not significantly different for aged, subfertile mares and young, normal mares at Day 2 after ovulation (Ball *et al.*, 1986). However, at Day 14 after ovulation, pregnancy rates were significantly lower in subfertile mares than in normal mares. The embryonic loss rate, when estimated as the difference in the Day-2 and the Day-14 pregnancy rates, was 73% in subfertile mares and 0% in normal mares (Ball *et al.*, 1986).

Possible causes of embryonic loss in aged, subfertile mares include (1) abnormalities of the uterine environment, (2) failure of oviducal transport or abnormalities of the oviducal environment, and (3) embryonic defects. In an earlier study, the hypothesis that the uterine environment of subfertile mares was a cause of early embryonic loss was tested (Ball *et al.*, 1987a). Morphologically normal, Day-7 or Day-8 embryos were transferred to the uteri of normal or subfertile recipient mares, and their respective embryo survival rates were compared. The survival rates of transferred embryos through Day 28 after ovulation were similar between normal and subfertile recipient mares; therefore, uterine environment did not appear to be a major cause of early embryonic losses in subfertile mares (Ball *et al.*, 1987a).

The objectives of the present study were to: (1) estimate the embryonic loss rates for subfertile mares between Days 4 and 14 after ovulation, (2) determine the fertilization rates for 'young,

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normal' and 'aged, subfertile' mares, and (3) test the hypothesis that embryonic losses before Day 14 in subfertile mares were due to embryonic defects. Preliminary results have been presented by Ball *et al.* (1987b).

Materials and Methods

Experimental animals

Normal donor (N = 25) and recipient (N = 38) mares were primiparous or multiparous Standardbred mares with a mean age of 5.7 ± 0.3 years and were obtained at public auction. Subfertile donor mares (N = 22) were Standardbred or Thoroughbred mares with various histories of reproductive failure for the previous 2 years. Subfertile mares were obtained by donation from several breeding farms and had a mean age of 19.4 ± 1.0 years. All mares were fed grass hay *ad libitum* and were maintained in drylots in groups of 20–25 mares. The experiment was conducted during June through August of 1986.

Endometrial smears for cytological evaluation (Knudsen, 1964) and endometrial biopsies for histopathological examination (Kenney, 1978) were obtained from all mares 6 weeks before the start of the experiment. Endometrial smears were stained with a modified Wright's stain (Diff-Quik, American Scientific Products, McCaw Park, IL, U.S.A.) and were considered indicative of inflammation if > 1 neutrophil per 5 fields ($\times 400$) was observed (Knudsen, 1964). Endometrial tissue sections were examined and categorized for inflammation or periglandular fibrosis (Kenney, 1978). Normal donor and recipient mares had no indication of inflammation based on cytological examination and had a Category I endometrial biopsy (minimal inflammation and/or fibrosis; Kenney, 1978). Subfertile mares included mares with or without inflammation according to cytological examination of the endometrium and a Category II or III endometrial biopsy (moderate to severe inflammation and/or fibrosis; Kenney, 1978).

Summary of experimental design

At the beginning of the investigation, normal and subfertile mares were inseminated and their Day-14 pregnancy rates were determined by transectal ultrasonography. After the Day-14 pregnancy examination, each mare received prostaglandin (PG) F-2 α , and the subsequent ovulations were detected. Normal and subfertile donor mares were again inseminated and Day-4 pregnancy rates were determined by oviducal embryo collections. Some subfertile donor mares were inseminated during a second ovulatory cycle, and additional Day-4 embryo collections were attempted. To test the hypothesis that embryonic defects were responsible for embryonic losses in subfertile mares, Day-4 embryos from normal mares and subfertile mares were transferred to the uteri of synchronized, normal recipient mares, and embryo survival rates were compared.

Ovulation detection

Donor and recipient mares were examined *per rectum* for follicular development at least 4 times per week and were examined daily whenever a follicle > 25 mm in diameter was detected. Whenever the largest follicle exceeded 30 mm in diameter, donor mares were artificially inseminated with $\sim 750 \times 10^6$ progressively motile spermatozoa from the pooled ejaculates of 3 stallions. All inseminations were performed by a single operator on a 4-times-per-week basis until ovulation was detected. The day of ovulation (Day 0) was defined as the day on which a previously detected follicle of > 30 mm in diameter was no longer detected. Ovulations were confirmed by transectal ultrasonography (Ginther, 1986).

