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In Vivo Embryo Production in Horse: First Successful Case in Indonesia

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ABSTRACT

In Indonesia, equine breeding has been largely limited to natural mating and artificial insemination (AI). Embryo transfer (ET) offers a more efficient reproductive approach by enabling the production of multiple offspring per year from genetically superior mares. This study evaluated the potential of *in vivo* embryo production in horses in Indonesia by examining the effects of embryo collection timing, oxytocin administration, and the duration of dominant follicle development on embryo recovery rates. Donor mares were selected based on physical examination and ovarian activity. Estrous cycles were synchronized using hormonal manipulation, and the estrous period was monitored by assessing ovarian follicular development and ovulation timing. AI was performed, and embryos were collected by uterine flushing on days 5, 6, and 7 after ovulation. No embryos were recovered on day 5, suggesting that embryos had not yet entered the uterus. In contrast, collections on days 6 and 7 resulted in recovery rates of 66.7% on both days, yielding embryos at the early blastocyst and blastocyst stages, respectively. Improved outcomes were observed in mares with dominant follicle development of ≤4 days and those receiving oxytocin. However, mares with mild reproductive disorders, such as endometritis, failed to produce embryos regardless of treatment. These results indicate that successful embryo recovery in horses depends on accurate collection timing, optimal follicular dynamics, and good uterine health of donor mares.

Keywords: embryo collection; embryo recovery; follicle development; mare; oxytocin

INTRODUCTION

Horses have long played an important role in human society and are now used more frequently in sports rather than for transportation. Breeding programs aim to produce genetically superior offspring; however, horses reach puberty relatively late. Mares reach peak fertility between 5 and 7 years of age, followed by a significant decline after 15 years of age (Masko *et al*., 2018; Rizzo *et al*., 2018). Considering an average equine lifespan of 25–30 years, mares may successfully produce healthy foals until 20 years of age or in some cases, later. Nevertheless, fertility rates decline significantly in mares older than 20 years, with a progressive annual reduction, indicating that reproductive success is highly individual-specific (Scoggin, 2015). This age-related decline in fertility is primarily associated with diminished oocyte quality rather than impaired uterine function (Arango *et al*., 2019). Reproductive disorders such as silent estrus, conception failure, and early embryonic loss further reduce reproductive efficiency (Marteniuk *et al*., 1998). These challenges are exacerbated in elite sport mares, in which athletic careers often delay genetic conversion efforts beyond the optimal reproductive period. Factors including performance-related stress, elevated body temperature, the use of estrus-suppressing hormones, and difficulties transitioning from athletic to reproductive roles all contribute to reduced fertility (Elhay *et al*., 2007).

In Indonesia, a tropical region, equine breeding practices have traditionally been limited to natural mating and artificial insemination (AI). Several ongoing challenges persist, particularly the breeding of elite mares at advanced ages, when reproductive performance has already declined. Furthermore, mares with injuries or reproductive constraints often have reduced ability to conceive or carry pregnancies to term, thereby limiting their overall reproductive potential.

Assisted reproductive technologies, particularly embryo transfer (ET), provide effective solutions to these challenges. ET allow genetically valuable mares to maintain their athletic careers while contributing to genetic improvement within the equine population. Although AI shows relatively high success rates in

Indonesia, its overall efficiency remains limited. In contrast, ET is a more efficient reproductive strategy because it enables the production of multiple offspring per year from genetically valuable mares through *in vivo* embryo production, uterine flushing, and transfer to recipient mares. Successful outcomes depend on the precise timing of insemination and embryo flushing, as well as oxytocin administration to enhance uterine contractions (Gutjahr *et al*., 2000). Furthermore, flushing techniques and other procedural factors may influence embryo recovery. However, the primary determinants of successful embryo recovery from the uterus have not yet been clearly defined (Ayala *et al*., 2024).

Follicular development is influenced by seasonal variation in day length. In equatorial regions such as Indonesia, day length remains relatively constant throughout the year, resulting in minimal seasonal effects and allowing year-round breeding. Despite this advantage, *in vivo* embryo production in horses remains less successful than in cattle due to greater physiological variability and a less predictable superovulatory response (Carnevale & Ginther, 1992). This study represents the first application of equine ET in Indonesia. Accordingly, it aimed to optimize *in vivo* embryo production by evaluating embryo recovery rates under local conditions.

