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Embryo Collection, Transfer and Pregnancy of Riding Horses : First Successful Case in Korea

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ABSTRACT

Embryo transfer (ET) could be a relevant tool for genetic improvement programs in horses similar to those already underway in other species and produce multiple foals from the same mare in one breeding season. However, there have been no reports describing equine embryo transfer performed in Korea. In the present study, we performed an equine embryo collection and transfer procedure for the first time. We examined the embryo collection and pregnancy, size of embryo during the incubation period after collection, and progesterone (P4) and estradiol-17ß (E2) concentrations in mare's serum at embryo collection and transfer. A total of 16 donors responded to estrus synchronization; estrus was induced in 12 donors and 4 recipients, and artificial insemination was successful in 10 donors and six blastocysts were collected from donors. Of these blastocysts, we monitored the size of blastocysts for 3 day during incubation and transferred 2 blastocysts to a recipient, with 1 successful pregnancy and foal achieved. The dimensions of equine embryo at day 7 to day 9 were 409 μm, 814 μm and 1,200 μm. The serum P4 and E2 concentrations were 7.91±0.37 ng/μL and 45.45±12.65 ng/μL in the donor mare, and 16.06±3.27 ng/μL and 49.13±10.09 ng/μL in the recipient mare.

(Key words: Equine, Embryo collection, Embryo transfer, Progesterone, Estrogen)

INTRODUCTION

The first successful horse offspring produced by embryo transfer (ET) was reported by Oguri and Tsutsumi (1974). During the past 40 years, ET has been successfully adapted for use in horse reproduction. The international Embryo Transfer Society newsletter committee on statistics suggested that 41,652 embryo collections and 27,497 transfers were reported for 2010 (Stroud and Callesen, 2012). ET could produce a multiple foals from the same mare in one breeding season (Coutinho, 2008) and a relevant tool for genetic improvement programs in horses similar to those already underway in other species. With this technique, embryos can be collected from mares still active in competition and multiple foals can be produced from mares with high genetic value. ET also helps maximize the production of foals from mares unsuitable to carry a pregnancy to term (Squires et al., 2003).

Several variables influence the success of ET. The rates of embryo recovery and pregnancy are influenced by the recipient and donor mare (Hinrichs and Choi, 2005). The synchronization of ovulation between the donor and recipient is fundamental. The commonly accepted and used protocol is to induce recipient ovulation 1 day before and 3 days after the donor's ovulation (Carnevale et al., 2000; Jacob et al., 2012). Synchrony between embryonic age and the uterine environment is essential for pregnancy due to progesterone (P4)-induced changes in the uterine environment (Kaercher et al., 2013). The success of ET is also influenced by the transfer method, the technician's skill; size, age, and morphology of the embryo, season of year, embryo storage, and age and reproductive history of the donor (Clark et al., 1987; Squires et al., 1999; Carnevale et al., 2000; Squires et al., 2003; Hinrichs and Choi, 2005).

To the best of our knowledge, there have been no reports

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describing equine ET performed in Korea. In the present study, we successfully performed an equine embryo collection and transfer procedure for the first time. We examined the embryo collection and pregnancy, size of embryo during the incubation period after collection, and P4 and estradiol-17ß (E2) concentrations in mares at embryo collection and transfer.

MATERIALS and METHODS

Animals

The experiment was conducted at Korea Racing Authority Jangsu Farm and Jeju Aewoul Farm, Korea. Sixteen donor and four recipient mares aged 3 - 7 years were used. Mares had no reproductive tract abnormalities, as confirmed by ultrasonography (HS-1500, Honda, Japan). Their estrous cycles were monitored using ultrasonography in order to synchronize ovulation between the donor and recipient mares. The desired synchronization was achieved by administering 5.0 mg/head of dinoprost trimethamine (Lutalyse, Pfizer, USA) as a luteolytic agent and 2,500 IU/head human Chorionic Gonadotropin (hCG, Daesung, Korea) as an ovulatory agent. Mares that were selected to be a recipients or donors had acceptable uterine and luteal echodensity and uterine and cervical tone compatible with diestrus.