Day-14 pregnancy rates

Normal and subfertile donor mares were given 10 mg PGF-2 α (Lutalyse; Upjohn Co., Kalamazoo, MI, U.S.A.), and follicular development was monitored. Mares were then inseminated, ovulations were detected, and pregnancy rates were determined *in situ* at Day 14 after ovulation. Mares from both groups were examined with a 5-MHz, linear-array ultrasound unit (Equisonics-300, Equisonics, Inc., Elk Grove, IL, U.S.A.) for the presence of an embryonic vesicle on Days 10, 11, 12 and 14 after ovulation (Ginther, 1986) to establish their Day-14 pregnancy rates. The uteri of the normal and subfertile donor mares were examined by transectal ultrasonography at Day 7 after ovulation to identify fluid-filled uterine cysts which might confuse early detection of the embryonic vesicle (Ginther & Pierson, 1984). The location and diameter of detected uterine cysts were noted, and the ultrasound scan was recorded on videotape for later reference (Ginther, 1986). In 3 subfertile mares with multiple uterine cysts, ultrasound examinations were conducted on alternate days through Day 25 after ovulation to ensure that embryonic vesicles were not present.

After the Day-14 pregnancy examination (Day 25 in 3 subfertile mares), all donor mares were given PGF-2 α , and their subsequent ovulations were detected. At 7–9 days after these ovulations, donor and recipient mares were given PGF-2 α in preparation for the Day-4 embryo collection and transfer.

Embryo collection

Normal mares and subfertile mares were inseminated with semen from the same 3 stallions, and ovulations were detected. At 4 days after ovulation, each donor mare was anaesthetized with sodium thiamylal (Bio-Tal, Bio-Ceutic Laboratories, St Joseph, MO, U.S.A.) and maintained under inhalant anaesthesia with halothane (Fluothane: Fort Dodge Laboratories, Fort Dodge, IA, U.S.A.). The oviduct ipsilateral to ovulation was excised through a flank laparotomy, and the number of ovulations was confirmed by direct observation of the ovary either *in situ* or after ovariectomy.

Embryos and oocytes were recovered by retrograde flush of the excised oviduct (Ball *et al.*, 1986) with modified Dulbecco's phosphate-buffered saline with 10% heat-inactivated fetal calf serum (Gibco, Grand Island, NY, U.S.A.). Recovered embryos were evaluated and photographed at $\times 120$ under a stereomicroscope (Wild M420 Macroscope, Wild-Heerbrugg Ltd, Switzerland) for stage of development and morphology. Oocytes were evaluated as recent, unfertilized oocytes or aged, degenerating oocytes based on the criteria of Betteridge *et al.* (1982). Embryos were graded on morphological characteristics (Linares & King, 1980; Shea, 1981; Linder & Wright, 1983) based on retrospective analysis of photographs taken at the time of embryo recovery.

Embryo transfer

Day-4 embryos recovered from normal and subfertile donor mares were transferred via standing flank laparotomy to randomly assigned, normal recipient mares that had ovulated within 1 day of the donor mare. Recipient mares were sedated with xylazine HCl (Rompun: Bayvet, Shawnee, KS, U.S.A.) and morphine sulphate, and local anaesthesia was induced with 2% lidocaine. Embryos were transferred (Squires *et al.*, 1982; Ball *et al.*, 1987a) to the left uterine horn regardless of the side of the recipient mare's ovulation except for 7 recipient mares that received embryos on 2 separate occasions and 4 normal mares that had previously served as donor mares. In these mares, the second embryo was transferred to the uterine horn opposite that of the previous surgery. The embryo transfer time was defined as the time from oviduct excision in the donor mare until transfer into the recipient mare. In 15 subfertile donor mares, a 2nd embryo recovery and transfer attempt was performed. The survival of transferred Day-4 embryos was determined by transrectal ultrasonography of the recipient mare's uterus at Days 10, 11, 12 and 14 after the donor mare's ovulation.