MATERIALS AND METHODS

Ethical Approval

This study was conducted at the Reproductive Rehabilitation Unit, School of Veterinary and Biomedical Medicine, Bogor Agricultural University, between January 2024 and March 2025. Ethical approval was obtained from the Ethics Commission (No. 262/KEH/SKE/X/2024).

Donor Mare Selection

Seven donor mares were included in this study, comprising one Warmblood, one Thoroughbred, two Sumba, and three KPI mares. The mares were 4–5 years old and selected based on overall health and reproductive suitability. Reproductive health screening included a physical examination and ultrasonographic evaluation. Physical examination confirmed normal external reproductive anatomy, including appropriate conformation of the vulvar labia, rectovulvar and rectovaginal areas, and cervix.

Ultrasonography was used to verify that the ovaries were cyclically active and that the vagina and uterus were free of abnormalities. Ovarian activity was confirmed by follicular development and ovulation, as indicated by the presence of a corpus luteum.

Nutrition and Housing Management of Mares

This study was conducted in Bogor, West Java, Indonesia (31.1704˚ N, 72.7097˚ E), which has a tropical climate. All mares received balanced nutrition consisting of a complete feed provided at 2% of their body weight, with a forage-to-concentrate ratio of 3:2, and *ad libitum* access to fresh drinking water. Mares were housed in individual stalls and managed under regular deworming and vaccination programs. Sanitary and hygienic conditions of housing facilities, feeding areas, and water troughs were consistently maintained. Electric fans were installed in stalls when needed to mitigate heat stress, and ambient temperature was maintained between 25 °C and 27 °C.

Mares Donor Preparation

Donor mare synchronization was achieved using prostaglandin F2α (PGF2α) containing dinoprost (Lutalyse® 12.5 mg/mL, Zoetis, Ireland), administered on day 7 after ovulation or when the corpus luteum reached a maximum diameter of 2.5 cm. Ovulation was confirmed by transrectal ultrasonography. The response to prostaglandin treatment is typically characterized by the appearance of estrus signs within 2–3 days after injection, depending on the stage of the estrous cycle and individual ovarian condition. Mares that enter estrus following spontaneous luteolysis were classified as having natural estrus.

All mares were monitored via ultrasonography to determine the optimal timing for insemination. Examinations were performed using a linear transducer (Edan® DUS 60 VET, 100–240 V, 50/60 Hz) operating at 7.5 MHz. Ovarian monitoring included assessment of corpus luteum presence, number and size of developing follicles, number of ovulating follicles, and duration of preovulatory dominant follicle development. Monitoring began 3 days after prostaglandin administration and continued throughout estrus until day 1 after ovulation.

Intensive ultrasonographic monitoring started when the follicular diameter reached 3 cm and continued until ovulation of the preovulatory dominant follicle. When the dominant follicle reached a diameter of 40 mm and moderate to obvious endometrial edema was observed, donor mares received human chorionic gonadotropin (hCG) (Chorulon® MSD Animal Health, Unterschleissheim, Germany) at the time of insemination. If ovulation had not occurred, hCG administration was repeated every 24 h.

The duration of preovulatory dominant follicle development was analyzed based on changes in follicle diameter and ovulation timing. Ovulation was identified by the disappearance of the preovulatory follicle and the subsequent formation of a hyperechoic structure at the same site, known as the corpus rubrum. Uterine status was further evaluated by examining estrus mucus and its ultrasonographic pattern. During estrus, the uterus exhibited a characteristic “cart wheel” appearance with a thickened endometrium, while the presence of diffusely patterned uterine mucus indicated the end of estrus.

Artificial Insemination

Donor mares were inseminated with chilled semen from a single 4-year-old Warmblood stallion.

Fresh semen was first evaluated to ensure high quality before processing. Semen assessment included both macroscopic and microscopic examinations. Chilled semen was prepared at a concentration of 500 × 10^6 spermatozoa with a minimum progressive motility of 60%. The semen extender used was skim milk supplemented with penicillin-streptomycin antibiotics.

