

Factors affecting the success of oocyte transfer in a clinical program for subfertile mares

E.M. Carnevale^{a,*}, M.A. Coutinho da Silva^a, D. Panzani^b,
J.E. Stokes^a, E.L. Squires^a

^aDepartment of Biomedical Sciences, Colorado State University, Fort Collins, CO 80523, USA

^bDipartimento di Clinica Veterinaria, Università di Pisa, Pisa 56010, Italy

Abstract

Oocyte transfer is a potential method to produce offspring from valuable mares that cannot carry a pregnancy or produce embryos. From 2000 through 2004, 86 mares, 19.2 ± 0.4 yr of age (mean $\pm$ S.E.M.), were used as oocyte donors in a clinical program at Colorado State University. Oocytes were collected from 77% (548/710) of preovulatory follicles and during 96% (548/570) of cycles. Oocytes were collected 21.0 ± 0.1 h after administration of hCG to estrous donors and cultured 16.4 ± 0.2 h prior to transfer into recipients' oviducts. At 16 and 50 d after transfer, pregnancies were detected in 201 of 504 (40%) and 159 of 504 (32%) of recipients, respectively, with an embryo-loss rate of 21% (42/201). Pregnancy rates were similar ($P > 0.05$) for cyclic and noncyclic recipients and for recipients inseminated with cooled, fresh or frozen semen. One or more recipients were detected pregnant at 16 and 50 d, respectively, for 80% (69/86) and 71% (61/86) of donors. More donors <20 than ≥ 20 yr (mean ages $\pm$ S.E.M. of 15.5 ± 0.4 and 23.0 ± 0.3 yr, respectively) tended ($P = 0.1$) to have one or more pregnant recipients at 50 d (36/45, 80%; 28/45, 62%, respectively). Results of the program confirm that pregnancies can consistently be obtained from older, subfertile mares using oocyte transfer.

© 2005 Elsevier Inc. All rights reserved.

Keywords: Equine; Oocyte; Assisted reproduction; Oocyte transfer; Aging

* Corresponding author. Present address: Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, CO 80523, USA. Tel.: +1 970 491 5834; fax: +1 970 491 3557.

E-mail address: emc@colostate.edu (E.M. Carnevale).

1. Introduction

Commercial oocyte transfer involves the collection of a donor mare's oocyte from the preovulatory follicle. The oocyte is transferred into the oviduct of an inseminated recipient. Fertilization and development of the embryo and fetus occur within the recipient. Because the donor is required only to develop a preovulatory follicle with a viable oocyte, many reproductive problems, e.g. ovulation failure, oviductal blockages, or uterine infections, are circumvented. Therefore, subfertile mares that cannot provide viable embryos are frequently good candidates for oocyte donors.

Although the first foal was born after an experimental oocyte transfer in the late 1980s [1], efficiency of the procedure was not sufficient for commercial use, with only one foal produced from 15 oocytes (1/15, 7%). In 1995, Carnevale and Ginther [2] reported experimental transfers with high embryo-development rates (11/12, 92%) after oocytes were collected from young donors and cultured *in vitro* prior to transfer into inseminated recipients' oviducts. Oocyte transfer was not used for clinical purposes until the late 1990s [3,4]. In 2001, Carnevale et al. [3] reported using oocyte transfer to obtain offspring from mares with various reproductive abnormalities, including persistent endometritis, ovulatory failure, and scarring of the cervix; for some mares, no definitive cause for subfertility was determined, but embryo-collection attempts were unsuccessful.

The present study is a retrospective analysis of the commercial oocyte transfer program at Colorado State University from 2000 through 2004. Objectives of the study were to review the success of oocyte transfer in a clinical setting and compare potential factors affecting pregnancy rates.

2. Materials and methods

2.1. Oocyte donors and reproductive monitoring

Oocyte collections and transfers occurred during the breeding seasons (February through September) from 2000 through 2004. Donor mares were of numerous, light-horse breeds that were housed at Colorado State University for variable intervals. Some donors were in the program for >1 yr; in these cases, data for the donors were included for each breeding season.

