Use of oocyte transfer in a commercial breeding program for mares with reproductive abnormalities

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During the past decade, embryo transfer has become a valuable clinical tool for obtaining offspring from mares that are incapable of maintaining a pregnancy. However, embryo transfer will be successful only if the reproductive tract of the donor mare can successfully ovulate an oocyte, transport the oocyte from the ovulatory follicle into the uterine tube (oviduct), transport the embryo from the oviduct into the uterus, and provide a suitable environment for fertilization and embryo development. Problems that develop prior to the sixth to eighth day after ovulation will prevent collection of an embryo from the donor mare. Therefore, in some mares with lesions of the reproductive tract, embryo collection and survival rates are low, or collection of embryos is not feasible. For these mares, oocyte transfer has been proposed as a method to obtain pregnancies.

Oocyte transfer involves collection of an oocyte from the follicle of a donor and transfer of that oocyte into a recipient's oviduct. The recipient is inseminated, and fertilization and embryo development occur within the recipient's reproductive tract. Oocyte transfer has resulted in pregnancy in research mares.[1,2] However, results of oocyte transfer in a large group of clinically subfertile mares have not been published. In the present report, a method for oocyte transfer in mares and results of oocyte transfer performed over 2 breeding seasons, using mares with long histories of subfertility and various reproductive lesions, are described.

Description of the Technique

Ovarian activity was monitored by means of transrectal ultrasonography to determine follicular development. Ovaries were scanned at daily or hourly intervals, as indicated, during the follicular phase of the cycle. **Human chorionic gonadotropin (hCG**; 2,000 to 2,500 units, IV) or an implant containing a gonadotropin-releasing hormone analog[a] (deslorelin acetate; 2.1 mg, SC), or both was used to initiate follicular and oocyte maturation. When possible, hCG or deslorelin acetate was administered when donors demonstrated the following signs: behavioral estrus, relaxed uterine and cervical tone, uterine edema, and a follicle ≥ 35 mm in diameter. Oocytes were collected by means of transvaginal ultrasound-guided follicular aspiration,[3] using a curvilinear or linear 5-MHz ultrasound transducer[b] and a double-lumen oocyte collection needle.[c] For collection of oocytes, donor mares were sedated with xylazine hydrochloride (0.33 mg/kg [0.15 mg/lb] of body weight, IV) and butorphanol tartrate (0.01 mg/kg [0.005 mg/lb], IV). Propantheline bromide[d] (0.04 mg/kg [0.02 mg/lb], IV) was administered to promote relaxation of the rectum. The ultrasound transducer was placed in a casing containing a needle guide and positioned in the anterior portion of the vagina while the ovary was manipulated per rectum. The dominant follicle was positioned over the transducer face, the collection needle was advanced, and the vaginal and follicular walls were punctured under ultrasound guidance. The contents of the follicle were gently aspirated (150 mg Hg), using a pump,[e] and the follicle was lavaged with 50 ml of flush medium (Dulbeccos phosphate-buffered saline solution with 1% fetal calf serum, 10 U of heparin/ml, 100 U of penicillin/ml, and 0.1 mg of streptomycin/ml). For 1 donor, transvaginal aspiration was not performed because of melanomas and vaginal fluid. In this mare, follicular aspiration was performed through a needle positioned through a flank trocar.[4]

Following follicular aspiration, **cumulus oocyte complexes (COC)** were evaluated for cumulus expansion (graded from compact to fully expanded) and signs of atresia. Oocytes were considered to be in a stage of atresia if the COC was clumped or sparse, the corona radiata was fully expanded, or the ooplasm was shrunken and dark or severely mottled. Oocytes with a fully expanded cumulus (ie, marked separation of cumulus cells with expansion of the corona radiata) were transferred as soon as possible into a recipient's oviduct. Oocytes with a moderately expanded cumulus complex (ie, translucent COC with moderate separation of cumulus cells and incomplete expansion of corona radiata) were placed in culture medium (**tissue culture medium [TCM]** 199 with 10% fetal calf serum, 0.2 m*M* pyruvate, and 50 µg of gentamicin sulfate/ml) for approximately 12 to 17 hours prior to transfer. Immature oocytes (ie, compact COC with little or no separation of cumulus cells) were cultured in maturation medium (culture medium to which 1 µg of estradiol/ml, 1 µg of luteinizing hormone/ml, and 15 ng of follicle-stimulating hormone/ml had been added) for 18 to 26 hours at 38.5 or 39.0 C in an atmosphere of 5% CO_2 and air.

