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Intrafollicular oocyte transfer in the horse: effect of autologous vs. allogeneic transfer and time of administration of ovulatory stimulus before transfer

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Abstract

Purpose To assess meiotic and developmental competence after transfer of immature cumulus-oocyte complexes (COCs) to the preovulatory follicles of mares (intrafollicular oocyte transfer (IFOT)).

Methods In Experiment 1, mares received an ovulatory stimulus at IFOT. Thirty hours later, COCs were recovered from the follicle, and mature oocytes underwent ICSI and embryo culture. In Experiments 2 and 3, autologous vs. allogeneic COCs were used. The mares were inseminated and embryos were recovered. In Experiment 3, the ovulatory stimulus was administered 9 h (autologous) and 15 h (allogeneic) before IFOT. In Experiment 4, only allogeneic COCs were used; the ovulatory stimulus was administered 9 or 15 h before IFOT. Excess embryos (autologous) and parentage-verified embryos (allogeneic) were considered IFOT-derived.

Results In Experiment 1, 36/54 IFOT oocytes (67%) were recovered, of which 56% were mature, vs. 49% of in vitro matured oocytes ($P > 0.1$). After ICSI, blastocyst rates were 25% and 18%, respectively ($P > 0.1$). In Experiment 2, 0/6 autologous and 2/6 allogeneic IFOT yielded IFOT-derived embryos. In Experiment 3, 0/7 autologous and 2/5 allogeneic IFOT yielded IFOT-derived embryos. The proportion of mares yielding IFOT-derived embryos was lower after autologous vs. allogeneic IFOT (0/13 vs. 4/11; $P < 0.05$). In Experiment 4, 1/8 9-h and 1/7 15-h IFOT yielded IFOT-derived embryos.

Conclusions Transferred oocytes mature within the follicle and can maintain developmental competence. Allogeneic IFOT was more efficient than was autologous IFOT. The time of ovulatory stimulation did not affect embryo yield. The IFOT procedure is still not repeatable enough to be recommended for clinical use.

Keywords Equine · Oocyte · Follicle · Intracytoplasmic sperm injection · Embryo

Introduction

Intrafollicular oocyte transfer (IFOT) offers a unique method to study oocyte maturation, as it allows the in vivo maturation of oocytes recovered from follicles of varied status and so provides a tool to separate the effects of oocyte origin from the effects of the maturation system (in vitro vs. in vivo). The IFOT procedure was first attempted in cattle and baboons [1]. Hinrichs and DiGiorgio [2], working in the horse, reported the first conclusive oocyte maturation and embryo production after IFOT of immature oocytes. The horse may provide an excellent model for this procedure, as the equine preovulatory follicle is large (~ 35–45 mm diameter) and is covered by a thick tunica albuginea which may resist damage during puncture.

In the horse, IFOT also has clinical potential as a method for foal production from isolated oocytes, as standard in vitro fertilization is not yet successful in this species [3] and its alternative, intracytoplasmic sperm injection (ICSI), is costly and available only in some locations. In addition, mares are monovulatory (although, as in women, mares may sometimes

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ovulate two or more follicles), and effective superovulation methods have not been developed in this species.

Despite the scientific and clinical potential of IFOT, few reports are available on this technique. In the study of Hinrichs and DiGiorgio [2], immature cumulus-oocyte complexes (COCs) were transferred to the preovulatory follicles of inseminated mares, then the mares' uteri were later flushed for embryo recovery. Four of 12 mares (33%) produced embryos in excess of the number of ovulated follicles, with the most effective procedure yielding 6 excess embryos after transfer of 15 COCs to the follicle. These results indicated that some transferred oocytes matured within the follicle, were delivered to the oviduct at ovulation, and underwent successful fertilization and embryo development.

Subsequently, Goudet et al. [4] transferred COCs to the dominant preovulatory follicle, then recovered oocytes from the follicle 34 h later. These authors transferred both immature and possibly mature oocytes; the recovery rate of transferred oocytes from the follicle was 49% (42/86). Of oocytes known to be immature at the time of IFOT, 7/29 (24%) were mature when recovered. Deleuze et al. [5] aspirated immature COCs from mares' subordinate follicles at the time that the mare received a gonadotropic ovulatory stimulus and transferred the immature COCs (3 to 14) to the preovulatory follicle of the same mare. Mares were inseminated and the uterus was later flushed for embryo recovery. Four of 10 mares yielded excess embryos (1 to 2 excess per mare). It should be noted that in the two equine IFOT studies yielding embryos, no parentage testing was performed; embryos in excess of the number of observed ovulations were assumed to originate from IFOT. Recently, IFOT has been investigated in cattle. A 17% morula/blastocyst yield was reported after transfer of immature oocytes [6]. Embryos in excess of one were considered to be from IFOT.

The results of these studies suggest that the IFOT of immature COCs has potential as a method to investigate factors affecting oocyte meiotic and developmental competence, as it can yield excellent embryo production results (for example, 6 blastocysts from 15 oocytes [2]). However, the procedure has had poor repeatability [2, 5, 6]. The cause for this poor repeatability is unclear. Inefficiencies could occur at essentially every step of the IFOT procedure: oocytes may not be delivered to or remain in the follicle, they may not mature or gain developmental competence within the follicle, they may not be released from the follicle at the time of ovulation, they may be released but fail to enter the oviduct, or they may enter the oviduct but not be fertilized.

An additional factor is the length of time the oocytes are within the preovulatory follicle before ovulation. This period must be adequate for the oocytes to undergo nuclear maturation to metaphase II (MII) and gain optimal cytoplasmic maturation [7], but not prolonged, as oocytes may undergo postmaturation aging which could affect their developmental competence. Thus, the timing of administration of the ovulatory stimulus, which governs the time at which the follicle ovulates after IFOT, could influence the development of the transferred oocytes. The most appropriate timing for IFOT is not clear; in the horse, the preovulatory follicle ovulates 36 to 42 h after administration of an ovulatory stimulus (pituitary extract, hCG, or GnRH analog; [8–10]). However, for oocytes recovered from immature follicles and subjected to in vitro maturation (IVM), optimal blastocyst rates after ICSI have been reported after only 24 to 36 h IVM [7, 11]. The duration of maturation needed for optimal development may be shorter if the oocytes are held overnight at room temperature before the onset of maturation [12], a common procedure in equine-assisted reproduction to allow scheduling of subsequent manipulations [13, 14]. Deleuze et al. [5] administered the ovulatory stimulus (crude equine pituitary gonadotropin) at the time of transfer. In the study of Hinrichs and DiGiorgio [2], excess embryos were recovered from mares that received hCG at the time of transfer to 16 h before transfer.

The relationship of the transferred oocytes to the host mare may be a factor in clinical application of IFOT. Hinrichs and DiGiorgio [2] transferred allogeneic oocytes, whereas Deleuze et al. [5] transferred autologous oocytes. For clinical use, autologous transfer avoids recovery of an undesired embryo from an allogeneic host mare. However, allogeneic transfer might be preferred to avoid excessive manipulation of a valuable donor mare or when the donor mare has reproductive pathology that prevents her from providing embryos.