Transrectal ultrasonography was used to monitor ovarian activity in donors. During estrus, ovaries of most mares were scanned daily to determine the optimal day for induction of ovulation. Typical criteria for induction of ovulation included: (1) follicle ≥ 35 mm in diameter; (2) relaxed uterine and cervical tone; (3) endometrial edema consistent with estrus; (4) estrous behavior. Because of the variety of mares in the program with atypical cycles, criteria were modified for individual mares. Availability and timing of semen delivery also contributed to the final decision of when to induce ovulation. To reliably induce follicle and oocyte maturation, a GnRH analog (deslorelin acetate; Ft. Dodge Animal Health, Ft. Dodge, IA, USA, or BET Pharm, LLC, Lexington, KY, USA) was administered approximately 4 h prior to administration of hCG (1500–2500 IU, i.v.; Chorulon[®], Intervet Inc., Millsboro, DE, USA).

2.2. Oocyte collections

Oocytes were collected by ultrasound-guided, follicular aspirations [5–7]. For the transvaginal aspirations, an ultrasound scanner with a linear or curvilinear 5 mHz transducer (Aloka Co. Ltd., Wallingford, CT, USA) was used. The transducer was placed in a plastic casing containing a needle guide and positioned within the anterior vagina, lateral to the posterior cervix and ipsilateral to the preovulatory follicle. The ovary was positioned by transrectal manipulation until the follicular apex was juxtaposed to the ultrasound transducer and needle guide. The needle was advanced within the needle guide to puncture the vaginal and follicular walls. Follicular contents were aspirated with a pump (Cook Veterinary Products, New Buffalo, MI, USA) using approximately 150 mmHg. Follicular antra were lavaged with 50–150 mL of medium (EmCare Embryo-flush Medium, ICP, Auckland, New Zealand), supplemented with 10 IU/mL of heparin (Calbiochem[®], La Jolla, CA, USA).

2.3. Handling and culture of oocytes

Supplies and media used in handling the oocyte were maintained at approximately body temperature (38–39 °C). Oocytes were assessed by degree of cumulus expansion and, to some extent, appearance of ooplasm. Oocytes were graded based on morphology (scores from 1, excellent to 4, poor). Cumulus expansion was graded as slight, moderate, good, complete and atretic. However, critical evaluations of oocytes were difficult, because cumulus cells frequently obstructed direct imaging of the oocyte under a stereomicroscope.

Approximately 14 h after hCG administration, follicles were imaged by ultrasound. If indications of imminent ovulation or hemorrhage (irregular shape, echogenic debris in antrum, thick and echodense border) were imaged, follicles were aspirated; the collected oocytes were evaluated and transferred based on morphology. The majority of oocytes were collected between 20 and 24 h after hCG administration to the donor and cultured *in vitro* for the completion of maturation before transfer. Oocytes were cultured in Tissue Culture Medium 199, with additions of 10% fetal calf serum, 0.2 mM pyruvate, and 25 µg/mL gentamicin at 38.2–39 °C and in an atmosphere of 5 or 6% CO₂ and air.

2.4. Oocyte recipients

Recipients for transfers were primarily between 3 and 10 yr of age, with a normal reproductive tract imaged with ultrasound. Oocyte recipients were cyclic or noncyclic. Cyclic recipients were synchronized with the donor, and received hCG (2500 IU, i.v.) on the same day as the donor. Recipients' oocytes were collected by follicular aspirations from the dominant follicle(s). Noncyclic mares had minimal follicular activity and included anestrus, transitional, and follicle-suppressed mares. A high-dose of a GnRH agonist (4.2 mg, deslorelin acetate; Ft. Dodge Animal Health, Fort Dodge, IA, USA) [8] was used during the ovulatory season to induce follicular suppression. For noncyclic recipients, estradiol (3 mg daily for 3–7 d; Sigma Chemical Co., St. Louis, MO, USA) was administered before insemination and oocyte transfer, and progesterone (Sigma Chemical Co.) in

cottonseed oil (150–200 mg daily) was administered after transfer. Exogenous progesterone or progestin was required to maintain pregnancies [3].