Monitoring ovarian activity

Ovarian activity of the mares was monitored ultrasonographically using a 5-0 MHz linear transducer. During estrus, the ovaries were scanned daily to determine the day for ovulation induction and day of ovulation, designated as day 0. Criteria used to determine the time of induction were as follows: (1) presence of follicle(s) with a diameter ≥35 mm; (2) relaxed uterine and cervical tone; and (3) endometrial edema consistent with estrus. Uterine edema was scored on a scale of 1 (minimal) to 4 (maximal), as described in previous literature (Samper, 2009).

Stallions and artificial insemination

A warmblood stallion aged 7 years and of proven fertility was used. Semen was collected using standard procedure with a Colorado model artificial vagina for horses (Colorado AV, ARS, USA). After collection, the gel fraction was removed and the semen was filtered through sterile filter. Semen from the stallion was evaluated to determine progressive motility and concentration was determined with a CASA (SpermVision, Minitube, Germany). Inseminations were performed using cooled semen containing a minimum of 100 $\times 10^6$ viable sperm, approximately 24 h after induction of ovulation. If ovulation did not occur after administration of hCG, then the mare was inseminated again 16h after the first insemination procedure.

Collection and evaluation of embryos

The embryo collection procedure was performed according to the guidelines described previously (Jacob et al., 2012), with some modifications. Uterine flushes for embryo recovery were performed at day 7 post-ovulation using nonsurgical uterine lavage. Mares were restrained and the perineum cleaned with mild soap and povidone/iodine solution. Then, a 32-French silicon Foley catheter (Vortech, Agtech, USA) was inserted into the vagina and through the cervix, guided manually with the index finger. Once in the uterine body, the cuff on the end of the catheter was inflated with 60 mL of air, and the catheter was pulled caudal to ensure a tight seal against the internal os of the cervix. Embryo collection solution (Embryo collection medium, Agtech, USA) was infused through the catheter and into the uterus and immediately drained into a collection cup fitted with a 75-μm filter (ECE056, Bioniche Animal Health, Canada). This procedure was performed 3 times. During the third flush, the uterus was massaged transrectally to boost suspension of the embryo into the flushing medium and to remove as much fluid as possible from the uterus. After the uterus was drained, the air cuff was deflated, and the catheter was removed from the mare. The approximately 50 mL of the solution remaining in the collection cup was placed in a petri dish and analyzed under a stereomicroscope at 40× magnification. The retrieved embryos were washed 5times each in embryo holding medium (Holding & Transfer medium, Agtech, USA) to remove cellular debris. Embryos were then incubated in TCM199 +5% fetal calf serum in a 37°C, 5% CO_2 and maximum humidified incubator. On each day of incubation, the embryo diameter was measured using Eclipse microscope system (Ni, Nikon, Japan).

Embryo transfer

Embryos were transferred using a nonsurgical technique. Embryos were drawn into a straw (0.5 mL) and placed into a disposable artificial insemination (AI) gun to prevent contamination. Recipient mares were chosen to receive

embryos based on the stage of reproductive synchronization, with preference given to mares within the +1 to -1 window of synchrony. Before transfer, each washed embryo was placed into a sterile AI pipette (Insemination unslit sheaths, Continental, USA) with a minimum volume (0.5 mL) of holding medium. Uterine contamination was minimized by enclosing the AI pipette in a sterile sheath. The pipette was passed transvaginally through the cervix while performing transrectal palpation to ensure that the pipette had entered the uterine body. After entry into the cervix confirmed by placing the index finger and pipette/sheath into the cervical lumen, the sheath was slowly pulled caudally while the pipette remained in place. The embryo was deposited into the uterine horn, and the pipette and sheath were withdrawn.

Pregnancy diagnosis

Pregnancy in recipient mares was evaluated using transrectal ultrasonography. Examinations were performed 15 and 45 days post-ovulation.