Insemination was performed deep into the uterine horn to maximize success, following protocols from previous studies (Hannan *et al*., 2020). Mares were inseminated during estrus, confirmed by ultrasound and behavioral signs. Insemination was initiated when the dominant follicle reached a diameter of 4.0 cm and continued until ovulation occurred.

Embryo Collection

Embryos were collected using the uterine flushing technique on days 5, 6, and 7 post-ovulation. Mares were restrained in a horse stock and sedated intravenously with detomidine at 0.02–0.04 mg/kg. Oxytocin (Intracin® Interchemie Holland, Waalre, Netherlands) was administered intramuscularly at a dose of 50 IU per mare in selected groups. An embryo flushing catheter (CH-28, ref. 19909/0032, Minitub GmbH, Germany) connected to a Y-junction with a high-flow connector spike port (19982/0202 Mintube USA) was used. A balloon cuff with a maximum capacity of 100 mL was inserted intravaginally and positioned inside the cervix to secure the catheter. The flushing medium consisted of lactated Ringer's solution enriched with estrous mare serum and penicillin-streptomycin antibiotics, warmed to a stable temperature of 37°C. The uterus was flushed with up to 1 liter of this medium. The solution was automatically expelled by gravity into the Emsafe embryo collection system (Ref. 19010/600 Minitub GmbH, Germany). This flushing procedure was repeated up to three times for each embryo collection session.

Collection and Vitrification of Embryos

Embryos were collected by filtering the flushing medium through a gridded Petri dish with a 65-µm mesh, suitable for capturing embryos of various sizes. Embryo identification and assessment of developmental stage and quality were performed using a stereomicroscope. Embryos were then immediately vitrified using the fast-freezing method (vitrification) based on Haq (2019).

First, embryos were equilibrated in a solution containing 10% ethylene glycol (EG) for 5–10 min. Next, they were swiftly transferred into a vitrification medium consisting of 15% EG, 15% dimethyl sulfoxide, and 0.5 M sucrose, with total exposure time kept under 1 min. Following equilibration, embryos were loaded into 0.25-mL hemi-straws. Vitrification was performed by plunging the hemi-straws directly into liquid nitrogen (−196 °C). The hemi-straws were simultaneously placed inside 0.5-mL outer straws for protection during storage.

Data Analysis

The chi-square test was used to assess the relationship between embryo collection timing (days 5, 6, and 7). Embryo recovery rates were analyzed using one-way analysis of variance. Independent t-tests compared recovery rates based on dominant follicle duration and assessed the effect of oxytocin administration versus no administration on embryo recovery.

RESULTS

Embryo Collection

The *in vivo* embryo production study using the uterine flushing technique showed the highest embryo recovery rates in healthy mares when embryos were collected on days 6 and 7 post-ovulation (Table 1). Statistical analysis revealed a significant difference ($p<0.05$) between these groups and the day 5 collection group (Table 2). However, no statistically significant difference ($p>0.05$) was found between the day 6 and 7 collection groups. Embryos flushed on day 6 were at the early blastocyst stage, whereas those collected on day 7 were at the blastocyst stage (Figure 1). Chi-square analysis confirmed a significant association between embryo collection timing and recovery rate, indicating successful collection ($Chi^2 = 8.89$; $p = 0.031$).

Effect of Oxytocin Administration and Follicle Development

Oxytocin administration during uterine flushing significantly increased the embryo recovery rate ($p<0.05$) (Table 3). Furthermore, mares with a dominant follicle development period of less than or equal to 4 days before ovulation showed a higher embryo recovery rate than those beyond 4 days. This difference was statistically significant ($p<0.05$) (Table 4).

DISCUSSION

This study reinforces that *in vivo* embryo production followed by ET is more efficient than traditional breeding methods in Indonesia, such as natural mating and AI alone. The ET protocol involves reproductive examination, estrus synchronization, ovarian activity monitoring, AI, and embryo collection via uterine flushing. This approach allows donor mares to maintain their athletic careers while producing offspring during peak reproductive and performance years. In this study, donor mares required only a 1-week recovery period after insemination before returning to competition, as recipient mares carried the pregnancy and provided foal care. This process represents an advancement in Indonesian equine breeding practices, enabling AI without compromising the donor mare's athletic career.