The purpose of the present study was to determine (A) the proportion of transferred oocytes that could be recovered from the follicle after IFOT, (B) the maturation rate and developmental competence of transferred and recovered oocytes, (C) the ovulation rates and rates of IFOT-derived embryo production after autologous and allogeneic IFOT in inseminated mares, and (D) the effect of timing of administration of the ovulatory stimulus on pregnancy and IFOT-derived embryo production rates. The parentage of embryos recovered after allogeneic IFOT was determined by genetic analysis to confirm their origin.

Materials and methods

Experimental design

For all experiments, oocyte donor mares were subjected to transvaginal follicle aspiration (TVA) for oocyte recovery from all ovarian follicles ≥ 8 mm diameter approximately once every 14 days. Oocyte donor mares were not treated with hormones for manipulation of the cycle. Host mares undergoing IFOT were designated as Allo (recipients of donor (allogeneic) oocytes) or Self mares (recipients of their own (autologous) oocytes) and were used during the follicular

phase, either of natural cycles or after treatment with a prostaglandin analog to shorten the length of the previous diestrus.

Experiment 1. Meiotic and developmental competence of oocytes recovered from the follicle after intrafollicular transfer

This experiment was conducted to determine the proportion of transferred oocytes that could be recovered from the follicle after IFOT, the maturation rate of IFOT oocytes within the follicle, and their developmental competence after ICSI and embryo culture. Allogeneic IFOT was performed. On the day before IFOT, mares in estrus with one dominant follicle ≥ 30 mm in diameter were selected as host mares. Oocyte donor mares were subjected to TVA, and the COCs recovered were pooled and held overnight at room temperature [13]. On the day of IFOT, 6–12 held COCs were randomly assigned to IFOT and the remainder were placed in culture for in vitro maturation. The COCs assigned to IFOT were transferred transvaginally to the host mare's dominant follicle. Ovulatory agents (hCG and deslorelin) were administered to the host mare immediately before the IFOT procedure. The host follicle was aspirated via TVA 30 h after IFOT. The presumptive native oocyte was differentiated from the IFOT oocytes by its characteristic cumulus, as described below, and was used for a separate study. The IFOT oocytes were denuded of cumulus and assessed for the presence of a polar body, and those that were mature were subjected to ICSI and embryo culture. Maturation, cleavage, and blastocyst development rates were compared with those for the oocytes matured in vitro.

Experiment 2. Embryo recovery after autologous or allogeneic IFOT (ovulatory stimulus at the time of transfer)

This experiment was conducted to determine the rate of embryo recovery from the uterus of inseminated mares that had undergone IFOT. Both autologous and allogeneic IFOT were performed. To reduce the possibility that luteinization of subordinate follicles after aspiration [11, 15] would interfere with normal ovulation of the preovulatory follicles of Self mares, IFOT was performed within 1 h of the time that the COCs were recovered (i.e., COCs were not held overnight before IFOT). For autologous transfers, a mare in estrus with one dominant follicle ≥ 32 mm in diameter was identified (Self mare). The mare's subordinate follicles were aspirated by TVA and the recovered COCs were immediately transferred to the dominant follicle. For allogeneic transfers, host mares were also selected to have a dominant follicle ≥ 32 mm diameter on the day of IFOT. For each allogeneic IFOT, COCs were recovered by TVA of a single donor mare and were immediately transferred to the dominant follicle of the host Allo mare. Ovulatory agents were administered to Self and Allo mares at the time of IFOT. The mares were inseminated the day after IFOT and were monitored via transrectal ultrasonography for evidence of ovulation. The uterus was flushed for embryo recovery 8 days after ovulation. Embryos recovered after allogeneic IFOT were biopsied or bisected and cells submitted for genetic analysis of microsatellites to determine parentage.

Experiment 3. Embryo recovery after autologous or allogeneic IFOT (ovulatory stimulus before transfer)

This experiment was conducted to determine the embryo recovery rate after IFOT when the time of residence of IFOT oocytes within the follicle was shortened by administering ovulatory agents prior to IFOT. Oocytes for allogeneic transfer were recovered by TVA of donor mares (one to two donor mares per IFOT) and held overnight before transfer to the preovulatory follicle of an Allo mare, to mimic the situation that might be encountered clinically when oocytes were shipped for IFOT; oocytes for autologous transfer were recovered from subordinate follicles of Self mares and transferred immediately to the preovulatory follicle of the same mare. The ovulatory agents were administered 9 h before IFOT for the Self mares and 15 h before IFOT for the Allo mares, under the hypothesis that immediately transferred oocytes may require a longer duration of maturation than would oocytes held overnight [12]. The mares were inseminated and monitored for ovulation. Eight to 9 days after ovulation, their uteri were flushed for embryo recovery. Embryos recovered after allogeneic IFOT were bisected and cells submitted for parentage testing.

Experiment 4. Embryo recovery after allogeneic IFOT in host mares receiving the ovulatory stimulus 9 or 15 h before transfer

Only allogeneic IFOT was performed. Oocytes were recovered from donor mares by TVA (one to two donor mares per IFOT) and held overnight. Host mares were randomly assigned to receive ovulatory agents either 9 or 15 h before IFOT. Mares were inseminated and monitored for ovulation. Their uteri were flushed 9 days after ovulation, and recovered embryos were bisected and cells submitted for parentage testing.

General methods

Experimental animals

Donor, Allo, or Self mares were selected from a herd of 30 American Quarter Horse and Quarter-type mares weighing

400–550 kg and aged 10–20 years. Donor oocytes for Experiment 1 were recovered from a separate herd of 16 similar mares, used for oocyte collection only. All mares were housed outside in paddocks and were fed hay ad libitum for the duration of the study. Fresh extended semen from one 16-year-old American Quarter Horse stallion of proven fertility was used for insemination. The stallion was housed in a stall and fed coastal hay and grain. All experimental procedures were performed according to the *United States Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training* and were approved by the Laboratory Animal Care Committee at Texas A&M University (IACUC AUPs 2015-0282 and 2015-0026).

Transvaginal follicle aspiration for recovery of immature COCs

Immature COCs were obtained by TVA, as described by Jacobson et al. [11] with minor modifications. Briefly, mares were restrained in stocks with tails wrapped and tied. The perineal region was cleansed with povidone-iodine scrub. Immediately prior to the procedure, 3–4 mg detomidine (Dormosedan, Zoetis, Parsippany, NJ, USA), 10 mg butorphanol (Torbugesic, Overland Park, KS, USA), and 120 mg *N*-butyl hyoscine bromide (Buscopan, Boehringer Ingelheim, Ingelheim, Germany) were administered i.v. A transvaginal ultrasound probe was lubricated with sterile lubricant (Therio-gel, AgTech Inc., Manhattan, KS, USA) and placed in the vagina, and the ovary was manipulated per rectum. All follicles ≥ 8 mm in diameter were aspirated using a 12-ga double-lumen needle (Oocyte Collection Set, MILA International Inc., Florence, KY, USA) and were flushed four to six times using ViGRO Complete Flush Medium (Vetoquinol, Pullman, WA, USA) with 8000 IU heparin (Sigma-Aldrich, St. Louis, MO, USA) added per 500 mL. An additional 5 mg butorphanol and 60 mg *N*-butyl hyoscine bromide were administered before performing TVA on the second ovary. Additional detomidine (1–3 mg, IV) or xylazine (50–150 mg, IV, Xylamed, Vetone, Boise, Idaho, USA) was administered as needed for additional tranquilization. The recovered fluid was poured through an embryo filter (EM Con Filter, Immune Systems Inc., Spring Valley, WI, USA) and the contents of the filter washed into Petri dishes and searched using a dissection microscope at ×40–60 to locate the COCs.