Statistical analysis

Embryo recovery rates from normal and subfertile donor mares (first collection attempt) were used to determine Day-4 pregnancy rates. Embryonic loss rates were estimated by the difference in the Day-4 and Day-14 pregnancy rates for normal and subfertile mares. Fertilization rates were determined by the number of cleaved ova (embryos) recovered per total number of recently ovulated ova (cleaved and uncleaved ova).

Proportional data were compared by the appropriate paired or independent samples χ^2 analysis, and continuous data were analysed based on the appropriate paired or independent samples *t* test (Ryan *et al.*, 1976; Snedecor & Cochran, 1980).

Results

Pregnancy rates were significantly higher in normal mares than in subfertile mares at Day 4 ($P < 0.05$) and at Day 14 ($P < 0.01$) after ovulation (Table 1). In normal mares, there was no significant difference ($P < 0.5$) in the Day-4 and the Day-14 pregnancy rates; however, in subfertile mares, the Day-4 pregnancy rate was significantly higher ($P < 0.05$) than the Day-14 pregnancy rate (Table 1). Therefore, the estimated incidence of embryonic loss was significantly lower ($P < 0.01$) in normal than in subfertile mares (Table 1).

The fertilization rates for subfertile mares were not significantly different ($P > 0.25$) between the 1st and 2nd collection attempts, and these rates were combined for comparison with the fertilization rate of normal mares. The estimated fertilization rate tended ($P < 0.1$) to be higher in normal mares (27/28; 96%) than in subfertile mares (22/27; 81%). In addition, the Day-4 embryo recovery rates per mare or per detected ovulation were significantly higher ($P < 0.01$) for normal than for subfertile mares (Table 2).

Significantly more ($P < 0.05$) Day-4 embryos from normal mares than from subfertile mares survived until Day 14 after transfer to the uteri of normal recipient mares (Table 3). For subfertile donor mares, the survival rates of embryos transferred from the first or second embryo recovery attempts were similar (3/14 or 2/8, respectively). Significantly more ($P < 0.05$) Day-4 embryos recovered from normal than from subfertile donor mares following single ovulations survived after

Table 1. Pregnancy rates at Day 4 and Day 14 and estimated embryonic loss rates for young, normal and aged, subfertile donor mares

Pregnancy rate‡	Mare group	
	Normal§ (%)	Subfertile¶ (%)
Day 4	23/25 (92)	13/20*† (65)
Day 14	21/25 (84)	5/20** (25)
Estimated losses	2/23 (9)	8/13** (62)

* $P < 0.05$, ** $P < 0.01$ compared with value for normal mares.

† $P < 0.05$ compared with Day-14 value for this group.

‡Expressed as no. of mares with embryos detected/no. mares examined.

§Includes two twin pregnancies and one triplet pregnancy detected at Day 14 after ovulation.

¶Two subfertile mares were not mated for the Day-14 pregnancy determination and were not included in this table.

Table 2. Day-4 embryo recovery rates and fertilization rates for normal and subfertile mares

	Donor mares	
	Normal (%)	Subfertile (%)
Fertilization rate†		
First collection attempt	27/28	14/16
Second collection attempt	—	8/11
Combined collection attempts	27/28 (96)	22/27* (81)
No. of embryos/no. of attempts (mares)		
First collection attempt	27/25	14/22**
Second collection attempt	—	8/15
Combined collection attempts	27/25 (108)	22/37** (59)
No. of embryos/no. of ovulations		
First collection attempt	27/30	14/26
Second collection attempt	—	8/15
Combined collection attempts‡§	27/30 (90)	22/41** (54)

* $P < 0.1$, ** $P < 0.01$ compared with value for normal mares.

†Estimated at Day 4 by: no. of embryos/(no. of embryos + no. of recently ovulated unfertilized ova).

‡Four pairs of twin embryos and one single embryo from 10 synchronous, double ovulations in normal mares. Four single embryos from 8 synchronous double ovulations in subfertile mares.

§One subfertile mare with ovulations at Days 3 and 4 had one recently ovulated unfertilized ovum (Day 4) and one 8–16-cell embryo that was judged to have resulted from the Day-3 ovulation. This embryo was not included in the recovery or fertilization rates.

transfer to normal recipient mares, but sample size prevented meaningful comparisons of survival rates for embryos recovered following single or double ovulations within each mare group (Table 3).

