Use of parentage testing to determine optimum insemination time and culture media for oocyte transfer in mares

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Abstract

Parentage identification was used to test the developmental competence of oocytes cultured under different conditions and fertilized in vivo after oocyte transfer. Oocytes were collected transvaginally from follicles of estrous mares approximately 22 h after administration of human chorionic gonadotropin. Oocytes were cultured for approximately 16 h in one of three media, with or without addition of hormones and growth factors. Groups of three or four oocytes, cultured in different media, were transferred into the oviduct contralateral to a recipient’s own ovulation. Recipients were inseminated with semen from two different stallions at 15 h before and 2.5 h after oocyte transfer. Sixteen days after transfer, embryos were recovered from uteri and submitted for parentage testing. The percentage of oocytes resulting in embryonic vesicles was nearly identical (P > 0.05) for transferred oocytes (32/44, 73%) versus ovulated oocytes of recipients (9/13, 69%). More (P < 0.01) oocytes were fertilized by sperm inseminated before (35/38, 92%) versus after (3/38, 8%) oocyte transfer. Tissue culture medium (TCM)-199 was superior to equine maturation medium I (EMMI; a SOF-based medium) for culturing oocytes (P < 0.05), although addition of hormones and growth factors during culture did not improve (P > 0.05) development of embryos.

Introduction

Oocyte transfer involves transfer of an oocyte collected from the follicle of a donor mare into the oviduct of an inseminated recipient. This technique has been used commercially to produce offspring from valuable mares considered infertile using other reproductive methods, including embryo transfer (Carnevale et al. 2001b). Oocyte transfer can also be used to obtain information about the interactions of the equine oocyte, sperm and oviduct.

Oocytes are often collected from dominant follicles of donors within 16 h of expected time of ovulation (Carnevale & Ginther 1995, Hinrichs 1998, Carnevale et al. 2000, Hinrichs et al. 2000). Ovulation is synchronized by an injection of human chorionic gonadotropin (hCG) during mid-estrus, and ovulation occurs approximately 36 h later (Duchamp et al. 1987). Most oocytes are in metaphase I (Carnevale et al. 1999) at 24 h after hCG administration. In 1995, Carnevale and Ginther (1995) collected oocytes approximately 24 h after administration of hCG to donors and cultured them in tissue culture medium (TCM)-199 without supplementation of hormones and/or growth factors for approximately 16 h before transfer into recipients. This method resulted in high rates of embryo development, and future studies (Hinrichs 1998, Carnevale et al. 2000, Hinrichs et al. 2000, Coutinho da Silva et al. 2002) were conducted using similar culture conditions. Thus effects of different media and addition of hormones and growth factors have not been investigated during culture of oocytes recovered from pre-ovulatory follicles after follicular aspiration.

In vitro fertilization has not been very successful in the horse. Therefore, one of the most accurate methods of assessing oocyte viability and developmental competence is through transfer of oocytes into a recipient mare’s oviduct. Although oocyte transfer is expensive, it provides a method of studying the process of fertilization, including the interactions of the oocyte, sperm and oviduct. To reduce the expense of transfers, multiple oocytes are often transferred per recipient (Carnevale & Ginther 1995, Carnevale et al. 2000, Scott et al. 2001, Coutinho da Silva et al. 2002) – reducing costs but increasing the effect of an individual recipient on the results. Methods of minimizing the effect of each recipient after oocyte transfer would be valuable for future research.

Recipients of oocytes are often inseminated before and after transfers, a procedure with demonstrated success...
Materials and Methods

Management of mares and oocyte collections

Light-horse mares (Equus caballus, n = 40), between 3 and 15 years of age and weighing 400–600 kg, were housed in a dry lot and provided with free-choice hay. Groups of mares were synchronized through the use of prostaglandin F2α (10 mg dinoprost, i.m.; Lutalyse, Pharmacia and Upjohn Co., Kalamazoo, MI, USA). Reproductive tracts of mares were examined with transrectal ultrasound. Mares were injected with hCG (2500 IU, i.v.; Intervet, Inc., Millsboro, DE, USA) when the following criteria were observed: (1) follicle > 35 mm in diameter; (2) uterine edema; and (3) relaxed tone of cervix and uterus. Approximately 22 h after administration of hCG, oocytes were collected from preovulatory follicles by ultrasound-guided, transvaginal follicular aspirations (Carnevale & Ginther 1995). The first insemination, at approximately 12 h before transfer, should be sufficient to establish a sperm reservoir within the oviduct (Scott 2000). However, the effect of oocyte transfer on the oviduct and on sperm release and transport is not known. Therefore, recipients have been inseminated a second time, approximately 2 h after transfer, to ensure that adequate numbers of sperm are available for fertilization. The value of a second insemination has not been determined.