Oocyte preparation

In Experiment 1, collected COCs were washed and held overnight at room temperature (22–25 °C) in ViGRO Holding Plus (Vetoquinol) or SYNGRO holding media (Vetoquinol) [14]. In Experiments 3 and 4, COCs that were held overnight were held in SYNGRO holding medium. Whether held overnight or transferred immediately after recovery, the COCs were washed in M199 with Hanks' salts and HEPES (Invitrogen, Carlsbad, CA, USA) with 20% added frozen-thawed equine follicular fluid, recovered previously via TVA from a follicle > 25 mm diameter. The COCs were then loaded in this medium into the needle apparatus for transfer.

Intrafollicular oocyte transfer

IFOT was performed via transvaginal ultrasound-guided follicle puncture, as described above for follicle aspiration, but using a needle system that was modified from equipment used for TVA in cattle (WTA Reproduction Technologies, Cravinhos, Brazil) and consisted of a replaceable single-lumen 20-ga 1.5-in trans-hub needle fitted with 20-ga plastic tubing. This tubing was run through a metal cylinder (8 mm outer diameter). The needle hub was attached to the proximal end of the cylinder and an 18-ga needle, attached to a 1-mL all-plastic syringe, was inserted into the plastic tubing at its distal end.

To minimize the volume of material transferred to the follicle, the COCs, in < 300 μL of medium, were loaded into the tubing from the needle tip. The tubing was first filled with medium, and then, 300 μL air was aspirated into the tubing, then the oocytes were loaded, followed by ~ 50 μL of medium. This method was modeled after a recent work on bovine IFOT [6, 16].

For transfer, Allo host mares were sedated with 150–250 mg xylazine and 5–10 mg butorphanol i.v. The mare's tail was wrapped and the perineum cleansed with povidone-iodine scrub. The transvaginal probe, with the needle system containing the oocytes already in the biopsy guide channel, was placed into the mare's vagina. The dominant follicle was visualized on the ovary and the ovary and probe handle manipulated so that the needle was inserted into the dominant follicle. Contents of the needle and tubing were slowly injected into the follicle. Once a flash of echogenicity, representing the air entering the follicle from the needle, was visualized on the ultrasound, the injection was stopped and the needle was removed from the ovary. The ovary was immediately released. The probe was withdrawn from the vagina, and the apparatus was taken into the laboratory, where the tubing was flushed to determine if any oocytes remained within the tubing. If the host mare had two preovulatory follicles > 30 mm on the day of transfer, if sufficient oocytes were available (> 10), oocytes were transferred into both follicles.

For Self mares, the mare remained in the stocks after TVA for oocyte collection and during the time the COCs were identified and loaded into the IFOT apparatus (< 30 min). The mare was then administered additional xylazine, and the perineum re-cleaned if necessary, and IFOT was performed as described above.

Oocyte recovery after IFOT (Experiment 1)

Thirty hours after IFOT, the contents of the dominant follicle were aspirated as described for TVA, above, with the modification that first the follicular fluid was aspirated from the follicle and collected into an empty bottle. Then, the follicle was flushed and aspirated six times; this aspirated fluid was collected into a separate bottle. The contents of the follicular fluid and aspirate bottles were transported to the laboratory and kept warm during searching. Because the cumulus of these oocytes was anticipated to be expanded, the contents of the bottles were not filtered but instead were pipetted directly into Petri dishes for evaluation under a dissection microscope. The follicular fluid was briefly allowed to sediment; an aliquot was removed from the top of the fluid for submission for microbial culture and the cellular portion examined to locate the COCs. The presumptive native COC was differentiated from the IFOT COCs by its characteristic large, yellow mucoid expanded cumulus. In contrast, IFOT-derived COCs, as they were stripped of the majority of their cumulus cells during initial recovery from their native follicle (Fig. 1a), had smaller, less cellular cumulus masses. The cumulus of IFOT-derived COCs was expanded (Fig. 1b); however, it appeared to have clear rather than yellow intercellular matrix and was less mucoid in appearance.

In vitro embryo production

In vitro embryo production was performed as previously described by Salgado et al. [17] with minor modifications. Briefly, for oocytes designated for IVM, after overnight holding, COCs were transferred to maturation medium (M199 with Earle's salts (Invitrogen), 5 mU/mL FSH (Sioux Biochemicals, Sioux Center, IA), 10% FBS (Invitrogen), and 25 μg/mL gentamicin (Sigma)) and cultured in 150-μL droplets of this medium under oil at 38.2 °C in a humidified atmosphere of 5% CO_2 in air for 30 h.

After IVM, or for oocytes recovered after IFOT, oocytes were denuded of cumulus in 0.6 mg/mL hyaluronidase. Those oocytes having an intact membrane and a polar body were considered to be mature and underwent ICSI using a Piezo drill, as previously described [17]. Frozen-thawed semen from one stallion was used for all the ICSI procedures; this was the stallion used for fresh semen insemination in the main study. Presumptive zygotes were held for 30 min in M199/Earle's salts with 10% FBS in 5% CO_2 in air at 38.2 °C, then cultured in a commercial human embryo culture medium (GB; Global medium, LifeGlobal, Guilford, CT) supplemented with 10% FBS in droplets at 5 μL medium per zygote under oil in an atmosphere of 6% CO_2, 5% O_2, and 89% N_2 at 38.2 °C. Medium was changed to DMEM/F-12 (Sigma) with 10% FBS at Day 5. Embryos were evaluated on Days 7 to 10 of culture for blastocyst development.

Insemination (experiments 2–4)

Semen was collected from the stallion using a Missouri-model artificial vagina and was diluted 1:1 in INRA 96 semen extender (IMV Technologies, France). The insemination dose used contained a minimum of one billion progressively motile spermatozoa. Mares were inseminated the day before anticipated ovulation, i.e., the day after IFOT (Experiment 2), or were inseminated the day before or the day of IFOT (Experiments 3 and 4). In Experiment 2, if the mare had not ovulated within 48 h of the time of insemination, the mare was inseminated a second time with either fresh or cooled semen from the same stallion. After IFOT and insemination, the ovaries and uterus of host mares were evaluated by ultrasonography per rectum at least twice daily until ovulation was detected. A mare was considered to have ovulated if the follicle was not visible on the ovary, if the follicle was not visible and a

