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NOTE

Theriogenology

Successful embryo transfer from Hokkaido native pony after artificial insemination with frozen semen

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ABSTRACT. There has been no report of equine embryo transfer in Japan for the last 24 years. Our objective was to establish an effective protocol for embryo transfer in domestic horse. A Hokkaido native pony was bred by deep-horn artificial insemination with frozen semen from a Connemara pony. Embryo collection was performed using a non-surgical method on day 7. Two embryos were obtained from three flushes (67% recovery) and were transferred fresh into crossbred recipient mares. Both recipient mares were diagnosed pregnant (100% pregnancy rate) 5 days after embryo transfer and had normal progesterone levels until the end of the observation on day 35 of gestation. This is the first successful embryo transfer in Japan by artificial insemination using frozen semen. Embryo transfer technology could be extremely useful in improving the productivity of Japanese domestic and special riding horses.

KEY WORDS: artificial insemination, embryo transfer, frozen semen, hokkaido native pony, progesterone

Embryo transfer is an assisted reproductive technique where viable embryos are collected from valuable donor mares and transferred into recipient mares that will carry the pregnancy until foaling. This technique allows the donor mare to potentially produce multiple foals in a year, overcome subfertility especially from uterine pathology, and allows mares with active athletic careers to simultaneously produce foals [2, 8, 12, 13]. In order to obtain acceptable pregnancy rates from embryo transfer, recipient mares must ovulate from a day before and up to the 3rd day after the donor [8].

Japan is the pioneer of successful embryo transfer in horse with the birth of the world's first foal from embryo transfer in 1974 [10]. According to Japan Equine Affairs Association, the last embryo transfer in horse was registered in 1994, and unfortunately, the technology has not flourished in Japan since, whereas equine embryo transfer has continued to be developed in Europe and America and has been commercialized [3, 6, 11].

Behaviors among horse breeds are variable and a better behavioral trait was shown in Japanese native horse than Thoroughbreds [5]. The Hokkaido native pony is very calm and can manage to survive in cold weather, popular for horse riding. Connemara ponies have a good temperament and are well known for being excellent riding horses. Therefore, a resulted crossbreed of Hokkaido native pony and Connemara pony may produce an ideal riding breed for the region.

The aim of the present study was to establish a successful non-surgical embryo-transfer program in Japanese domestic horse by artificial insemination using frozen semen.

The study was approved by the Animal Welfare and Ethics Committees of Obihiro University of Agriculture and Veterinary Medicine. All horses remained in good health, during the study. A donor Hokkaido native pony (body weight, 347 kg; age, 6 years) and 2 recipient (#1 and #2: body weight, 414 and 421 kg, respectively; age, 13 and 10 years, respectively) crossbreed (Hokkaido native pony × Haflinger) mares were used. The mares were maintained at the Horse Research Farm, Obihiro University of Agriculture and Veterinary Medicine since spring 2017. The mares were kept on a pasture attached to a roofed shelter and fed with hay, compressed hay (Hokkaido Horse Feed, Hokkaido, Japan), and Stamm 30 (Hallway Feeds, Lexington, KY, U.S.A.).

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The reproductive tract of the mares was examined weekly by transrectal ultrasound (Hitachi Aloka, Tokyo, Japan) equipped with a 7.5 MHz linear array transducer from December 2017. Once the mares were found to be close to the end of the vernal transition in early spring 2018, they were checked daily to confirm the precise date of ovulation. The first ovulation of the season was allowed to occur spontaneously and not considered for artificial insemination (AI) or embryo transfer. Luteolysis was induced by intramuscular administration of 0.1 mg PGF2α analogue (Planate®, Nagase Medicals Co., Hyogo, Japan) during the luteal phase in both donor and recipient mares to synchronize the onset of estrus. Once the donor mare developed a dominant follicle that was at least 35 mm in diameter with concurrent uterine edema of at least grade 3 from a scale of 0–5, ovulation was induced by administering 2,000 IU of human chorionic gonadotropin (hCG) (Gonatropin®, Asuka Animal Health Co., Tokyo, Japan). Recipient mares were also given hCG one day after the donor to induce ovulation in the recipient within the acceptable recipient-ovulation synchrony window of 1 day before to 3 days after the donor mare.