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Supported in part by the benefactors of the Preservation of Equine Genetics Program and the Lucy Whittier Foundation.

Presented at the 45th Annual Convention of the American Association of Equine Practitioners, Albuquerque, NM, 1999.

The authors thank Dr. George Seidel, Mary O'Donovan, Ricardo Ramirez, and Dr. Marco Coutinho da Silva for technical assistance.

Prior to oocyte transfer, the recipient's ovary and oviduct were exposed through a flank laparotomy with the horse standing. The oocyte was placed in transfer medium (TCM 199 with 20 m*M* Hepes, 10% fetal calf serum, 5 m*M* bicarbonate, 0.2 m*M* pyruvate, and 50 µg of gentamicin/ml) and pulled into a fire-polished glass pipette with < 0.03 ml of medium. During transfer, the pipette was threaded 2 to 3 cm through the recipient's infundibulum and ampulla, and the oocyte was slowly deposited within the oviduct. Recipients were inseminated before or after oocyte transfer or both. Ideally, recipients were inseminated < 20 hours before and < 4 hours after oocyte transfer; however, timing and number of inseminations depended on availability of semen. Semen used for oocyte transfer was obtained from stallions at various locations and included fresh, frozen, and cooled semen.

Oocytes were transferred into recipients that were cycling, that were not cycling, that were in the early phase of estrus, and that had just ovulated. Recipients that were cycling were in estrus and had 1 or more follicles > 35 mm in diameter, uterine edema, and relaxed uterine and cervical tone. An injection of hCG (2,000 units, IV) was administered to the potential recipients and to the donor mare at approximately the same time of day. To prevent fertilization of the recipient's oocyte, the dominant follicles of the recipient were aspirated approximately 24 hours after administration of hCG. Only mares from which oocytes were collected from all follicles > 30 mm in diameter were used as recipients. Recipients that were not cycling were in anestrus or the spring or fall transitional period, did not have any follicles > 25 mm in diameter, and had a flaccid uterus. Prior to transfer, these recipients were given injections of estradiol in cottonseed oil (2 to 10 mg, IM) until moderate to prominent uterine edema and a relaxed cervix were evident. Recipients that were in the early phase of estrus were given injections of estradiol in cottonseed oil (2 to 10 mg, IM) to promote uterine edema and cervical relaxation; they did not have any detectable corpora lutea or follicles > 27 mm in diameter. The largest follicle was typically aspirated from these recipients during oocyte implantation to remove the immature oocyte. One recipient had just ovulated. Estradiol (9 mg, IM) was administered on the day of ovulation, and the oocyte was transferred 24 to 48 hours after the recipient had ovulated. The recipient was inseminated after transfer of the donor's oocyte. Recipients that were cycling, that were not cycling, and that were in the early phase of estrus were given daily injections of progesterone in cottonseed oil (150 mg, IM) after oocyte transfer until they were determined to be pregnant. If pregnant, recipients were given a synthetic progesterone (altrenogest[f]; 0.044 mg/kg [0.02 mg/lb], PO) until at least 120 days of gestation.

During the 1999 breeding season, 2 additional oocyte transfers were performed, using oocytes from mares that were euthanatized for humane reasons. Both mares had severe metabolic disorders. The ovaries of 1 mare were inactive. Immediately after euthanasia, the ovaries were collected from this mare, rinsed in sterile saline (0.9% NaCl) solution (28 C [88 F]), and packaged in a plastic bag. Ovaries were shipped in a container[g] packed with bags containing saline solution (25 to 28 C [77 to 88 F]) to Colorado State University. The other mare was euthanatized at Colorado State University. Oocytes were collected by slicing and rinsing ovaries.[5] Recovered oocytes were evaluated and placed in maturation medium for 24 hours. Oocyte transfer was performed, as described, for the other mares.