The present study was designed to use genotyping for parentage identification to evaluate the effect of different media on the developmental potential of oocytes after oocyte transfer and to determine whether more oocytes are fertilized by sperm inseminated before versus after transfer of oocytes.

Oocyte transfer

Recipients were cyclic mares that were injected with prostaglandin F2α and hCG at the same time as donors. Transfers were done through standing flank laparotomies. Recipients were placed in a stock and administered pre-surgical sedation (xylazine HCl, 0.3 mg/kg; Sedazine and butorphanol tartrate (0.01 mg/kg, i.v.; Torbugesic). The surgical site was scrubbed and blocked with 2% lidocaine HCl (Abbott Laboratories, North Chicago, IL, USA). Immediately before surgery, detomidine HCl (9 mg/kg, i.v.) and butorphanol tartrate (0.1 mg/kg, i.v.) were administered. An incision, approximately 15 cm in length, was made midway between the last rib and tuber coxae. The muscle layers were separated by blunt dissection, and the ovary and oviduct were exposed through the incision. Oocytes were transferred contralateral to the recipient’s own preovulatory follicle or side of ovulation. For transfer, oocytes were loaded into a glass pipette with a fire-polished end. The pipette was gently threaded 2–3 cm into the infundibulum of the recipient’s oviduct, and the oocytes were expelled with <0.2 ml of transfer medium (EmCare embryo holding medium, ICP).

Semen collection and insemination

Stallions (n = 3) used for the study had histories of good fertility. In the morning before an anticipated oocyte transfer, semen from two stallions was collected at approximately 10 h before the recipient’s first insemination and 24 h before the second insemination. Stallions were grouped so that equal numbers of all combinations of stallions were used for inseminations before and after transfers. Semen was evaluated and packaged for cooling as previously described (Squires et al. 1999) at 25 × 10⁶ to 50 × 10⁹ total sperm per milliliter. Each insemination dose contained 1 × 10⁸ progressively motile sperm, and semen was extended to between 40 and 50 ml in a non-fat milk extender containing amikacin (E-Z Mixin, CST, Animal Reproduction Systems, Chino, CA, USA). Semen was packaged in a container (Equitainer, Hamilton Research, Inc., South Hamilton, MA, USA) for passive cooling, with an initial cooling rate of 0.3 °C/min and a steady-state temperature of 4–6 °C (Squires et al. 1999). Recipients were cooled for approximately 16 h (16–18 h) in an atmosphere of 6% CO₂ and air at 38.5 °C. All oocytes (three to four) collected on a day were transferred into the same recipient.

Oocyte culture

Oocytes were identified and rinsed twice in flush medium and once in the respective culture medium before transfer into a petri dish containing 2.5 ml of one of the following culture media: (1) TCM (Bio Whittaker, Walkersville, MD, USA (TCM-199 with the additions of 10% fetal calf serum, 0.2 mM pyruvate and 25 μg/ml gentamicin sulfate)); (2) TCM+ (TCM with additions of 1 μg/ml luteinizing hormone, 15 ng/ml follicle stimulating hormone, 1 μg/ml estradiol, 500 ng/ml progesterone, 10 ng/ml insulin-like growth factor and 100 ng/ml epidermal growth factor); and (3) equine maturation medium I (EMMI), formulated as described previously (Macellman et al. 2001) with the exception that bovine serum albumin was replaced with 10% fetal calf serum; EMMI contained the same hormones and growth factors as TCM+. Oocytes collected on a given day were randomized for culture in: (1) TCM or EMMI or (2) TCM or TCM+. Oocytes were cultured for approximately 16 h (16–18 h) in an atmosphere of 6% CO₂ and air at 38.5 °C. All oocytes (three to four) collected on a day were transferred into the same recipient.
were inseminated with semen from one stallion at 15.3 ± 0.2 h (mean±S.E.M.) before oocyte transfer and with semen from a second stallion 2.5 ± 0.3 h after oocyte transfer.