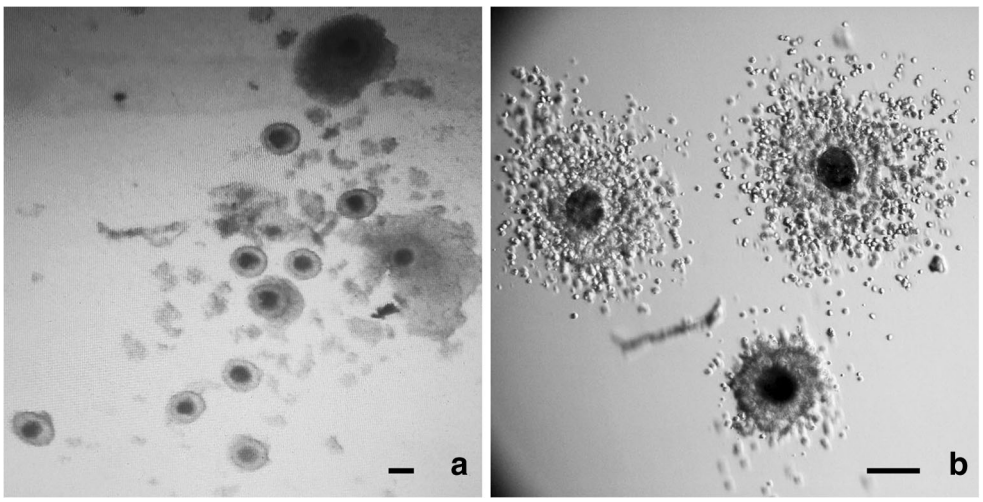

Fig. 1 Appearance of COCs on their initial recovery from immature follicles of the donor mare (**a**), showing the compact cumulus, typically three to four layers; and on recovery from the dominant stimulated follicle 30 h after IFOT (**b**) showing varying levels of cumulus expansion. Bar = approximately 150 μm

solid echogenic area was visible on the ovary, or if an echogenic area with a small central non-echogenic region was visible on the ovary. The day that ovulation was detected was designated Day 0. If uterine fluid was present when mares were evaluated, the mares were treated with oxytocin, 20–40 IU i.m. If the fluid appeared echogenic, the uterus was flushed with 2 L Lactated Ringer's solution. If a follicle > 28 mm was seen in addition to the host follicle, examinations continued until the follicle was seen to ovulate or regress.

Uterine flush for embryo recovery (Experiments 2–4)

Eight to 9 days after confirmed ovulation of the IFOT follicle, the mare's uterus was flushed for embryo recovery. For this, mares were restrained in stocks with tails wrapped and tied. The perineal region was cleansed with povidone-iodine scrub. Sedation, 150–200 mg xylazine i.v., was given intravenously as needed. The mare's uterus was flushed four times using ViGRO Complete Flush Medium (1 L per flush), and the flush fluid was run through an embryo filter. During the last flush, the mare was treated with 20 units of oxytocin intravenously to aid in expulsion of fluid.

The contents of the embryo filter were rinsed into a Petri dish. Any embryos recovered were evaluated on a Zeiss Axiovert inverted microscope at ×100–200 and embryo diameter was measured. Embryos from allogeneic IFOTs were either biopsied using a micromanipulator, as previously described [18], or were bisected. The cells or demi-embryos were frozen and submitted to the Veterinary Genetics Laboratory at the University of California, Davis, for parentage testing using loci for one sex-associated gene (AME), one X-linked marker (LEX3), and 16 microsatellite identification markers [19].

Statistical analysis

Fisher's exact test (GraphPad; https://www.graphpad.com/quickcalcs/contingency1.cfm) was used to compare maturation, cleavage, and blastocyst rates between IFOT and IVM oocytes (Experiment 1) and incidence of ovulation, overall embryo production, and excess (Self mares) and IFOT-derived (Allo mares) embryo production between autologous and allogeneic IFOT mares (Experiments 2 and 3) or between treatment groups having different timing of administration of the ovulatory stimulus (Experiment 4). For Experiments 2, 3, and 4 combined, the diameter of native vs. IFOT embryos was compared for each day of uterine flush using Student's *t* test (GraphPad; https://www.graphpad.com/quickcalcs/ttest1.cfm).

Results

Experiment 1. Meiotic and developmental competence of oocytes recovered from the follicle after intrafollicular transfer

The cumulus of the COCs recovered from donor mares for IFOT varied in size but was generally three to four layers of cells (Fig. 1a). Five IFOTs were performed. Transferred oocytes were recovered from the follicle in each case. All oocytes recovered, both native and IFOT-derived, were found in the initial follicular fluid aspirate, rather than in the fluid recovered after flushing the follicle. Microbial cultures of the five samples of follicular fluid collected during recovery of the oocytes 30 h after IFOT yielded no bacterial growth. From the five follicles, four native oocytes were recovered and were used for a separate study. The cumulus of the IFOT oocytes (Fig. 1b) was expanded but was smaller, less yellow, and less mucoid than typical for the cumulus of a native oocyte. Of the 54 oocytes transferred via IFOT, 36 (67%) were recovered on aspiration of the follicle (Table 1). The rates of recovery of IFOT oocytes for the individual mares were 5/6, 7/12, 6/12, 9/12, and 9/12. Of the 36 recovered IFOT oocytes, 20 (56%) were found to be mature after denuding, compared to 22/45 (49%) of in vitro matured oocytes ($P > 0.1$). Overall, 20/54 transferred oocytes resulted in recovered MII oocytes (37%). After ICSI, there were no significant differences in cleavage (80% and 77% for IFOT and IVM oocytes, respectively) or blastocyst rates (25% and 18%, respectively; $P > 0.1$) between IFOT and IVM treatments.

For these five mares, the host follicle was measured before IFOT and then again 24 h after IFOT. Four of the mares had minimal reduction of follicle diameter (< 10%), and one mare (PS) had a marked (33%) reduction in follicle diameter (Table 2). The recovery rate of IFOT oocytes for mare PS was 50%, compared to 58%, 75%, 75%, and 83% for the other four mares. Notably, the maturation rate for IFOT oocytes from mare PS was 17%, compared to 44% to 86% for the other four mares. This difference in maturation rate (1/6 vs. 19/30) tended toward significance ($P = 0.07$).

Experiment 2. Embryo recovery after autologous or allogeneic IFOT (ovulatory stimulus at the time of transfer)

Ultrasonographic monitoring after IFOT demonstrated that the preovulatory follicle appeared to ovulate in 4/6 Self mares and in 5/6 Allo mares. Ultrasonographic images of follicles after IFOT are presented in Fig. 2. Two of five Self mares and three of five Allo mares showed a reduction in follicle size of > 10% on the day after IFOT; no IFOT-derived embryos were obtained from these mares (Table 3). One Allo mare ovulated

Table 1 Recovery and maturation rates, and cleavage and blastocyst rates after ICSI, for oocytes recovered after IFOT or matured in vitro

Treatment	No. recovered (%)	No. mature (%)	No. cleaved after ICSI (%)	No. blastocysts (%)
IFOT	36/54 (67%)	20/36 (56%)	16/20 (80%)	5/20 (25%)
IVM	NA	22/45 (49%)	17/22 (77%)	4/22 (18%)

IFOT intrafollicular oocyte transfer, *IVM* in vitro maturation

a secondary follicle. Host follicles that failed to ovulate after IFOT presented some characteristics of hemorrhagic anovulatory follicles (HAF; [20]) on ultrasonographic examination, in that they gained a thick echogenic rim as well as echogenic strands within the lumen (Fig. 2i); thus, we designated them HAF. Uterine flush was performed on the three mares that were classified as having developed HAF; none of these mares yielded any embryo.

One of four ovulating Self mares yielded an embryo (Table 3). Four of five ovulating Allo mares yielded at least one embryo. In Self mares, only embryos in excess of the number of ovulations were presumed to result from IFOT; thus, no Self mare was considered to have yielded embryos from IFOT. In Allo mares, genetic analysis for parentage identification demonstrated that two mares yielded embryos resulting from fertilization of transferred oocytes (IFOT embryos). One mare (FR) yielded one IFOT embryo after transfer of nine donor oocytes, as well as native embryos from each of two ovulations, and one mare (LD) yielded five IFOT embryos after transfer of nine donor oocytes (Fig. 3) and no native embryos.