Results

Oocyte transfer was attempted in 18 mares during the 1998 breeding season and 20 mares during the 1999 breeding season. Mares were between 9 and 30 years old, but 15 of the 18 examined during the 1998 breeding season and 14 of the 20 examined during the 1999 season were ≥ 18 years old. All mares had a history of reproductive failure while in breeding and embryo transfer programs. For the 14 mares from which oocytes were collected and records were available, the last date of a successful pregnancy or embryo collection ranged from 3 to 15 years previously (mean ± SEM, 6.7 ± 1.1 years). Three mares (9, 18, and 22 years old) had not produced a foal despite repeated attempts at insemination or embryo transfer.

Most mares had 1 or more abnormalities of the reproductive tract, including persistent endometritis or pyometra, intermittent or repeated ovulatory failure (luteinized or hemorrhagic follicles), or scarring of the cervix. In some mares, no definitive cause for subfertility was determined; however, previous repeated embryo transfer attempts had been unsuccessful.

Results for the 1998 breeding season—Oocyte transfer was attempted between May and September 1998 for 18 mares ranging in age from 17 to 30 years (mean ± SEM, 22.1 ± 1.2 years). Some mares did not cycle (n = 2) or cycled only intermittently (4). Number of oocyte collection attempts per mare ranged from 1 to 6. Mean times from administration of hCG or deslorelin acetate to oocyte collection were 22.8 ± 0.8 hours and 30.0 ± 1.0 hours, respectively. For 7 oocyte collections, follicular changes consistent with impending ovulation were observed, and no treatment was used to induce follicular maturation. Oocytes were collected during 39 of 46 (85%) cycles and from 39 of 56 (70%) follicles. Nine oocytes with a fully expanded cumulus were transferred into a recipient < 1.5 hours after collection. Sixteen oocytes with moderate cumulus expansion were cultured (mean ± SEM duration of culture, 12.6 ± 1.5 hours) to allow maturation prior to transfer. One immature oocyte was collected and cultured in maturation medium for 25 hours prior to transfer. Four oocytes were not transferred, because they were atretic; 3 oocytes were not transferred, because they were damaged during collection. In 6 instances, 2 oocytes from the same donor were transferred into the same recipient.

Pregnancy rate per donor cycle was 13% (6/46). Six (23%) pregnancies resulted from the 26 oocyte transfers and from 38% (6/16) of the cycling mares. In 6 instances, 2 oocytes from a single donor were transferred per recipient. Three of these recipients did not become pregnant, 2 became pregnant with a single

embryonic vesicle, and 1 became pregnant with 2 embryonic vesicles. In 5 of these instances, 1 of the 2 oocytes transferred was immature (n = 2) or atretic (3). In the recipient with 2 embryonic vesicles, 1 of the vesicles was delayed in development, and the pregnancy reduced to a singleton; the recipient had a normal foal. Pregnancies were obtained with fresh (1/1) and cooled (5/23; 22%) semen but not with frozen semen (0/2). Pregnancies were established in recipients that were cycling (3/21; 14%), that were not cycling (2/4, 50%), and that had just ovulated (1/1). Four of the recipients had live healthy foals. Two pregnancies were lost approximately 30 days after oocyte transfer.

Problems encountered during oocyte transfer included an atretic appearance to the COC (n = 5), an immature oocyte (1), inadequate semen quality (low motility or numbers; 4), and a delay in semen shipment (1). Six (40%) pregnancies resulted from the 15 transfers in which the oocyte and sperm appeared viable.