2.5. Oocyte transfer and recipient insemination

Ovarian exposure was accomplished through standing flank laparotomies, with surgical procedures similar to previously described methods for surgical embryo transfer [9]. After the peritoneum was punctured, the ovary was located and gently exteriorized through the incision. Lidocaine on a gauze pad was placed on the broad ligament prior to exposure if the recipient was uncomfortable with ovarian manipulations. The oocyte was loaded into the end of a fire-polished, glass pipette with <0.05 mL of medium and gently deposited within the oviduct 2–3 cm past the infundibular o.s. For cyclic recipients, most transfers were into the oviduct contralateral to follicle aspiration.

Recipients were inseminated before and/or directly after oocyte transfer with fresh, cooled or frozen semen from desired stallions. Quality and number of sperm varied. Uteri of recipients were scanned for intrauterine fluid accumulations prior to transfer and between 6 and 24 h after insemination. If fluid was imaged using ultrasound prior to transfer, oxytocin (10–40 IU, i.v.) was administered before sedation of the recipient or approximately 30 min prior to transfer. If intraluminal fluid accumulations were imaged after transfer, recipients were treated as needed for fluid retention or uterine infection. Beginning on the day of transfer, recipients received phenylbutazone (2 g, i.v. or p.o.) for 3 d and an antibiotic (procaine penicillin G, 20,000 IU/kg, i.m. daily or trimethoprim sulfa, 24 mg/kg, p.o., twice daily) for 6 d. Uteri of recipients were scanned with ultrasound for the detection of pregnancy beginning on Day 11 and at various intervals until recipient departure from our facility. For the present study, pregnancy rates at Days 16 and 50 were used (Day 0 = day of oocyte transfer).

2.6. Statistical analyses

Statistical analyses were performed using SAS software (SAS Institute Inc., Cary, NC, USA). The effects of the interval from hCG to oocyte transfer, oocyte grade, oxytocin, lidocaine, age of donor mare, multiple transfers, year, recipient type, semen type, and quality on pregnancy rates were compared by Fisher's Exact test. Mean oocyte grade for young and old mares was compared by Student's *t*-test.

3. Results

From 2000 through 2004, light-horse mares ($n = 86$) of various breeds were oocyte donors. On average, 27 donors were admitted to the program per year, with some mares participating during multiple years. Mean age of donors was 19.2 ± 0.4 yr (mean $\pm$ S.E.M.; range of 4–29 yr). For statistical analyses, comparisons were made for mares <20 yr (15.5 ± 0.4 yr, $n = 70$ mare-years) and ≥ 20 yr (23.0 ± 0.3 yr, $n = 67$ mare-years). Potential causes of reproductive failure included ovulation failure, uterine pathology, cervical pathology, and no specific diagnosis. The requested number of pregnancies varied

for donors. Some donors were housed at our facility for short intervals, while others were maintained for extended periods. Feeding and management varied based on the needs of individual mares.

Donors had 4.8 ± 0.3 follicle aspirations per breeding season with 3.7 ± 0.2 oocytes recovered per donor. Oocytes were collected from 96% (548/570) of cycles and 77% (548/710) of follicles. The mean interval from administration of hCG to the donor until oocyte collection was 21.0 ± 0.1 h, with oocytes cultured for 16.4 ± 0.2 h prior to transfer. The mean interval from hCG to transfer was 37.5 ± 0.2 h. The interval from hCG to oocyte transfer into a recipient's oviduct affected pregnancy rates, with an interval of <32 h resulting in fewer ($P < 0.05$) pregnancies than intervals of 32–40 h and >40 h (Table 1). Oocyte grades differed ($P < 0.01$) by mare age, with higher grades representing worse morphology scores (<20 yr, 2.2 ± 0.0 , $n = 180$ and ≥ 20 yr, 2.4 ± 0.0 , $n = 162$); however, oocyte grade and degree of cumulus expansion was not predictive of pregnancy ($P > 0.05$). Administration of oxytocin <1 h before transfer and placement of lidocaine on the broad ligament prior to transfer did not significantly affect pregnancy rates (Table 1).