Experiment 3. Embryo recovery after autologous or allogeneic IFOT (ovulatory stimulus before transfer)

The mean time of administration of ovulatory agents to Self mares was 7.7 h (range 7 to 8.75 h) and to Allo mares was 15.2 h (range 14.5 to 15.5 h) before IFOT. One Allo mare (SQ) presented two follicles > 30 mm at the time of IFOT. Oocytes from a different donor mare were transferred to either follicle (11 and 5 oocytes). One of five Self mares and none of three Allo mares that could be evaluated showed a reduction in follicle size of > 10% on the day after IFOT (Table 4). The time of ovulation of Self and Allo mares after IFOT is presented in Table 4. Of seven Self mares, five ovulated. In the other two Self mares, the preovulatory follicle developed into a HAF. All five Allo mares ovulated. The two mares that were classified as having developed HAF were examined by transrectal ultrasonography of the uterus 14 days later; neither were pregnant.

Of the five ovulating Self mares, one (MW) yielded two expanded blastocysts on uterine flush. This mare had been observed to ovulate only one follicle; however, after the embryos were recovered, review of videos of ultrasonographic examinations at the time of ovulation of the IFOT follicle revealed a second, 25 × 30-mm diameter, follicle present on the ovary. It is possible that the second embryo originated from ovulation of this secondary follicle, and thus, it was not considered to be an IFOT embryo.

Three of the five Allo mares yielded embryos, two of which included IFOT embryos (Table 4). The first mare to undergo embryo recovery in this experiment yielded two IFOT embryos and no native embryo on Day 8 after ovulation. One IFOT embryo was a morula (190.5 μm), and the other was a blastocyst of 528-μm diameter, similar in size to a Day 7 blastocyst [19]. Because these embryos appeared small for Day 8 after ovulation, subsequent embryo recoveries were performed on Day 9 after ovulation, to ensure that all embryos had time to enter the uterus before recovery was attempted. The second Allo mare that yielded IFOT embryos (SQ) was the mare in which oocytes were transferred to both of two large preovulatory follicles (31 and 35 mm). Both follicles had ovulated within 22 h after IFOT. Three blastocysts and an early morula were recovered from this mare on uterine flush. On parentage testing, one blastocyst was shown to be IFOT-derived, from one of the five donor oocytes transferred to the smaller-diameter preovulatory follicle. The other two blastocysts and the morula were found to be native embryos. The presence of the morula indicates a late third ovulation

Table 2 Results for parameters associated with oocytes recovered after IFOT, by mare, in Experiment 1

Mare	% Reduction in follicle size	No. oocytes recovered (%)	No. mature (%)	No. cleaved after ICSI (%)	No. blastocysts (%)
LY	5.1	5/6 (83%)	4/5 (80%)	4/4 (100%)	1/4 (25%)
BF	1.3	7/12 (58%)	6/7 (86%)	5/6 (83%)	1/6 (17%)
TN	8.1	9/12 (75%)	4/9 (44%)	3/4 (75%)	1/4 (25%)
PS	33	6/12 (50%)	1/6 (17%)	0	0
CH	−1.5	9/12 (75%)	5/9 (56%)	4/5 (80%)	2/5 (40%)

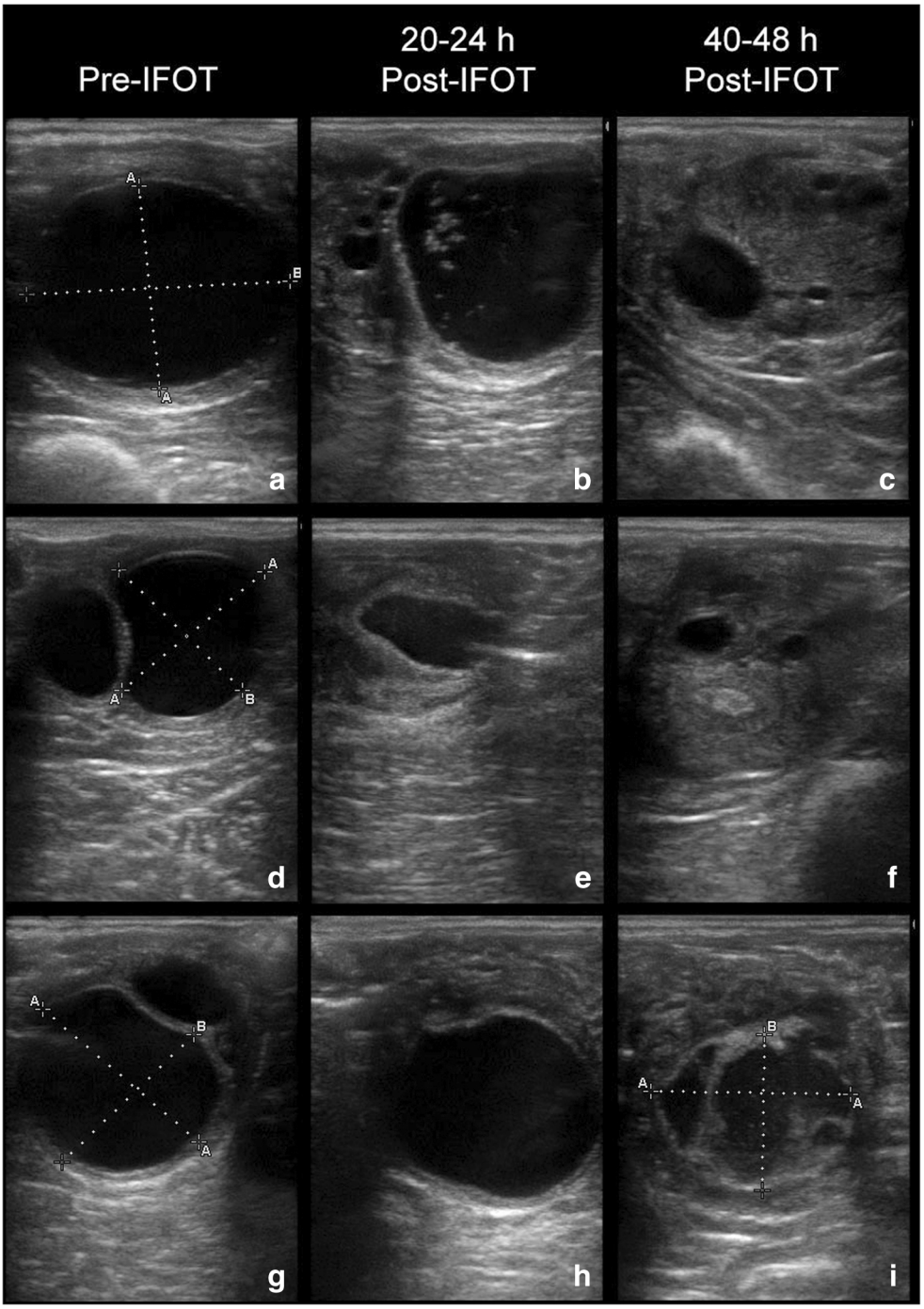

Fig. 2 Ultrasonographic images of follicle progression after IFOT. Appearance of follicles before IFOT (**a**, **d**, **g**), 20 to 24 h after IFOT (**b**, **e**, **h**), and 40–48 h after IFOT (**c**, **f**, **i**). **a**–**c** Minimal change in follicle diameter followed by ovulation. **d**–**f** Marked reduction in follicle diameter followed by ovulation. **g**–**i** Minimal change in follicle diameter followed by formation of an apparent hemorrhagic anovulatory follicle

from this mare. No other follicle > 20 mm was seen on the ovaries of this mare at the time of ovulation of the two large preovulatory follicles.