Embryo collection performed on days 5, 6, and 7 post-ovulation revealed variable success rates, highlighting the importance of optimal timing in *in vivo* embryo production. Notably, one group included donor

Table 1. Details of the embryo recovery rate per group in mares

Groups (day post-ovulation)	Mare [age (years), breed]	Duration of preovulatory dominant follicle development (days)	Number of corpus luteum	Embryo recovery number	Embryo stage
5	Melody (12, KPI)	>4	1	0	-
5	Boo (15, WB)	≤4	1	0	-
5	Hindun (5, Sumba)	≤4	1	0	Unfertilized oocyte (UFO)
5	Nagita (4, Sumba)	>4	1	0	-
5	Arecta (10, KPI)	≤4	1	0	-
6	Boo (15, WB)	>4	2	0	-
6	Arecta (10, KPI)	≤4	2	1	Early blastocyst
6	Hindun (4, Sumba)	≤4	1	1	Early blastocyst
7	Scarlet (12, THB)	>4	1	0	-
7	Arecta (10, KPI)	≤4	1	1	Blastocyst
7	Nagita (3, Sumba)	≤4	1	1	Blastocyst
Reproduction disorder	Boo (15, WB)	≤4	2	0	-
Reproduction disorder	Boo (15, WB)	≤4	1	0	-
Reproduction disorder	Sion (10, KPI)	≤4	1	0	-
Reproduction disorder	Melody (12, KPI)	>4	1	0	-
Reproduction disorder	Arecta (10, KPI)	≤4	1	0	-

Note: Kuda Pacu Indonesia (KPI), Warmblood (WB), and Thoroughbred (THB).

Table 2. Embryo recovery rate per group based on differences in collection time in mares

Group	Embryo collection (n)	Embryo recovery rate ± SD (%)
5th	5	0/5 (0)[a]
6th	3	2/3 (66.7 ± 0.58)[b]
7th	3	2/3 (66.7 ± 0.58)[b]
Reproduction disorder	5	0/5 (0)[a]

Note: [a,b] shows a significant difference ($p<0.05$).

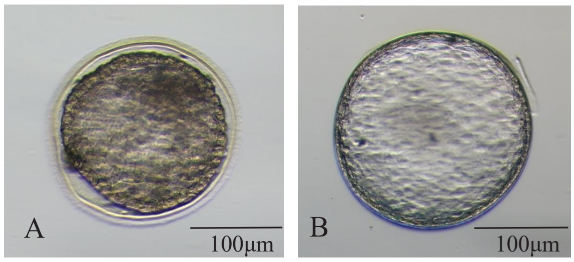

Figure 1. Mare embryo collection on days 6 and 7 post-ovulation. (A) An early blastocyst stage (180 μm), (B) a blastocyst stage (210 μm).

Table 3. Embryo recovery rate per group based on differences in oxytocin usage in mares

	Embryo collection (n)	Embryo recovery rate ± SD (%)
Oxytocin administration	6	3/6 (50 ± 0.54)[a]
Without oxytocin administration	5	1/5 (20 ± 0.44)[b]

Note: [a,b] shows a significant difference ($p<0.05$).

Table 4. Embryo recovery rate per group based on the duration of follicle preovulatory development in mares

Duration of follicle preovulatory development	Embryo collection (n)	Embryo recovery rate ± SD (%)
<4	7	2/7 (28 ± 0.49)[a]
≥4	4	0/4 (0)[b]

Note: [a,b] shows a significant difference ($p<0.05$).

mares with underlying reproductive disorders despite initial health screening.

No embryos were recovered from collections performed on day 5 post-ovulation, likely because embryos had not yet entered the uterus. The timing of embryo entry into the uterus is critical for successful uterine flushing. These findings align with those of Stout (2012), who reported that equine embryos at the late morula to early blastocyst stage enter the uterine lumen between 144 and 168 h post-ovulation (days 6–7). Thus, embryos collected on day 5 may not have reached an appropriate developmental stage for recovery, even with oxytocin support during uterine flushing.