Embryo-development rate per transferred oocyte was 38% (207/548) at 16 d. In 44 mares, two oocytes were transferred into the same recipient; 43% (19/44) of these recipients became pregnant. Twin pregnancies were detected in 14% (6/44) of the recipients that received two oocytes, and one vesicle was manually crushed. At 16 and 50 d, 40% (201/504) and 32% (159/504) of recipients, with one or two transferred oocytes, were diagnosed as pregnant. There was no difference in pregnancy rates between recipients receiving one or two oocytes (182/460, 40%; 19/44, 43%, respectively). The overall embryo-loss rate was 21% (42/201). Year, age of donor, and type of recipient did not significantly affect pregnancy rates at 16 and 50 d (Table 2). Pregnancy rates were similar ($P > 0.05$) at 16 d for mares inseminated with cooled, fresh and frozen semen (188/458, 41%; 10/21, 48%; 2/9, 22%, respectively) and for semen with a total motility $<$ or $\geq 50\%$ (14/29, 48%; 126/286, 44%).

Table 1

Effects of interval from hCG administration to oocyte transfer and use of oxytocin or lidocaine at oocyte transfer on pregnancy rates at 16- and 50-d in mares

Endpoint	Day of pregnancy	
	15	50
Interval (h) from hCG to transfer		
<32	5/31 a, 16%	4/31 a, 13%
32–40	170/386 b, 44%	134/386 b, 35%
>40	8/16 b, 50%	6/16 b, 38%
Oxytocin <1 h before OT		
Oxytocin	74/160, 46%	64/160 c, 40%
No oxytocin	49/127, 39%	37/127 d, 29%
Lidocaine on broad ligament		
Lidocaine	14/38, 37%	11/38, 29%
No lidocaine	79/180, 44%	67/180, 37%

(a and b) Values within columns for individual endpoints are different ($P < 0.05$). (c and d) Values within columns for individual endpoints tend to be different ($P = 0.06$).

Table 2

Effect of year, donor age and type of recipient on pregnancy rates at 16 and 50 d in mares

Endpoint	Day of pregnancy	
	15	50
Overall pregnancy rate per recipient	201/504, 40%	159/504, 32%
Pregnancy rate per year		
2000	45/112, 40%	31/112, 28%
2001	29/92, 32%	23/92, 25%
2002	34/72, 47%	27/72, 38%
2003	46/103, 45%	38/103, 37%
2004	47/125, 38%	40/125, 32%
Donor age		
<20 yr	105/266, 39%	88/266, 33%
≥ 20 yr	96/238, 40%	71/238, 30%
Type of recipient		
Cyclic	33/82, 40%	27/82, 33%
Noncyclic	166/405, 41%	132/405, 32%

Values for endpoints were not different ($P > 0.05$).

One or more recipients were pregnant at 16 and 50 d, respectively, for 80% (69/86) and 71% (61/86) of donors. The number of donors with at least one pregnant recipient at Day 16 did not differ for mares <20 and ≥20 yr (39/45, 87%; 34/45, 76%, respectively). However, the number of donors <20 yr with at least one pregnant recipient at 50 d tended ($P = 0.1$) to be higher than for donors ≥20 yr (36/45, 80%; 28/45, 62%, respectively).

4. Discussion

A clinical program using oocyte transfer was established at Colorado State University in 1998, and results from 1998 and 1999 have been previously been published [3]. In this review, we retrospectively examined transfer results from 2000 through 2004. Mares admitted to the program were considered infertile using standard breeding practices or embryo transfer. Reasons for infertility varied and were, in some cases, undiagnosed.

Rates for oocyte collections were high: 77% per aspirated follicle and 96% per cycle. Collections were done by one of two experienced clinicians. Oocytes were recovered, on average, 21 h after administration of hCG to the donor. Timing and response to hCG is critical for successful oocyte collections. Prior to follicle aspirations, a combination of deslorelin acetate and hCG was administered to donors. In previous years, administration of hCG alone resulted in the failure of some follicles to respond, and no oocytes or immature oocytes were collected [3]. Maturation of the follicle and oocyte after deslorelin and hCG was reliable.