Results for the 1999 breeding season—The 20 mares examined during this season ranged from 9 to 29 years old (mean ± SEM, 19.2 ± 1.1 years). Some mares did not cycle (n = 3) or cycled only intermittently (2). Number of oocyte collection attempts per mare ranged from 1 to 7. Mean times from administration of hCG or deslorelin to oocyte collection were 23.1 ± 0.4 hours and 27.3 ± 0.3 hours, respectively. For 2 oocyte collections, follicular changes consistent with impending ovulation were observed, and no treatment was used to induce follicular maturation. Oocytes were collected during 41 of 53 (77%) cycles and from 51 of 67 (76%) follicles. Nine oocytes had a fully expanded cumulus and were cultured 1 to 5 hours (mean ± SEM, 2.8 ± 0.5 hours) prior to transfer. Twenty-two oocytes had a moderately expanded cumulus and were cultured 14 to 20 hours (mean ± SEM, 17 ± 0.4 hours) prior to transfer. Four oocytes had a compact or immature cumulus and were cultured 18 to 26 hours (mean ± SEM, 23.5 ± 1.9 hours) prior to transfer. Culture times were not recorded for 3 oocytes. Eight oocytes were not transferred, because they were fragmented (n = 2), atretic (5), or immature (1). In 5 instances, 2 oocytes from the same donor were transferred in a recipient.

Fourteen (37%) pregnancies resulted from the 38 oocyte transfers, and at least 1 pregnancy was obtained for 81% (13/16) of the cycling mares from which at least 1 follicular aspiration was attempted; 2 pregnancies were obtained for 1 donor mare. In 5 instances, 2 oocytes from a single donor were transferred per recipient. Three of these recipients became pregnant with a single embryo; the other 2 recipients did not become pregnant. Pregnancies were obtained in recipients inseminated with fresh (4/5; 80%), cooled (9/30; 30%), and frozen (1/3; 33%) semen and in recipients that were cycling (4/12; 33%), that were not cycling (9/23; 39%), and that were in the early phase of estrus (1/3; 33%). Four pregnancies were lost approximately 13, 23, 50, and 55 days after oocyte transfer. Ten recipients delivered live healthy foals.

Problems during oocyte transfer included mottled (degenerating) ooplasm (n = 1), an immature oocyte (4), inadequate semen quality (motility < 10%; 1), insemination delayed > 9 hours after transfer because of delayed semen shipment (3), and poor positioning of the oocyte during transfer (2). Fourteen (54%) pregnancies resulted from 26 transfers performed under ideal conditions.

Oocytes were collected from the 2 mares that were euthanized (9 oocytes from the ovaries transported to Colorado State University; 21 oocytes from the mare euthanized at the university). Oocytes (n = 4 and 19) that did not show signs of advanced atresia were transferred into a single recipient per donor. One pregnancy resulted from the mare euthanized at Colorado State University, even though semen quality was poor (< 10% progressive motility). However, the embryonic vesicle was delayed in development and was lost after approximately 25 days of gestation.

Discussion

During these 2 consecutive breeding seasons, oocyte transfers were attempted for 38 mares with histories of subfertility. Previous breeding and embryo transfer attempts had been unsuccessful in these mares. Oocyte collection rates were high, with oocytes recovered during 80% of cycles. The oocyte collection procedure was well-tolerated by donor mares without any complications. Manipulation and aspiration of the preovulatory follicle required approximately 5 minutes. Most oocytes were collected approximately 12 hours prior to expected ovulation. Therefore, oocytes should have been in the process of nuclear and cytoplasmic maturation with the cumulus moderately expanded (ie, approx 1- or 2-mm thick and translucent). Occasionally, immature oocytes with a compact cumulus were collected from follicles of donors that did not respond to hCG. During subsequent cycles, donors not responding to hCG were treated with deslorelin acetate or a combination of deslorelin acetate and hCG to initiate oocyte maturation.

Pregnancies were obtained with oocytes from mares with a wide range of reproductive abnormalities, including persistent endometritis, pyometra, cervical fibrosis, and repeated ovulatory failure (luteinized unruptured follicles or hemorrhagic follicles). Three mares (9, 18, and 22 years old) had previously failed to produce a foal or viable embryo; however, at least 1 pregnancy was established with oocyte transfer for each of these mares. In some mares, no definitive cause of reproductive failure was diagnosed; however, breeding and embryo transfer had failed to result in a pregnancy. During the 1999 breeding season, 2 mares underwent repeated embryo collection attempts. The mares were then switched to the oocyte transfer program. Pregnancies were produced after 1 oocyte transfer procedure for each mare, using semen from the same stallions as had been used for the embryo collection attempts.