Oxytocin-induced uterine contractions apply mechanical force to the embryo, increasing the likelihood of it being flushed out during uterine flushing. Oxytocin is released in pulses with increasing frequency and amplitude, and when it binds to certain myometrium oxytocin receptors, it triggers myometrial contractions (Moberg, 2024). However, this does not completely rule out embryo recovery on day 5. According to McCue (2017), equine embryos usually enter the uterus at the early morula or blastocyst stage on days 6–7 post-ovulation. Nevertheless, embryos that have already entered the uterus can be mechanically displaced backward toward the anterior uterine horn, particularly when oxytocin-induced contractions are strong. Thus, oxytocin use on day 5 has not yet been shown to improve embryo recovery rates optimally.

This study demonstrated that the highest embryo recovery rates were achieved when collection was performed on days 6 or 7 after ovulation. Based on the transport timeline of equine embryos through the oviduct, this process is divided into three stages: the pre-transport period (120–128 h post-ovulation), the transport period (130–142 h post-ovulation), and the post-transport period (144–152 h post-ovulation) (Freeman *et al*., 1991). Embryo collections on days 6 or 7 correspond to the transport and post-transport stages, indicating that embryos generally reach the uterus at an optimal time. In these groups, oxytocin administration did not significantly affect recovery rates, supporting the idea that embryos can be successfully recovered without oxytocin when collection timing is optimal. Carnevale *et al*. (2000) reported high success rates for embryos collected on days 6 or 7, which are typically at the early blastocyst and blastocyst stages. Similarly, Jacob *et al.* (2012) found embryo recovery rates of 56%–66% when flushing was performed between days 7 and 10 post-ovulation, and Park *et al.* (2017) reported a 60% recovery rate with day 7 uterine flushing.

Both groups demonstrated comparable embryo recovery rates. However, the main difference was in the developmental stage of the embryos collected: day 6 collections yielded early blastocysts, whereas day 7 collections yielded more advanced blastocysts. Choosing the optimal collection time depends on the intended use of the embryos—whether for fresh transfer or cryopreservation. More developed blastocysts contain greater intracellular fluid volume, which can complicate freezing and reduce survival rates during cryopreservation.

Timing is also crucial because embryos collected later tend to be larger (>300 μm) or enter the expanding blastocyst phase, increasing the risk of damage during handling. Embryos smaller than 300 μm are easier to handle, as larger embryos (particularly after day 8) pose challenges during aspiration and release with pipettes, risking mechanical stress. Larger or zona pellucida–absent embryos have a thinner protective layer, making them more vulnerable to pressure or friction during manipulation. Current protocols recommend embryos be ≤300 μm in diameter for successful vitrification, avoiding blastocoel puncture or aspiration (Couto *et al*., 2023). Embryos at days 9–10 have more complex internal structures and are prone to rupture from excessive pipette pressure or rough handling (Squires, 2020). Carnevalle *et al.* (2000) also noted that embryo collection on days 8 or 9 is particularly challenging in subfertile or older mares due to increased embryonic loss.

The physiological aspects of follicular development were analyzed by comparing the duration of preovulatory dominant follicular development (<4 vs. ≥4 days). Preovulatory follicles are identified by ultrasonography based on changes in diameter and morphology. Follicles entering the preovulatory phase typically measure 3–3.5 cm in diameter and shift from a spherical to a nonspherical shape. Immediately before ovulation, the follicle diameter generally reaches approximately 4.5–5 cm. In mares, the extended follicular phase is associated with a mean dominant follicle growth rate of 0.3 cm per day. Prolongation of preovulatory follicle development beyond 4 days adversely affects oocyte quality. Delayed ovulation increases the duration that the oocyte remains within the follicle, which can impair oocyte maturation, fertilization capacity, and the interovulatory interval (Demond *et al*., 2016). In aged mares, prolongation of the estrous cycle has been attributed to a reduced dominant follicle growth rate compared with younger mares (Marinone *et al*., 2015). These alterations also negatively influence subsequent embryo developmental potential (Bittner *et al*., 2011). Demond *et al*. (2016) further reported that intrafollicular aging caused by delayed ovulation reduces fertilization rates, increases polyspermy, promotes chromosomal abnormalities, and elevates embryo mortality.