Experiment 4. Embryo recovery after allogeneic IFOT in host mares receiving the ovulatory stimulus 9 or 15 h before transfer

The mean time of administration of ovulatory agents to the 9-h group was 8.9 h (range 8.25 to 9.5 h) and to the 15-h group was 14.2 h (range 13.75 to 15) before IFOT. One mare (LB) in the 15-h group presented two follicles > 30 mm at the time of IFOT, and oocytes from one donor mare were divided and transferred into the two follicles (five and six oocytes). The time of ovulation of the mares in each group is presented in Table 5. In the 9-h group, 7/8 mares ovulated after IFOT, and in the 15-h group, 6/7 mares ovulated after IFOT. The remaining two mares developed HAF. The two mares that were classified as having developed HAF were examined by transrectal

Table 3 Results for parameters associated with embryo production for Self and Allo mares that ovulated after IFOT in Experiment 2

Type	Host mare	Size follicle at IFOT (mm)	No. oocytes transferred	% Reduction in follicle size	Ovulation time[a] (h)	No. follicles ovulated	No. total embryos recovered	No. IFOT embryos
Self	TN	37	3	41.9	18–43	1	0	0
	CS	33	7	36.4	40–42	1	0	0
	BO	42	5	9.5	24–40	1	0	0
	RX	35	2	5.7	29–43	1	1	0
Allo	FR	36	9	8.3	43–50	2	3	1
	LD	42	9	6	20–40	1	5	5
	MW	40	11	36.3	24–40.5	1	1	0
	PS	38	7	26	41–43	1	1	0
	CS	37	6	23	18–40	1	0	0

Self mare received autologous oocytes, *Allo* mare received allogeneic oocytes

[a] Ovulation of the IFOT follicle occurred between ultrasound examinations performed at these times. The ovulatory stimulus was administered at the time of IFOT

ultrasonography of the uterus 14 days later; neither were pregnant. In the 9-h group, 3/7 ovulating mares yielded embryos on uterine flush, and one of these mares yielded two IFOT embryos after transfer of 11 oocytes, from two donor mares, to one preovulatory follicle. The two IFOT embryos were from different donor mares. In the 15-h group, 5/6 ovulatory mares produced embryos, and one of those mares produced an IFOT embryo.

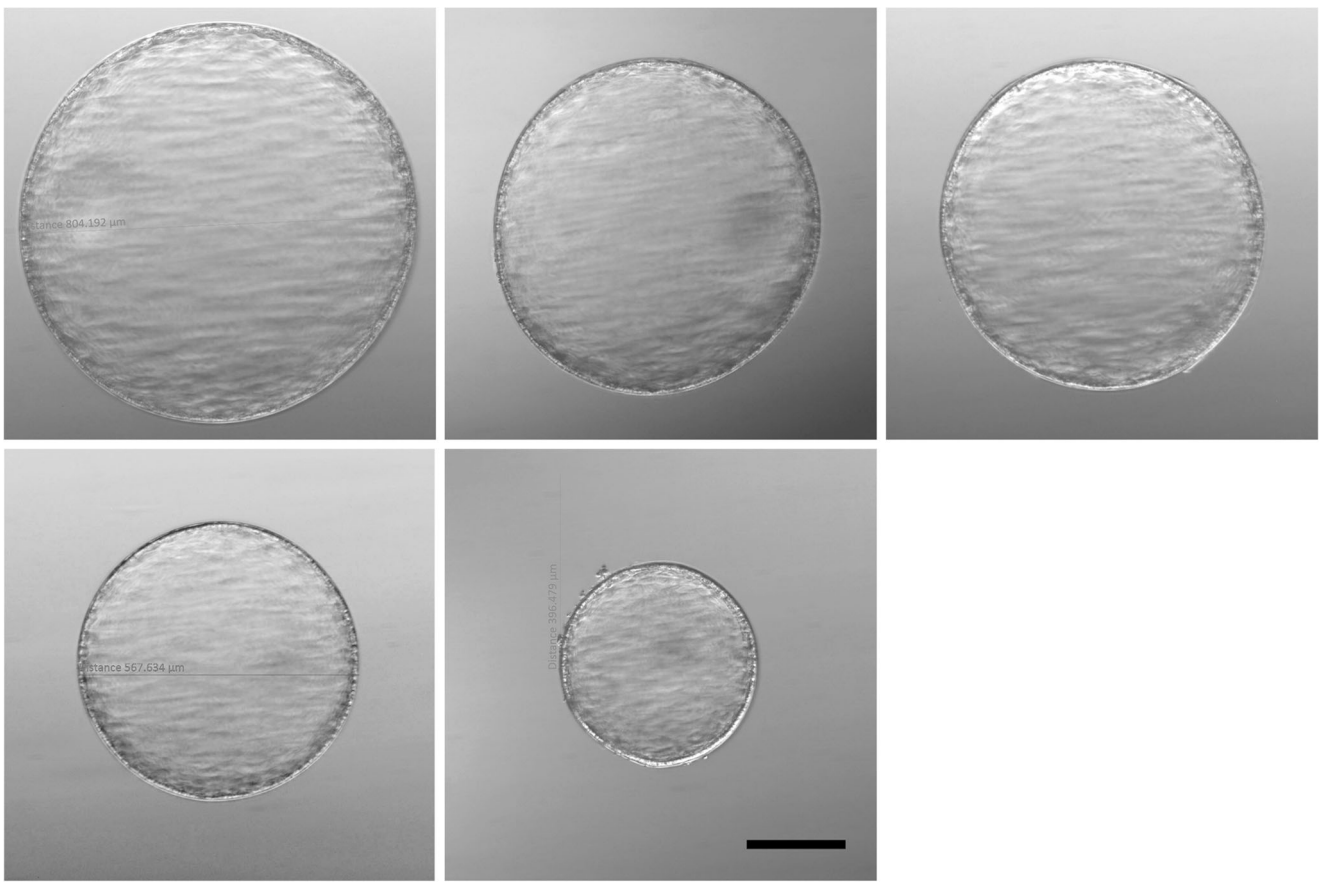

Fig. 3 Expanded blastocysts confirmed by parentage testing as having resulting from IFOT, recovered from the uterus of mare LD on Day 8 after ovulation (Experiment 2). The appearance of the follicle of this mare is shown in Fig. 2**a**–**c**. The mare yielded five IFOT embryos and no native embryos, after transfer of nine donor oocytes to the preovulatory follicle. Bar = 200 μm

Table 4 Results for parameters associated with embryo production after IFOT for the ovulating Self and Allo mares in Experiment 3