**Parentage testing and statistical analyses**

Recipient were scanned using transrectal ultrasound with a 5 mHz transducer for detection of pregnancy on days 12, 14 and 16 after oocyte transfer. On day 16, uteri were flushed with lactated Ringer solution to collect embryonic vesicles. Vesicles were washed in lactated Ringer solution (Abbott Laboratories, North Chicago, IL, USA) and frozen (−6 °C) until submission for parentage identification through analysis of microsatellites using the polymerase chain reaction (Veterinary Genetics Laboratory, University of California, Davis, CA, USA) (Bowling et al. 1997). Sexes of embryonic vesicles were determined by examination of a genetic marker (Lex 3) located on the X chromosome. When a single genetic marker was observed in an embryo, the embryo was determined to be male if the sire and dam did not contain the same allele. Embryos with a single genetic marker and with similar markers for the sire and dam could not be differentiated between male and homozygous female; therefore, they were excluded from comparisons for sex ratio. Fisher’s exact test was used to detect significant differences (P < 0.05) in the number of oocytes developing into embryonic vesicles with different media and insemination treatments and to compare the number of male and female embryos after oocyte transfer and natural ovulation.

**Results**

With the exception of one recipient, all mares ovulated prior to oocyte transfer. At least two embryonic vesicles were imaged in the uterus of each recipient. The recipient’s own oocytes resulted in embryos after 9 of 13 ovulations (69%), and 32 of 44 (73%) transferred oocytes (P > 0.1) developed into embryos. In the four recipients that did not have an embryonic vesicle from their own oocyte, at least two embryonic vesicles from transferred oocytes were present. No recipient had embryonic vesicles resulting only from their ovulated oocyte.

More (P < 0.05) embryonic vesicles from transferred oocytes and ovulated oocytes resulted from the insemination before than after oocyte transfer (transferred oocytes: 28/30 (93%) and 2/30 (7%); ovulated oocytes: 7/8 (88%) and 1/8 (12%), for insemination before and after transfer respectively). Conclusive laboratory results were not available for two embryonic vesicles. Embryo development rates overall were different (P < 0.05) among culture media (Table 1). In recipients (n = 7) that received oocytes cultured in TCM or EMMI, more (P < 0.05) embryonic vesicles resulted from oocytes cultured in TCM versus EMMI (Table 1). In recipients (n = 6) that received oocytes cultured in TCM or TCM+, similar numbers of oocytes resulted in embryonic vesicles (Table 1). Values with different superscripts within a column, are significantly different (P < 0.05).

Of the population of mares available for oocyte donors (n = 32), 6 mares were homozygous for Lex 3 (19%). Identification of the Lex 3 marker was not available for 1 donor, and 3 of 26 (12%) embryos with a single genetic marker could not be differentiated between male and homozygous female. One embryonic vesicle, cultured in TCM+, was diagnosed as triploid from fertilization of a diploid oocyte. Of the vesicles for which sex could definitively be determined (n = 27), percentages of males were similar (P > 0.05) among groups (TCM: 9/17 (53%); TCM+: 3/6 (50%); and EMMI: 2/4 (50%) but tended (P = 0.1) to be different between ovulated and transferred oocytes (7/8 (88%) and 14/27 (52%) respectively).

**Discussion**

To be successful, oocyte transfer requires proper insemination of recipients. Although methods to optimize pregnancy rates have been defined in ovulating mares, less information is available regarding insemination of oocyte transfer recipients. Within 4 h after insemination of the ovulating mare, motile sperm were found within the oviduct (Scott et al. 1995). Although pregnancy has resulted in mares that ovulate more than 6 days after insemination (Burkhardt 1949), insemination of mares at least every other day during estrus has been recommended for optimal fertility (Pickett et al. 1987). Because the mare has a variable and long estrus (mean of 6.5 days and range of 4.5–8.9 days) (for review see Ginther (1992)), coordination of ovulation and sperm transport is essential for fertility. In other species, the preovulatory follicle and the site and time of ovulation have been suggested as being important in the synchronization of ovulation with release of sperm from the isthmus (Hunter 2001). Factors such as follicular fluid or progesterone (Hunter et al. 1999) could signal release of sperm from the isthmus and their subsequent movement to the site of fertilization (Hunter 1988). With oocyte transfer, the events surrounding fertilization are manipulated. In the present study, oocytes were transferred contralateral to the side of ovulation; therefore, any effect of follicular fluid or of the preovulatory follicle on the ipsilateral oviduct was eliminated. In other species, local vasculature between the ovary containing an oocytes was manipulated to carry a signal, potentially progesterone, to the oviduct that can affect sperm release (Hunter 1988). In contrast to the ewe and cow, the mare’s ovarian artery has only
limited contact with the utero-ovarian vein (Ginther 1992); therefore, less direct apposition between arterial and venous blood supplies in the mare could minimize local interactions between ovary and oviduct.