Prolonged estrus may cause oocytes to remain in an immature state, thereby reducing their viability and fertilization ability. This condition is typically associated with elevated follicle-stimulating hormone concentrations without corresponding increases in luteinizing hormone. Such a hormonal imbalance is more common in older mares and reflects age-related dysregulation of endocrine control (Carnevale, 2008).

Five embryo collection cycles were classified as the reproduction disorder group and included donor mares that had previously been enrolled in other groups. Mild endometritis was identified before uterine flushing through ultrasonographic examination, and a cloudy appearance of the recovered flushing medium further indicated uterine pathology. None of the donors in this group yielded embryos (recovery rate: 0%). These findings suggest that although the same donors were able to produce embryos in previous cycles on days 6 and 7 post-ovulation, unstable uterine conditions—such as subclinical endometritis or an exaggerated immunological response to AI—can result in complete failure of embryo recovery in subsequent cycles. Despite oxytocin administration in four of the five mares, embryo recovery was unsuccessful, confirming that oxytocin is ineffective when uterine conditions are unfavorable.

During breeding, the introduction of semen, bacteria, and cellular debris into the uterus normally induces transient endometritis, which is typically resolved within 48 h through an effective inflammatory response and uterine clearance. In mares predisposed to persistent post-breeding endometritis, uterine immune defense and clearance mechanisms are impaired, preventing resolution of inflammation within this timeframe and leading to continued intrauterine fluid accumulation beyond 48 h post-breeding. This condition is associated with increased rates of early embryonic loss and reduced pregnancy success compared with unaffected mares. This condition may interfere with fertilization, impair embryo development, or reduce embryo survival within the uterine environment (Maischberger *et al*., 2008).

Uterine inflammatory responses accompanied by fluid accumulation are commonly observed in mares older than 15 years. Although the uterine structure may appear normal, post-insemination echogenic fluid is more frequently associated with an immunological response to antigenic stimulation from semen rather than with bacterial infection. Spermatozoa and semen extenders activate the complement system and induce polymorphonuclear cell chemotaxis within the uterus. This response is further exacerbated by reduced capacity for uterine clearance (Troedsson *et al*., 2001). Age-related changes contribute to this impairment, as old mares (≥14–16 years) often exhibit decreased uterine muscle tone and reduced elasticity of the uterine wall, limiting effective uterine contractions that are essential for expelling inflammatory fluid after mating or AI (Le Blanc, 2010).

Uterine clearance is mediated by increased frequency and intensity of myometrial contractions. Although endometritic reactions do not necessarily prevent embryo recovery, they substantially reduce the likelihood of embryo survival following transfer to recipient mares. An endometriotic uterine environment compromises embryonic defense mechanisms and increases the risk of embryonic loss (Derbala *et al*., 2024). Furthermore, the physiological inflammatory response following insemination reduces fertility in approximately 15% of mares (Scarlet *et al*., 2023). Based on the ability to resolve inflammation or infection within 48 h post-insemination, mares are classified as either susceptible or resistant to persistent breeding-induced endometritis (Canisso *et al*., 2020). These observations are consistent with the findings by Jacob *et al.* (2012), who identified reproductive health status and embryo collection timing as primary determinants of successful embryo recovery in donor mares.

In vivo embryo production in horses is an assisted reproductive technology that offers a practical solution to reproductive limitations and enhances reproductive efficiency, particularly in genetically superior mares. This approach includes donor mare selection, estrus cycle synchronization, ovulation induction, AI, and embryo recovery from the uterus via flushing at optimal time points. The success of *in vivo* embryo production is strongly influenced by multiple factors, including the timing and method of embryo collection, the physiological and reproductive status of the donor mare, and the effectiveness of the uterine flushing technique.

CONCLUSION

In vivo equine embryo production is feasible in tropical regions. Optimal outcomes are achieved through the application of assisted reproductive technologies, combining AI with embryo collection on days 6 or 7 after ovulation, which yields the highest embryo recovery rates.

CONFLICTS OF INTEREST

A. Boediono serves as editor of the Tropical Animal Science Journal but has no role in the decision to publish this article. The authors certify that there are no conflicts of interest, including any financial, personal, or other relationships with individuals or organizations, related to the material discussed in this manuscript.

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DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the writing process, none of the authors used generative AI or AI-assisted technologies.

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