Determination of P4 and E2

From both recipients (*n*=4) and donors (*n*=4), blood samples (jugular vein) were collected at embryo collection in donors or at 7 day after ovulation in recipients. P4 and E2 concentration were analyzed by radioimmunoassay as described previously with some modification (Park et al., 2002). After collection, the blood plasma was removed by centrifugation at 1500 x g for 20 minutes and kept at -20℃ until analysis. The samples were analyzed using Coat-A-Count Progesterone(Simens, USA) and Coat-A-Count Estradiol(Simens, USA) by Gamma10(Sinjin, Korea) followed manufacturer's manual.

Statistical analysis

Difference in proportions of embryo recovery rates was analyzed using χ^2 analysis. P4 and E2 concentrations were analyzed using mean ±SE and *t*-test. Significance was defined at $P < 0.05$.

RESULTS

A total of 16 donors responded to estrus synchronization; estrus was induced in 12 donors, and artificial insemination was successful in 10 donors (Table 1). Six singleton blastocysts were collected from the 10 successful donors. Of these blastocysts, we monitored the size of 3 during incubation and transferred 2 blastocysts to a recipient, with 1 successful pregnancy and foal achieved. The dimensions of equine embryo at day 7 to day 9 were 409 µm and 1,200 µm (Fig. 1). The serum P4 and E2 concentrations were 7.91±0.37 ng/mL and 45.45±12.65 pg/mL in the donor mare (*n*=4), and 16.06±3.27 ng/mL and 49.13±10.09 pg/mL in the recipient mare (*n*=4) (Table 2). P4 concentration was significantly differ between donor and recipient mares (p<0.05).

DISCUSSION

It is current practice in the equine breeding industry to recover an embryo by uterine flush 7 or 8 days post-ovulation. The equine embryo remains in the oviduct and usually does not enter the uterus until day 6 (Freeman et al., 1991). Flushing before day 7 may result in a lower recovery rate (Jacob et al., 2012; Battut et al., 1998; Freeman et al., 1991). Embryo recovery rates were similar on days 7, 8, 9, and 10 and ranged between 56 – 66% (Jacob et al., 2012). Our current findings agree, as the Day 7 recovery rate (60%) was similar recovery rate compared that of previous studies. The embryo recovered on days 7 or 8 post-ovulation was either be immediately transferred into a recipient or cooled for short-term storage. Morphology and suitability of embryos for transfer are evaluated before ET.

Examining donor and recipient mares to determine ovulation dates and degrees of synchrony is regarded as one of the most time-consuming activities performed in an ET

Table 1. The result of estrous induction, artificial insemination, and embryo collection in equine

No. of Donors	No. of Donors Induced Estrus	NO. of success Artificial Insemination	No. of Collected Embryo
16	12	10	6

program (Riera, 2009). In most equine embryo transfer programs, the generally accepted optimal time to perform an embryo transfer, based on the date of the donor mare's ovulation, is into a recipient that has ovulated 1 day before through 3 days after the donor mare (Jacob et al., 2012; Stout, 2006). In this experiment, we synchronized recipient and donor within 1 day and obtained a 50% pregnancy rate, but the number of cases evaluated was so small that further study is needed.

The pregnancy rate after ET is influenced by embryo grade and development stage, uterine tone, and mare body condition grades (Clark et al., 1987; Jacob et al., 2012). However, the age of recipient, freshness of embryo, cooling and transportation did not affect pregnancy rate (Carnevale et al., 2000; Carney et al., 1991)., A higher pregnancy rate was observed using day 7 embryos than 6-, 8-, 9- and 10-day embryos (Jacob et al., 2012; Wilsher et al., 2006). However, Carnevale et al.(2000) and Jacob et al.(2012) reported that pregnancy rates were similar for recipients mares that received an embryos on Day 5 through Day 9 (42% to 56%). Equine embryos with average diameters of 0.208 to 2.200mm can be recovered from the uterine lumen from Day 6 to Day 9 postovulation (Squires et al., 2003). The embryo increases significantly in size after it enters the uterus. The large embryos (>1,000μm) can be achieved to a good pregnancy (Fleury et al., 2001), but other report suggested that embryos over 1,200μm in diameter survived much lower than other reports (Camargo et al., 2013).