The donor mare was bred approximately 32 hr after administration of hCG by deep-horn artificial insemination using frozen semen from a Connemara pony stallion (body weight, 473 kg; insemination dose, 400×10^6 sperms with 50% post-thaw progressive motility). Deep-horn insemination was performed by inserting a flexible insemination pipette through the cervix and the tip of the insemination pipette was guided to the tip of the uterine horn ipsilaterally to the dominant follicle by transrectal palpation. In one of the estrous cycles monitored, the donor mare ovulated spontaneously. In that cycle, the mare was bred post-ovulation approximately 24 hr after the previous ultrasound examination.

A non-surgical transcervical procedure was used to collect the embryo from the uterine lumen of the donor mare at day 7 post-ovulation. The donor was mildly sedated by injecting 4 μg/kg medetomidine hydrochloride (Dorbane Vet, Kyorituseiyaku Co., Tokyo, Japan) approximately 10 min prior to the start of the uterine flush for embryo recovery. The embryo recovery setup included a sterile uterine catheter with a cuff (Jorgensen Laboratories, Inc., Loveland, CO, U.S.A.) attached to a sterile Y Junction W/Folly Connector (SPI-MFG, Canton, TX, U.S.A.) with clamps to regulate the inflow and outflow of a commercially prepared complete flush solution (VIGRO, Vetoquinol N. -A. Inc., Princeville, QC, Canada) in one end and with a sterile embryo filter (EZ-Way Filter, SPI-MFG) on the other. Using a sterile plastic sleeve to cover the hand and arm of the operator, the catheter tip was delicately passed through the cervical opening and into the posterior uterine body. A small amount of sterile, nontoxic water-soluble lubricant (K-Y jelly) (Reckitt Benckiser Healthcare Ltd., Hull, U.K.) was applied to the outside of the sleeve prior to attempting placement of the catheter in the uterus. The balloon cuff of the catheter was then filled with 50 m*l* of air and pulled posteriorly to establish a good seal at the junction of the uterine body and internal cervical os. The flush media was pre-warmed to 37°C and was allowed to enter the uterine lumen and pass through the embryo filter (68 μm nylon membrane) by gravity flow. The uterus of the mare was often massaged by trans-rectum during infusion and recovery of the solution to ensure that the fluid fills the entire area of uterine body and horns. The flushing was performed three times sequentially with a total of 6 *l*, 2 *l* each time. Oxytocin (Atonin®-O, Asuka Animal Health Co., Tokyo, Japan) was administered intramuscularly (25 IU) before the final flush to aid complete evacuation of the fluid. After that, the filter was rinsed several times to ensure that the embryo is not attached to the mesh and the filter dish was examined using a stereo microscope (Olympus Corp., Tokyo, Japan) to identify the embryo. Identified embryo was washed 6 times in holding media (VIGRO, Vetoquinol N. -A. Inc.,) in a petri dish before aspirating into a sterile 0.25 m*l* straw. A 2.5 m*l* syringe containing approximately 2 m*l* of air was first secured to the appropriate end of the straw, and then the medium and embryo was loaded into the straw using the following sequence: medium, air, medium, air, medium containing embryo, air, medium, and air. This embryo-filled straw was then loaded into the inner flexible tubing of the insemination gun (bovine YT gun, Yamanetech Co., Nagano, Japan) and fresh embryos were transferred non-surgically into the uterine body of the recipients which ovulated 1 day after donor for both embryos transferred. Five days after embryo transfer, pregnancy was diagnosed by ultrasonographic examination of the uterus.

Blood samples were collected into heparinized vacutainer (Terumo Corp., Tokyo, Japan) by jugular venipuncture from recipient mares on the day of ovulation (day 0), day 7, followed by twice weekly until the 5th week. The collected blood was immediately placed on ice and then centrifuged (3,000 rpm for 12 min at 4°C) to separate plasma. The plasma samples were stored at −30°C and thawed just prior to progesterone assay, which was done using a commercially available enzyme immunoassay kit (ST AIA-PACK PROG II, Tosoh Bioscience, Inc., San Francisco, CA, U.S.A.).