Type	Host mare	Size follicle at IFOT (mm)	No. oocytes transferred	% Reduction in follicle size	Ovulation time[a] (h)	No. follicles ovulated	No. total embryos recovered	No. IFOT embryos
Self	MW	41	2	18.5	23–39.5	2	2	0
	PS	35	9	− 2.9	28–41	1	0	0
	MM	45	7	7.9	22–38	1	0	0
	NY	39	4	1.3	20–40	1	0	0
	LD	34	3	− 7.5	19–40	1	0	0
Allo	BO	38	11	− 5.3	22–24	1	2	2
	LY	40	6	− 8.9	23–23.5	1	0	0
	CH	36	11	Ov	22.5–23	1	1	0
	SQ	35, 31	11, 5	Ov	4–22, 4–22	3	4	0, 1
	FG	36	4	− 1.4	23.5–26	1	0	0

Self mare received autologous oocytes, *Allo* mare received allogeneic oocytes, *Self* mares received ovulatory stimulus 8 h before IFOT, *Allo* mares received ovulatory stimulus 15 h before IFOT, *Ov* follicle appeared to be in the process of ovulating when evaluated on the day after IFOT

[a] Ovulation of the IFOT follicle occurred between ultrasound examinations performed at these times

Overall results

To evaluate overall efficiency of autologous and allogeneic transfers, data from Experiments 2 and 3 were combined. In these two experiments, the incidence of ovulation was 9/13 (69%) in Self mares and 10/11 (91%) in Allo mares. This difference was not significant ($P > 0.1$). Two of 13 (15%) Self mares yielded any embryos, vs. 7/11 (64%) Allo mares; this difference was significant ($P < 0.05$). None of the 13 Self transfers resulted in excess embryo production, vs. 4/11 (36%) Allo transfers resulting in parentage-verified IFOT embryo production ($P < 0.05$).

To evaluate the development of IFOT-derived embryos after fertilization, the diameters of confirmed native vs. IFOT embryos (Allo mares) in Experiments 2, 3, and 4 were compared (Table 6). For this analysis, the early native morula obtained from mare SQ in Experiment 3 was not included, as it was assumed to be from ovulation of a third follicle closer to the time of embryo flush. All other secondary follicles ovulated on the same day as did the host IFOT follicle. In uterine flushes performed at Day 8, the diameter of native embryos ($n = 4$) was 552.2 ± 113.6 μm (mean ± SD) and the diameter of IFOT embryos ($n = 8$) was 525.1 ± 179.4 μm. In flushes performed at Day 9, the diameter of native embryos

Table 5 Results for parameters associated with embryo production after IFOT for ovulating mares by treatment group in Experiment 4

Tx group[b]	Host mare	Size of follicle at IFOT (mm)	No. oocytes transferred	Ovulation time[a] (h)	No. follicles ovulated	No. total embryos recovered	No. IFOT embryos
9	GD	42	5	26–28.5	1	1	0
9	TU	38	11	26–30	1	1	0
9	SQ	39	19	25.5–30	1	0	0
9	FF	37	10	26–30.5	1	0	0
9	SK	35	11	27.5–28	1	3	2
9	LY	37	10	24–29	1	0	0
9	MW	38.5	13	34–46	1	0	0
15	LB	35, 42	5, 6	3–22.5, 3–22.5	2	1	0
15	LY	43	13	6–24	1	2	1
15	RX	34	10	22–24	2	2	0
15	HK	39	10	23	1	0	0
15	GD	37	11	23.5–27.5	1	1	0
15	FG	39.5	12	7–23.5	1	1	0

All mares received allogeneic oocytes

[a] Ovulation of the IFOT follicle occurred between ultrasound examinations performed at these times

[b] Ovulatory stimulus was administered 9 or 15 h before IFOT

Table 6 Diameter of native and IFOT embryos recovered from Allo mares in Experiments 2–4

Exp	Treatment	Host mare	Day embryo recovery	Native or IFOT embryo	Size of embryo 1 (μm)	Size of embryo 2 (μm)	Size of embryo 3 (μm)	Size of embryo 4 (μm)	Size of embryo 5 (μm)
2	Allo	FR	8	N	648.42	468.46			
	–	–	8	IFOT	402.11				
	Allo	LD	8	IFOT	804.19	656	655.12	567.65	396.48
	Allo	MW	8	N	440.3				
	Allo	PS	8	N	651.7				
3	Allo	BO	8	IFOT	190.50	528.46			
	Allo	CH	9	N	> 1800[a]				
	Allo	SQ	9	N	1862.49	1514.96	Morula		
	–	–	9	IFOT	755.82				
4	Allo 9-h	SK	9	N	1374.50				
	–	–	9	IFOT	1480.85	1070.79			
	Allo 9-h	GD	9	N	> 1200[b]				
	Allo 9-h	SQ	9	N	1732.6				
	Allo 15-h	GD	9	N	917.9				
	Allo 15-h	LY	9	N	769.7				
	–	–	9	IFOT	1986.95				
	Allo 15-h	RX	9	N	1800[a]	1345			
	Allo 15-h	LB	9	N	1920				
	Allo 15-h	FG	9	N	> 1900				

N native embryo, *IFOT* embryo originating from transferred oocytes, as confirmed by genetic analysis

[a] Embryo collapsed in dish

[b] Approximate size, embryo lost before measurement

($n = 12$) was 1511.4 ± 393.4 and the diameter of IFOT embryos ($n = 4$) was 1323.6 ± 532.6. There was no significant difference in embryo diameter between native and IFOT embryos at either day ($P > 0.1$).

Discussion

The results of this study confirm that immature oocytes can be transferred to the preovulatory follicle of mares, mature within the follicle, be ovulated from the follicle, and maintain meiotic and developmental competence. However, the cause of the poor repeatability of the IFOT procedure remains unclear, and currently, the procedure cannot be considered to be successful enough to be used clinically.

In previous studies in the horse [2, 4, 5], it was possible that the transfer method used could have affected the integrity of the host follicle. In these studies, the follicle was punctured, follicular fluid was aspirated from the follicle, then the oocytes were delivered through the needle apparatus into the follicle, and the apparatus flushed with follicular fluid. A relatively large-gauge needle was used (15 to 18 ga). The large diameters of these needles, and the manipulations involved, could have caused trauma to the follicle or leakage of follicle contents. We hypothesized that use of a smaller-gauge needle in which oocytes were loaded in a minimal volume at the tip, similar to that used in recent bovine studies [6, 16], would decrease damage to the follicle and potentially increase the repeatability of the procedure. However, this was not the case, as the incidence of HAF formation was not markedly lower in the current study (11.5%, vs. 15.8% and 20% [2, 5]) nor was the percentage of mares providing IFOT embryos higher. In mares that did ovulate, the IFOT procedure did not seem notably to perturb follicle dynamics, as native and/or IFOT embryos were recovered from 65% of ovulating Allo mares. Nevertheless, there was an indication that follicle damage was related to outcome. In Experiment 1, the one mare with a marked reduction in follicle diameter (33%) provided the lowest oocyte recovery (50%) and by far the lowest maturation rate (17%). In Experiments 2 and 3, in Allo mares for which follicle diameter could be evaluated the day after IFOT, 3/8 mares demonstrated a marked reduction in follicle diameter (23–36%). None of these mares yielded IFOT embryos, vs. 3/5 mares demonstrating minimal (< 10%) loss in follicle diameter. We used the smallest-gauge needle available for our apparatus (20 ga); while speculative, it is possible that use of an even smaller-gauge needle could help to decrease damage to the follicle and potentially increase IFOT embryo production.