Most mares selected as embryo recipients were between 3 – 10 years of age. In a previous study, pregnancy rates for recipients aged 2 – 9 and 10 – 18 years were not significantly different (Carnevale et al., 2000). However, embryo death

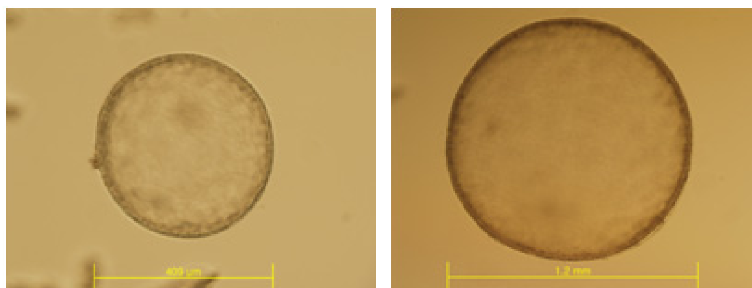

Fig. 1. Equine embryo blastocyst flushed and cultured at day 7(left), and day 9(right)

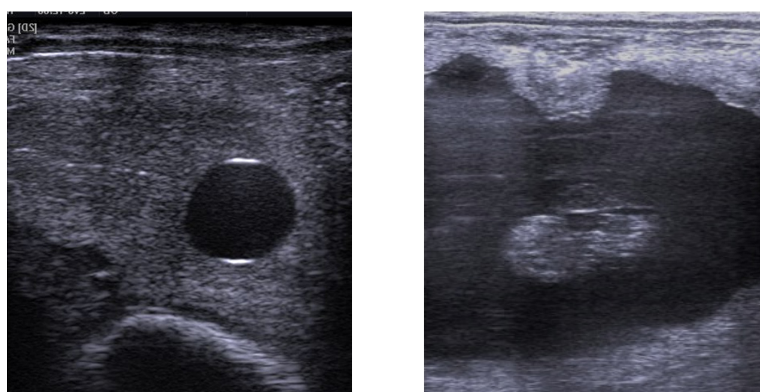

Fig. 2. The ultrasound images of pregnancies at day 15 (left) and day 45 (right) after embryo flushed and transfer (day 7) in equine

Table 2. Serum progesterone and estradiol concentrations during embryo collection and transfer

	Progesterone (ng/mL)	Estradiol (pg/mL)
Donor	7.91±0.37[b]	45.45±12.65
Recipient	16.06±3.27[a]	49.13±10.09

[a,b] Means in columns±SE with different superscripts differ ($p < 0.05$)

tended to be higher for the older group of recipients. Reduced uterine tone and quality scores for recipients were associated with reduced pregnancy rates and tended to be associated with increased embryo loss rates (Carnevale et al., 2000). Recipients that were older (≥10 years) or poor to fair uterine tone on day 5 tended to have more pregnancy loss than younger (≤9 years) recipients and recipients with good to excellent uterine tone. Uterine quality is associated with embryonic death.

The synchrony between embryonic age and the uterine environment is essential for pregnancy because progesterone (P4) changes the uterine environment. Exogenous P4 has been used to improve uterine tone in mares (Mckinnon et al., 1988), which induced thickening and uterine tone characteristic of diestrus (Backman et al., 2004). Circulating P4 concentrations were higher in mares that qualified as recipients than in those that failed to qualify (10.0±4.2 and 6.5±4.3 ng/mL); therefore, lower circulating concentrations of P4 appeared to correlate with reduced uterine and cervical tone (McCue et al., 1999). Recipients with a fair to poor uterine tone may have a lower circulating concentration of P4 that affects the uterine tone (Backman et al., 2004) and pregnancy maintenance (Vanderwall, 2011). Continued production of P4 by the corpus luteum allows secretion of uterine-specific protein (Sharp and McDowell, 1985) that may be important for pregnancy maintenance. However, direct comparison to other ET programs is difficult because of variability in donors, embryo quality, and embryo manipulations.

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