Reasons why oocyte transfer may have succeeded in subfertile mares when embryo transfer failed are numerous. Approximately 30% of donors in the oocyte transfer program had potential ovulatory problems. Ovulatory failure has been reported to occur more often in older than in younger mares.[6] Ovulatory prob-

lems for most donors were evident during sequential cycles and were primarily a result of luteinization of unruptured follicles.[7] Ultrasonographically, these follicles were irregular in shape and usually had a rent within the wall. Borders of the follicles were echogenic and thick. Echogenic debris, probably blood and exfoliated cells, was evident within the lumen. When aspirated, follicles contained blood-tinged follicular fluid, and the COC had changes associated with degeneration or atresia. However, when oocytes were collected before the appearance of echogenic debris in the follicular fluid from mares with histories of repeated abnormal ovulations, oocytes appeared viable and resulted in pregnancies. In mares with ovulatory problems, removal of oocytes prior to attempted ovulation and completion of maturation in vitro appeared to increase fertility. In donors with an atypical appearance to the ovulatory site, the oocyte is probably not released into the oviduct. In a previous study,[8] when recently ovulated ova (fertilized and unfertilized) were collected from oviducts 1.5 and 3 days after ovulation, the number of ova collected per ovulation was significantly reduced in mares > 20 years old versus mares 2 to 10 years old. The process of ovulation or movement of the oocyte into the oviduct appeared to be less successful in older mares. Transferring the oocyte from the donor's follicle to the recipient's oviduct can eliminate this cause of reduced fertility.

Endometritis is a major component of reduced fertility in mares. Uterine inflammation and infection is detrimental to survival of sperm and embryos. Therefore, mares with pyometra, recurrent bacterial endometritis, or fungal infections were considered good candidates for oocyte transfer. When the oocyte was transferred, the sperm and embryo were not exposed to the donor's uterine environment; in addition, costs for treatment of endometritis were minimized. In the present study, pregnancies were obtained from mares with persistent fungal infections, pyometra, recurrent endometritis, and endometritis associated with urine pooling. In these mares, oocyte transfer may be more economical than repeated uterine treatment and embryo transfer.

In the present study, the success of oocyte transfer was affected by oocyte and semen quality. During the 1998 and 1999 breeding seasons, semen was shipped from various stallions and farms. Therefore, quality of semen (motility and sperm numbers) was variable. Oocyte transfer was more successful when fresh semen (5/6; 83%) was used versus cooled or frozen semen (14/53 [26%] and 1/5 [20%], respectively). Selection of a stallion with high-quality semen is important to maximize success of oocyte transfer.

Success of oocyte transfer in the present study was affected by oocyte quality. Oocytes with signs of atresia (mottled ooplasm, clumped cumulus cells) did not result in pregnancies, and although high pregnancy rates can be achieved when transferring oocytes collected from preovulatory follicles, transfer of oocytes matured in vitro was not as successful, as has been reported previously.[9] In the present study, 5 oocytes were immature at the time of collection. The oocytes were matured in vitro and transferred into recipients, but no pregnancies resulted. On the other hand, transfer of oocytes matured in vitro from a euthanatized donor did result in a pregnancy that was lost at approximately 25 days. Research is ongoing to improve in vitro maturation of equine oocytes. With improved technology, transporting ovaries and salvaging oocytes from ovaries of valuable mares that have died may be feasible.