The results of Experiment 1 confirmed that immature oocytes transferred into the preovulatory follicle underwent nuclear maturation within the follicle; however, there was no significant advantage for the IFOT (in vivo) system over our in vitro maturation system (56% vs. 49% maturation, respectively). Goudet et al. [4] reported a nuclear maturation rate for recovered IFOT oocytes of only 24%; however, oocytes matured in vitro in that study also had an atypically low maturation rate (3/12, 25%), making these findings difficult to interpret.

In Experiment 1, the ovulatory stimulus was administered at the time of IFOT, and oocytes were recovered from the follicle 30 h later. These oocytes demonstrated developmental competence, as evaluated by blastocyst development after ICSI, equivalent to that for IVM oocytes matured for 30 h. In Experiment 2, we also administered the ovulatory stimulus at the time of IFOT and allowed the mares to ovulate. However, in this case, ovulation would typically have occurred 38–42 h after IFOT [8–10] and had occurred by 50 h in all ovulatory mares in that study. We hypothesized that the lower than expected rate of IFOT embryo production (0/7 Self mares and 2/5 Allo mares) in Experiment 2 may have been related to the prolonged time the transferred oocytes resided in the follicle. Thus, in Experiment 3, we administered the ovulatory stimulus before IFOT in an attempt to shorten the time that the oocytes resided in the follicle. In this experiment, for Allo mares, we collected COCs the day before IFOT and held them overnight, to replicate the clinical situation in which oocytes might be shipped overnight to a center that performs IFOT. We have previously shown that oocytes recovered by TVA and held overnight have equivalent blastocyst rates after ICSI when matured in vitro for 24 or 30 h (33% and 33% per injected oocyte; [11]). Thus, we administered the ovulatory stimulus to Allo mares 15 h before IFOT to result in ovulation approximately 24 h after IFOT. Self mares continued to receive their own COCs immediately after recovery, to limit the possibility that post-aspiration luteinization of follicles could interfere with normal development and ovulation of the IFOT follicle. Because oocytes matured immediately after collection (i.e., not held overnight) may take longer to mature [12], these mares were scheduled to receive the ovulatory stimulus 9 h before IFOT in an effort to have them ovulate approximately 30 h after IFOT. However, IFOT embryo production was still low in both groups. In Experiment 4, we directly compared administration of the ovulatory stimulus 9 or 15 h before IFOT in Allo mares and again found no difference between treatments. Although these results provide the first critical evaluation of the effect of time of administration of ovulatory stimulus on embryo production in IFOT, through all three experiments, there was no apparent relationship of ovulatory timing to the efficiency of the procedure. The mare providing the highest number of IFOT embryos (five embryos from nine transferred COCs) was in Experiment 2; this mare received oocytes immediately after their recovery from the donor mare and received the ovulatory stimulus at the time of IFOT. It is possible that placement of the oocytes into the follicle hours after the administration of the ovulatory stimulus caused them to miss important signaling, either from the stimulus itself or from signals from the mural granulosa cells in response to that stimulus. For example, mRNA for growth differentiation factor 9 and bone morphogenetic protein 15, factors associated with increased oocyte developmental competence, and mRNA for phosphodiesterase 3A, a factor important in release of the oocyte from meiotic arrest, have been shown to rise in native equine oocytes within 6 h of administration of recombinant equine LH [21].

The similarity in size of recovered native and IFOT embryos suggests that embryo development proceeded at a consistent rate between the two oocyte types. This lack of difference in embryo growth suggests that IFOT oocytes gained normal developmental competence during final maturation within the follicle and were fertilized at approximately the same time as the native oocyte.

One aspect of the procedure that is difficult to ascertain is whether the transferred oocytes are ovulated normally from the follicle at the time of ovulation. The COCs in our study were recovered from immature follicles by follicle aspiration, which in the horse results in collection of oocytes with only a few layers of cumulus (Fig. 1a) [22]. It is possible that lack of cumulus may impair adherence of the COC to the follicle wall after injection, as is seen in oocytes transferred to intact follicles in vitro [23] and which could be important for the follicle environment to convey enhanced developmental competence to the oocyte. An associated possible scenario is that the cumulus does not attach to the follicle wall, and the transferred COCs are free floating in the follicle; this possibility is supported by the finding that all oocytes recovered in Experiment 1 were found in the initial aspiration of follicular fluid, rather than in the fluid from follicular flushing. As most follicular fluid appears to be lost into the peritoneum at ovulation in the mare [24] and the (native) COC appears to leave the follicle after most fluid has been expelled [25], it is possible that transferred free-floating COCs could exit with the initial fluid and be lost into the peritoneum. Decreased cumulus size may also hinder capture of the oocyte by the oviduct during ovulation. Alternatively, the cumulus of the transferred COC may adhere too strongly or not detach appropriately, or be adhered in the wrong location, to be released from the follicle at ovulation.

Genetic analysis, performed here on embryos after IFOT for the first time, allowed accurate interpretation of parentage for embryos from Allo mares. Of the 15 Allo mares yielding embryos, 2 mares gave IFOT embryos only, 4 mares gave both IFOT and native embryos, and 9 mares gave only native embryos. As past reports have counted all "excess" embryos as resulting from IFOT, it is notable that in two cases in the

current study (one Self and one Allo), native embryos in excess of the number of recorded ovulations were recovered on uterine flush. In one of these cases (Mare MW), review of ultrasound videotapes taken at the time of ovulation of the IFOT follicle revealed a second large follicle present on the ovary. In the second case (Mare SQ), no additional periovulatory follicle was seen. From the early stage of development of this native embryo, it likely originated from an ovulation that occurred about 4 days after ovulation of the IFOT follicle.

Self IFOT would be beneficial for clinical use, as the mare serves as her own host and thus all embryos recovered would originate from the desired mare. However, in our study, no Self transfers yielded excess embryos. This result appears to be different from that reported by Deleuze et al. [5], in which 4/10 mares undergoing autologous transfer yielded excess embryos. As similar management was used for Self transfers in the two studies, the reason for this difference in result is not immediately apparent.

For clinical use, allogeneic transfer offers multiple benefits. Oocytes can be transferred immediately (as in Experiment 2) or held overnight before transfer (as in Experiments 3 and 4) as might be necessary for shipment to a central facility. Notably, use of an allogeneic host mare allows IFOT to be performed to produce embryos from donor mares with compromised reproductive tracts. To avoid recovery of unwanted native embryos, a host mare could be used whose embryo by the chosen stallion would also be valuable. Our data indicates that the probability of recovering the host mare's native embryo is essentially normal, even if IFOT-derived embryos are not recovered. Recovered embryos could undergo biopsy for parentage testing before transfer to recipients [18], or parentage could be determined after foals are born.

Overall, 6/26 Allo mares provided IFOT-derived embryos. A total of 236 allogeneic COCs were transferred, and 12 IFOT embryos were recovered (5.1% per transferred COC). Thus, while IFOT offers a method for production of embryos from isolated oocytes of mares in the absence of resources for in vitro embryo production, at the current rate of repeatability, IFOT does not appear to be successful enough for clinical use. An alternative procedure is oocyte transfer (surgical transfer of mature COCs to the oviducts of inseminated recipient mares), which has been shown to be effective for clinical production of foals [26, 27]. If surgery is possible, and especially if in vitro maturation can be performed, oocyte transfer may offer a more effective alternative for foal production from isolated oocytes in the absence of sophisticated laboratory facilities.

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