In the present study, similar fertilization rates were obtained for ovulated oocytes as for oocytes transferred into the contralateral oviduct (69 and 73% respectively), so the preovulatory follicle probably did not have a local effect on the oviduct for sperm release, transport and fertilization. Potentially, the large preovulatory follicle in the mare secretes significant amounts of steroids or other factors into the circulation to cause a systemic effect. In the present experiment, mares were inseminated with large numbers of sperm (1 x 10^9 sperm before and after oocyte transfer). After insemination of mares with 5 x 10^8 sperm in the uterine body, the numbers of sperm were high and were not different in the oviducts ipsilateral or contralateral to the preovulatory follicle (1499 ± 1019 and 1876 ± 1831 sperm respectively) (Rigby et al. 2000). Therefore, in the present experiment, insemination with large numbers of sperm could have negated any effect of the preovulatory follicle on the sperm reservoir within the ipsilateral oviduct. In addition, manipulation of the oviduct at surgery could have affected sperm release and transport within the oviduct.

In recent studies (Scott et al. 2001, Maclellan et al. 2002), embryo development rates were over 80% when mares were inseminated only before oocyte transfer with fresh semen. However, when recipients were inseminated with fresh semen only after oocyte transfer, pregnancies were also obtained (8/14, 57%) (Carnevale et al. 2000). In the present experiment, sperm from the insemination of recipients before transfers resulted in fertilization of 93% of transferred oocytes, indicating that inseminations only before oocyte transfer were sufficient when using adequate numbers of sperm from fertile stallions.

In a commercial program for oocyte transfer, pregnancy rates between 27 and 40% per transfer were obtained (Carnevale et al. 2001a,b). Two primary factors associated with oocytes and sperm were different between commercial and experimental transfers. Donors in the commercial programs were older, with histories of subfertility; in contrast, mares in experimental programs were typically young and probably highly fertile. In addition, recipients in previous oocyte transfer studies (Carnevale & Ginther 1995, Hinrichs 1998, Carnevale et al. 2000, Hinrichs et al. 2000, Scott et al. 2001, Coutinho da Silva et al. 2002, Maclellan et al. 2002) were inseminated with fresh semen from fertile stallions. In commercial programs, cooled, transported semen was used predominantly and fertility of stallions was not known. In the present experiment, cooled semen was used to inseminate recipients. Embryo development rates were high, demonstrating that cooling semen did not reduce the success of oocyte transfer when: (1) semen was collected from fertile stallions; (2) semen was properly handled for cooling; and (3) an adequate number of sperm were inseminated.

Embryo development rates for transferred versus ovulated oocytes (73 versus 69%) were not different; therefore, procedures for oocyte culture and transfer did not affect oocyte viability or fertilization. In 4 of the 13 recipients, the oocytes ovulated by recipients were not fertilized, although a minimum of two transferred oocytes developed into embryonic vesicles in each recipient. The results show that recipients' reproductive tracts were adequate to support fertilization and embryo development; therefore, other factors - such as quality of the recipient's own oocyte, ovulation failure or early embryonic death – could have caused the failure of embryo development.

Although multiple births are uncommon in the mare (Ginther 1992), the results of the present research show that embryo reduction does not occur within the oviduct. High fertilization rates and early embryo development occurred when multiple oocytes were transferred into the oviduct in the present experiment.