Most oocytes were probably collected at metaphase I [10]. Therefore, they were cultured for the completion of maturation to metaphase II, as previously described [2]. Ovulation occurs approximately 36 h after hCG or 40 h after deslorelin administration. The interval from hCG to transfer was 37.5 h, which would correspond to the approximate time

of ovulation after administration of hCG to a mare. In the present study, when oocytes were transferred <32 h after hCG was administered to the donor, pregnancy rates were lower than transfers after an interval of ≥ 32 h. However, reasons for these results may not be due to incomplete maturation. If ovulation or follicular hemorrhage appeared imminent, the follicle was aspirated to recover the oocyte. These oocytes were usually transferred within a short interval of time into recipients' oviducts. Some of these oocytes may not have been as viable; insemination of the recipient was more likely to occur only after transfer. Previous studies have demonstrated that transfer of mature, viable oocytes directly after collection is successful. Hinrichs et al. [11] timed oocyte recoveries from administration of hCG to donors; oocytes were collected at approximately 24 or 36 h after hCG. Oocytes collected at 24 h were cultured prior to transfer, while oocytes collected at 36 h were directly transferred into recipients' oviducts. Pregnancy rates were not significantly different, and the results agree with those from other experiments [12].

Compared to embryos, oocyte grading is more difficult, because the oocyte is surrounded by a mass of cumulus cells. In the present experiment, grades were assigned to oocytes after imaging under a stereomicroscope, but oocyte grades and degree of cumulus expansion did not correspond with embryo-development after transfer of oocytes.

Pregnancy rates were similar for cyclic and noncyclic recipients, consistent with a previous report [12]. Because cyclic recipients must be synchronized with donors and their oocytes must be recovered, use of noncyclic recipients allows for the use of fewer mares and better synchronization of appropriate recipients for particular donors. Cyclic and noncyclic recipients were maintained on progesterone or progestins after transfer. Although not critically studied, many noncyclic recipients were observed to form secondary corpora lutea, although this did not appear to consistently occur when transfers were done late in the breeding season. The embryo-loss rate was 21% between 11 and 50 d of gestation. Although not significantly different, the embryo-loss rate was numerically higher in 2000 (31%) versus 2001 through 2004 (range of 15–21%). In 2000, a progestin was administered once daily; beginning in 2001, the progestin was administered twice daily for the maintenance of pregnancy. Perhaps more consistent concentrations of hormones in recipients without luteal tissue were beneficial.

High pregnancy rates after experimental oocyte transfers have been obtained [12]. In a previous study [13], oocytes were collected from donors 3 to 15 yr of age and cultured in different media; and recipients were inseminated with 1×10^9 progressively motile, cooled sperm from fertile stallions. Recipients ovulated their preovulatory follicles at the approximate time of transfer, and embryos were collected for parentage testing at 16 d. Embryo-development rates were similar ($P > 0.05$) for transferred and ovulated oocytes (932/44, 73%; 9/13, 69%, respectively). Oocytes cultured in the same media as the clinical program had a high embryo-development rate (83%, 19/23). Previous results suggest that procedures for oocyte transfer were effective.

In the present study, two factors probably had a negative effect on pregnancy rates: quality and number of sperm and age of donors. Semen was obtained from numerous stallions. Numbers of sperm and fertility of stallions was often not known. Type of semen did not affect pregnancy rates, although the majority of recipients were inseminated with cooled, transported semen.

Oocyte quality is affected by age. Carnevale and Ginther [2] transferred oocytes from young donors (6–10 yr) and old donors (20–26 yr) into the oviducts of inseminated, young recipients. Embryo-development rates were higher ($P < 0.05$) for oocytes from young than old donors (11/12, 92%; 8/26, 31%, respectively). The study demonstrated an age-associated decline in oocyte viability. In 1998 and 1999, mares in the clinical program for oocyte transfer were 22.1 ± 1.2 yr and 19.2 ± 1.1 yr (mean $\pm$ S.E.M.) of age, respectively. In the first year (1998), the last date of a successful embryo-collection or pregnancy was 6.7 ± 1.1 yr prior, with a range of 3–15 yr [3]. From 2000 through 2004, the average age of oocyte donors was 19 yr; comparisons were made between mares <20 and ≥ 20 yr of age, with mean ages of 15.5 and 23.0 yrs in respective groups. Oocyte transfers for mares less than the younger mean age (<15 yr) resulted in a 50% pregnancy rate (28/56), while transfer of oocytes from donors greater than the older mean age (>23 yr) resulted in a 16% (12/77) pregnancy rate. One 16-d pregnancy and no 50-d pregnancies were achieved for mares ≥ 27 yr after 12 transfers. Age differences were probably muted by the paucity of young mares (<12 yr) in the program. The most apparent age difference was that 50-d pregnant recipients were less likely to be obtained for donors ≥ 20 versus <20 yr.