There was no significant difference ($P > 0.25$) in the distribution of Day-4 embryos with fair-to-good or poor morphology or in the mean diameter of Day-4 embryos between normal and subfertile donor mares (Tables 3 & 4). Embryos with poor morphology had a lower survival rate after transfer from both normal ($P < 0.05$) and subfertile ($P < 0.1$) donor mares (Table 3). Embryos with fair-to-good morphology from subfertile donor mares tended to have a lower survival rate ($P < 0.1$) after transfer than did fair-to-good quality embryos from normal donor mares (Table 3).

Table 3. Survival of Day-4 embryos transferred from normal and subfertile mares to normal recipient mares

Survival of transferred embryos:	Donor mares	
	Normal (%)	Subfertile (%)
Through Day 14§	14/27 (52)	5/22** (23)
From single vs double ovulations:		
Single	11/19	5/18**
Double	3/9	0/4
Fair-good vs poor morphology:		
Fair-good	13/19†	5/14*‡
Poor	1/7	0/8

* $P < 0.1$; ** $P < 0.05$ compared with value for normal mares.

† $P < 0.05$; ‡ $P < 0.1$ compared with value for poor embryos.

§Excludes one embryonic loss detected by ultrasonography between Days 12 and 14 in each group.

Donor–recipient mare synchrony was not significantly different ($P > 0.5$) between embryo transfers from normal or subfertile donor mares and had no effect ($P > 0.9$) on survival of embryos from normal donor mares (Table 4). However, donor–recipient mare synchrony tended ($P < 0.1$) to affect survival of embryos from subfertile donor mares (Table 4). The transfer time tended ($P < 0.1$) to be longer for embryos transferred from subfertile than from normal donor mares (Table 4). Embryo survival rates for recipient mares that had prior surgery were not lower ($P > 0.25$) than those for recipient mares without prior surgery (3/11 vs 16/38).

Table 4. Synchrony, transfer times and embryo diameters for Day-4 embryos transferred from normal and subfertile donor mares

	Donor mares	
	Normal	Subfertile
Donor–recipient synchrony† and embryo survival‡§		
–1	4/8	0/4
0	7/13	5/15
+1	3/6	0/3
Transfer time (min)	41.9 ± 1.6	47.7 ± 2.6*
Embryo diameter (µm)	152 ± 1.4	151 ± 3.6

* $P < 0.1$ compared with value for normal mares.

†Recipient mare ovulated on the same day (0), the day before (–) or the day after (+) the donor mare.

‡Survival of embryos (no. of embryos transferred/no. of embryos present at Day 14) for each degree of synchrony.

§Difference ($P < 0.1$) in distribution of embryo survival for subfertile mares.

Discussion

The estimated embryonic loss rate between Days 4 and 14 for aged, subfertile mares (62%) was consistent with that reported for other aged, subfertile mares between Days 2 and 14 (73%) (Ball

et al., 1986). Furthermore, the estimated embryonic loss rate for young, normal mares (9%) was consistent with that of other young, normal mares between Days 2 and 14 (0%) (Ball *et al.*, 1986). This high rate of embryonic loss for subfertile mares in both studies was also consistent with the significantly lower non-surgical recovery of embryos from the uteri of subfertile mares at Days 7–10 after ovulation (Douglas, 1982; Squires *et al.*, 1982; Pascoe *et al.*, 1985; Woods *et al.*, 1986). Other investigators have also indicated that embryonic losses in fertile mares occur before Day 12 and have suggested that many of these losses occur before the embryo reaches the uterus (Forde *et al.*, 1987). Comparative data from subfertile or repeat-breeder cows also indicated that many embryonic losses occur by Day 8 after breeding (Ayalon, 1978; Maurer & Echternkamp, 1985). Overall, these studies indicate a high rate of embryonic loss in subfertile mares and cows after fertilization.

Fertilization rates for young, normal mares (96%) at Day 4 in this study were comparable to those previously reported for embryos collected at Day 2 (91%) (Ball *et al.*, 1986) and at Day 4 (100%) (Peyrot *et al.*, 1987) from similar mares. The fertilization rate for subfertile mares (81%) was similar to that for subfertile mares (92%) in a prior study (Ball *et al.*, 1986). Fertilization failure therefore represented 8–19% of fertility losses in subfertile mares in these studies while embryonic loss represented a much larger proportion of fertility losses in subfertile mares.