Transfer of oocytes did not affect the expected sex ratio, as the percentages of male and female embryos that resulted were similar. However, the number of embryos from ovulated oocytes tended to have a higher incidence of males than females, potentially a chance effect of small numbers. Most recipients had ovulated by the time of oocyte transfer and had been inseminated 15 h previously; however, the effect of timing of insemination on sex ratio has not been determined in the horse.

Follicular and oocyte maturation was initiated in the present study through the administration of hCG to donors. Upon collection at this stage, equine oocytes were surrounded by an expanding mass of cumulus cells; and frequently, granulosa cells were also attached. The mass of cumulus and granulosa cells was typically >1 mm in diameter and moderately dense. Because of the attached cells, imaging morphology of the oocytes was difficult and unreliable. During culture, the cumulus cells continued to expand as demonstrated by a light, mucoid appearance and a loosening of the ring of corona cells. Cumulus cells were not removed from oocytes prior to transfer, as this could have affected the fertilization process. Therefore, although oocytes were rapidly imaged through a stereomicroscope prior to transfer, morphology of the ooplasm was impaired by the cellular mass and differences were not observed for the majority of oocytes, with an ooplasm appearance of homogeneous or heterogeneous grey.

One embryo was triploid. In this case, the polar body was not extruded and the diploid oocyte was fertilized. At the time of collection and transfer, the oocyte was noted as having an atypical morphology of the ooplasm. In this study and in a previous study (Coutinho da Silva et al. 2002), approximately 50 embryos from transferred oocytes were parentage tested. This is the only embryo that has been determined to have a chromosomal abnormality.

Collection, culture and transfer of preovulatory oocytes were first described in 1995 (Carnevale & Ginther 1995). Because the oocytes were collected from preovulatory
follcles (approximately 12 h before ovulation) and the resumption of meiosis had occurred in vivo, hormones were not added to the medium. In more recent studies (Hinrichs 1998, Carnevale et al. 2000, Hinrichs et al. 2000, Scott et al. 2001, Coutinho da Silva et al. 2002), similar culture methods have been used – a base medium of TCM-199 with 10% fetal calf serum and no hormones. However in these studies, the fetal calf serum could have provided some hormones and growth factors. Maclellan et al. (2002) cultured oocytes from preovulatory follicles in EMMI, a medium formulated specifically for equine oocytes; EMMI contained gonadotropins, steroids and growth factors (Maclellan et al. 2001). Use of EMMI resulted in high (7/8, 88%) embryo development rates. In the present study, the effect of media and additions were compared directly. Culture of oocytes in TCM-199 resulted in high (7/8, 88%) development rates to embryos, regardless of the addition of hormones and growth factors. Therefore, addition of hormones did not increase the incidence of completion of maturation during culture of preovulatory oocytes with 10% fetal calf serum. When compared with TCM, oocytes cultured in EMMI resulted in significantly lower development rates to embryos. EMMI has been used previously to culture preovulatory and immature equine oocytes (Maclellan et al. 2002). For the present experiment, EMMI was prepared off-site, and potentially, subtle differences in media preparation could have occurred. However, results of the present experiment suggest that TCM is a better medium than EMMI for the culture of preovulatory oocytes.

Based on this study, we conclude the following: (1) parentage testing was valuable for establishing treatment effects while minimizing the influence of individual recipients; (2) embryo development rates were similar for ovulated and transferred oocytes, and oocyte transfer was shown to be a highly effective assisted reproductive technique in the mare; (3) addition of hormones and growth factors to culture medium containing 10% fetal calf serum for preovulatory oocytes did not improve rates of embryo development; (4) insemination of recipients before oocyte transfer was adequate to fertilize a high percentage of transferred oocytes; (5) fertilization rates were similar in oviducts ipsilateral and contralateral to the preovulatory follicle; and (6) fertilization of transferred oocytes resulted in approximately equal percentages of male and female embryos.

Acknowledgements

An extended abstract of this manuscript was published in the Proceedings of the 8th International Symposium on Equine Reproduction (Carnevale et al. 2002). This study was funded by benefactors of the Preservation of Equine Genetics program. The authors would like to thank Pharmacia and Upjohn Co., Kalamazoo, MI, USA for the donation of Lutalyse. The following people assisted: K A Preis, J R Neves Neto and K M Leao.

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Received 13 February 2004
First decision 18 May 2004
Revised manuscript received 20 July 2004
Accepted 30 July 2004