The most important factor affecting oocyte viability in the present study was probably donor age. Mean age of donors was 21 years of age. Reduced fertility in mares ≥ 20 years old has been associated with reduced oocyte viability. Embryo development rates were significantly reduced when oocytes from old versus young donors were transferred into young recipients (31 vs 92%, respectively).[1] Evaluation of oocytes from old and young mares with light and electron microscopy demonstrated that although some oocytes from old mares are morphologically similar to oocytes from young mares, more oocytes from older than younger mares had morphologic anomalies such as large vacuoles and oblong shapes.[h] Therefore, viability of individual oocytes is probably reduced in some old mares, requiring more cycles per pregnancy than in younger mares. On a breeding farm, old mares were bred an average of 2.3 more cycles than young mares to produce a foal.[10] During the 1999 breeding season, pregnancies were established for 3 of the 4 youngest donors (≤ 16 years old) after a single oocyte transfer; in contrast, a pregnancy was established after a single oocyte transfer for only 1 of 6 donors ≥ 20 years old. However, with repeated transfers (2 to 4; mean, 2.75), pregnancies were established for 4 additional donors ≥ 20 years old. Among donors that had oocytes transferred, only the 2 oldest mares (25 and 26 years old) did not have a successful transfer. During 1999, oocytes were also collected from experimental donors < 13 years old and transferred into recipients. The studies used different oocyte treatments and insemination routines, but between 57 and 82% of transferred oocytes developed into embryonic vesicles. Although oocyte transfer has been shown to be a successful technique in producing foals from old mares, pregnancy rate per cycle will probably be lower when transferring oocytes from old versus young donors.

Success of the oocyte transfer program at Colorado State University improved from 1998 to 1999. Improvements were probably a result of using younger donors, placing more emphasis on obtaining high-quality semen, using more experienced personnel, and changing recipient selection. When problems (eg, poor quality semen, semen delayed during shipment, atretic changes in oocytes, immature oocytes) were not encountered, pregnancies resulted from approximately 50% of transfers. Therefore, success of oocyte transfer could be improved by altering a number of factors. Good quality semen is essential to maximize chances of a pregnancy. Reliability of shipments and the stallion's collection schedule should be considered. Earlier collection (≤ 24 hours after hCG administration) of oocytes from mares with ovulatory problems seemed to improve oocyte quality and fertility. Additional research is needed to determine whether age-related changes in oocyte and follicular quality can be treated.

Oocytes were transferred into cycling and noncy-

cling recipients. Use of noncycling mares provided a convenient method for obtaining a recipient without requiring synchronization of donor and recipient cycles and removal of the recipient's oocyte. Pregnancy rates appeared higher for noncycling versus cycling recipients (41% vs 21%). One recipient in early estrus and 1 recipient that had just ovulated became pregnant; however, both recipients lost the pregnancies. Prior to 70 days of gestation, all noncycling recipients had at least 1 corpus luteum. Pregnancies were lost from recipients that were cycling (n = 1; approx 55 days), that were not cycling (3; 23, 30, and 50 days), that were in the early phase of estrus (1; 13 days) and that had recently ovulated (1; 30 days). Overall incidence of early embryonic death was 30% (6/20). This rate was higher than anticipated but probably reflected the population of donor mares. Early embryonic losses increase as mares age, and rates of 33 and 62% have been reported for older mares.[11,12]

Results described in the present report suggest that oocyte transfer can be a successful method for obtaining pregnancies from subfertile mares in a commercial setting. Using this technique, pregnancies were established from mares with long histories of subfertility and abnormalities of the reproductive tract.

[a]Ovuplant, Fort Dodge, Fort Dodge, Iowa.
[b]Aloka 500V, Aloka Co Ltd, Wallingford, Conn.
[c]V-FOAD-1260-L, Cook Veterinary Products, Spencer, Ind.
[d]Propantheline bromide, Sigma Chemical Co, St Louis, Mo.
[e]Regulated vacuum pump, VMAR-500, Cook Veterinary Products, Spencer, Ind.
[f]Regumate, Hoechst Roussel Vet, Warren, NJ.
[g]Equitainer, Hamilton-Thorn, Beverly, Mass.

[h]Carnevale EM, Uson M, Bozzola JJ, et al. Comparison of oocytes from young and old mares with light and electron microscopy (abstr). *Theriogenology* 1999;15:299.

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