The fertilization rates for normal mares reported in this study and in our earlier studies appear higher than those previously reported by Betteridge *et al.* (1982), Forde *et al.* (1987) and Allen & Rowson (1975) (71, 75 and 82%, respectively) for embryos and recent ova collected from the oviduct between Days 1 and 6 after ovulation. One important difference between these earlier studies and our studies was the insemination of mares with the pooled ejaculates from 3 stallions. The effect of heterospermic inseminations on fertility in mares has not been reported; however, conception rates are significantly higher in cows with heterospermic compared to homospermic inseminations with frozen semen (Nelson *et al.*, 1975). Fertilization rates of ~90% for normal mares were consistent with results reported for normal heifers and cows (Sreenan & Diskin, 1986).

The hypothesis that embryonic defects were responsible for the high rate of embryonic loss in subfertile mares before Day 14 after ovulation was supported by the significantly higher survival rate of Day-4 embryos from normal than from subfertile donor mares. The reduced viability of embryos from subfertile donor mares was also suggested by a study with commercial horse embryo transfers in which Day-7 uterine embryos from subfertile mares tended to survive at a lower ($P < 0.1$) rate than did embryos from fertile donor mares (Iuliano & Squires, 1986). Another study indicated a significantly higher pregnancy loss rate between Days 35 and 50 in recipient mares with embryos from subfertile donor mares compared to recipient mares with embryos from fertile donor mares (Squires *et al.*, 1982). These studies indicate that embryonic factors reduce the viability of embryos from subfertile mares.

Defects in embryos from aged, subfertile mares might be inherent within the embryo or might arise due to the influence of the oviducal environment before Day 4 after ovulation. Woods *et al.* (1986) reported more morphological abnormalities in uterine embryos collected from subfertile compared to normal donor mares at Days 7–9 after ovulation. These abnormalities, however, may have been related to the uterine or oviducal environment as well as to primary embryonic defects. Although Henry & Vandeplassche (1981) reported a higher incidence of histopathological abnormalities in oviducts from aged mares, the relationship between the oviducal environment and embryonic loss in mares has not been established. Likewise, the occurrence of primary embryonic defects, such as chromosomal abnormalities, in embryos from aged, subfertile mares has not been investigated.

Our results show that maternal age is an important cause of embryonic loss in mares. This has also been shown in field studies in which a higher incidence of embryonic loss was detected in mares 10–13 years of age or older (Laing & Leech, 1975; Woods *et al.*, 1987). Commercial studies of cattle embryo transfers indicated a reduced viability of embryos collected from cows over 10 (Donaldson, 1984) or 15 (Hasler *et al.*, 1983, 1987) years of age. Several studies in laboratory animals also

supported a reduced viability of embryos collected from aged donor animals (Maurer & Foote, 1971; Blaha, 1975; Adams, 1975).

In the present study, there was no significant difference in the distribution of embryos with fair-to-good or poor morphology between subfertile and normal donor mares. Poor quality embryos from both groups of donor mares survived at lower rates than did fair-to-good quality embryos, as has been reported for bovine embryos (Linder & Wright, 1983; Hasler *et al.*, 1987). The reduced survival ($P < 0.1$) of fair-to-good quality embryos from subfertile mares compared to fair-to-good quality embryos from normal donor mares, however, indicated that factors other than those that were morphologically apparent may have been responsible for the reduced survival of embryos from subfertile mares. Results from commercial bovine embryo transfer studies indicated a reduced viability of embryos from subfertile donor cows in some studies (Elsden *et al.*, 1979; Hasler *et al.*, 1983) but not in others (Hasler *et al.*, 1987).

Embryonic losses secondary to apparent embryonic defects were responsible for a large part of the reduced fertility of aged, subfertile mares in this study, and many of these losses occurred before the time of initial pregnancy detection with ultrasonography. Although the role of the oviducal environment should be considered in these embryonic defects, it appears possible that many of these defective embryos may arise from defective ova in aged, subfertile mares.

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