Donors were in the clinical program for one or multiple cycles during a breeding season. For 71% of these donors, one or more recipients were pregnant at 50 d. Results of the program confirm that pregnancies can be obtained for mares with various reproductive abnormalities that are considered infertile using standard breeding procedures or embryo transfer.

Acknowledgements

Research in oocyte transfer has been funded by benefactors of Preservation of Equine Gametes, the Colorado Equine Racing Commission through the Research Council of the College of Veterinary Medicine and Biomedical Sciences at Colorado State University, and the Cecil and Irene Hylton Foundation. Authors would like to thank Steve Roman of Aloka Co. Ltd. for providing support for ultrasound equipment.

References

- [1] McKinnon AO, Carnevale EM, Squires EL, Voss JL, Seidel Jr GE. Heterogenous and xenogenous fertilization of in vivo matured equine oocytes. *J Equine Vet Sci* 1988;8:143–7.
- [2] Carnevale EM, Ginther OJ. Defective oocytes as a cause of subfertility in old mares. *Biol Reprod Mono* 1995;1:209–14.
- [3] Carnevale EM, Squires EL, Maclellan LJ, Alvarenga MA, Scott TJ. Use of oocyte transfer in a commercial breeding program for mares with reproductive abnormalities. *J Am Vet Med Assoc* 2001;218:87–91.
- [4] Hinrichs K, Provost PJ, Torello EM. Treatments resulting in pregnancy in nonovulating, hormone-treated oocyte recipient mares. *Theriogenology* 2000;54:1285–93.
- [5] Bruck I, Raun K, Synnvestvedt B, Greve T. Follicle aspiration in the mare using a transvaginal ultrasound-guided technique. *Equine Vet J* 1992;24:58–9.
- [6] Carnevale EM, Ginther OJ. Use of a linear ultrasonic transducer for the transvaginal aspiration and transfer of oocytes in the mare. *J Equine Vet Sci* 1993;13:331–3.

- [7] Cook NL, Squires EL, Ray BS, Cook VM, Jasko DJ. Transvaginal ultrasound-guided follicular aspiration of equine oocytes. *J Equine Vet Sci* 1993;15:71–4.
- [8] Carnevale EM, Checura CH, Coutinho da Silva MA, Maclellan LJ, Squires EL. Use of deslorelin acetate to suppress follicular activity in mares used as recipients for oocyte transfer. *Theriogenology* 2001;55:358.
- [9] Squires EL, Seidel GE. Collection and transfer of equine embryos. *Animal Reproduction and Biotechnology Laboratory Bulletin*, No. 08. Colorado State University, Fort Collins, CO, 1995. pp. 24–26.
- [10] Bezard J, Mekarska A, Goudet G, Duchamp G, Palmer E. Meiotic stage of the preovulatory equine oocyte at collection and competence of immature oocytes for in vitro maturation: effect of interval from induction of ovulation to follicle puncture. *Theriogenology* 1997;47:386.
- [11] Hinrichs K, Betschart RW, McCue PM, Squires EL. Effect of time of follicle aspiration on pregnancy rate after oocyte transfer in the mare. *J Reprod Fertil Suppl* 2000;56:493–8.
- [12] Carnevale EM, Maclellan LJ, Coutinho da Silva MA, Checura CM, Scoggin CF, Squires EL. Equine sperm–oocyte interaction: results after intraoviductal and intrauterine inseminations of recipients for oocyte transfer. *Anim Reprod Sci* 2001;68:305–14.
- [13] Carnevale EM, Coutinho da Silva MA, Maclellan LJ, Seidel Jr GE, Squires EL. Use of parentage testing to determine optimum insemination time and culture media for oocyte transfer in mares. *Reproduction* 2004;128:623–8.