Two embryos were recovered from 3 collection attempts (67% recovery) on a single donor mare. The intervals between the first and the second and the second and the third embryo flushings were 17 and 24 days, respectively. Both donor and recipients had a single ovulation in all cases. The embryos were at early blastocyst (230 μm; Fig. 1A) and hatched blastocyst (550 μm; Fig. 1B) stages at collection, possessing apparently good or excellent grades. Both recipient mares were diagnosed pregnant (100% pregnancy rate) by confirming the presence of the embryonic vesicle in the uterus using transrectal ultrasonogram at day 5 after embryo transfer. Transrectal ultrasonogram at pregnancy day 35 showed presence of embryo in between the allantoic and the yolk sacs in recipient mare #1 (Fig. 2A) and #2 (Fig. 2B). The plasma progesterone concentrations in the 2 recipient mares from the day of ovulation until the 5th week of pregnancy are shown in Fig. 3. Progesterone levels on the day of ovulation were below 1 *n*g/m*l* and then increased, maintaining high levels of at least 5 *n*g/m*l* until the end of our observation in both recipients.

According to the records of Japan Equine Affairs Association, embryo transfer in horse was last registered in 1994 in Japan. Embryo transfer was done after natural mating of donor mares with stallions, previously. This is the first report in the history of Japan of a successful embryo transfer after artificial insemination using frozen semen. Here, we report a non-surgical embryo collection and transfer technique followed by a successful pregnancy in horse.

In the present study, 2 embryos were recovered in 3 collection attempts on a donor Hokkaido native pony and pregnancy was confirmed in both the recipient mares, 5 days after embryo transfer. In our 2 successful attempts, hCG was administered in both the donor and the recipient mares for inducing ovulation and AI was performed approximately 32 hr after treatment, assuming

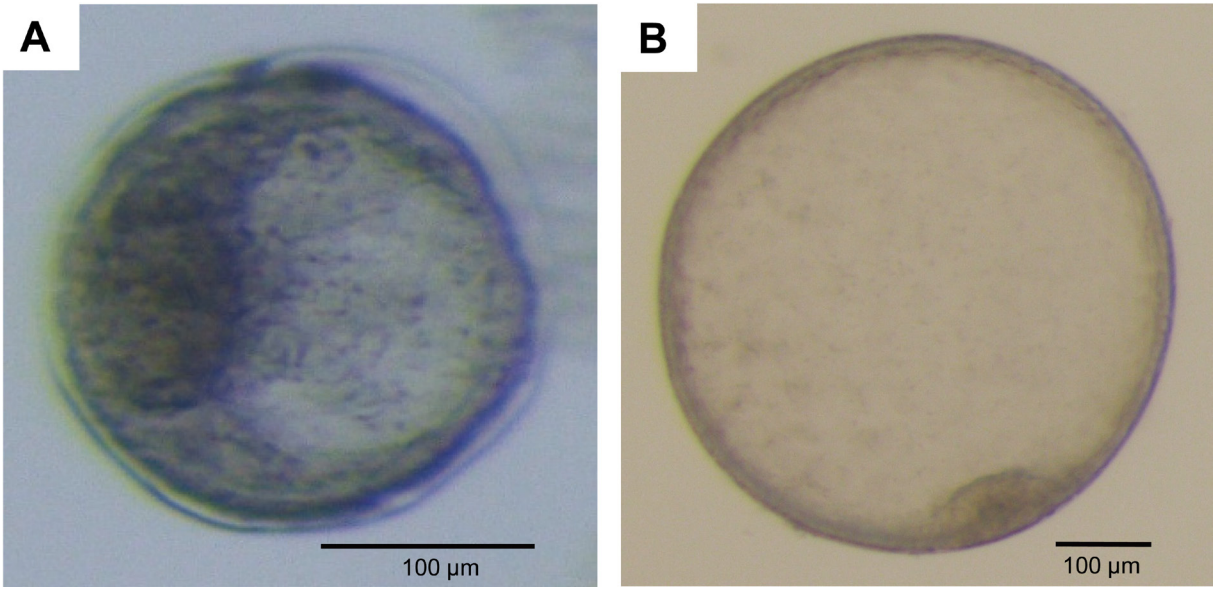

Fig. 1. Photomicrograph of an early blastocyst (a) and a hatched blastocyst (b) collected from the same donor (Hokkaido native pony) at day 7 after artificial insemination with frozen semen of a Connemara pony stallion.

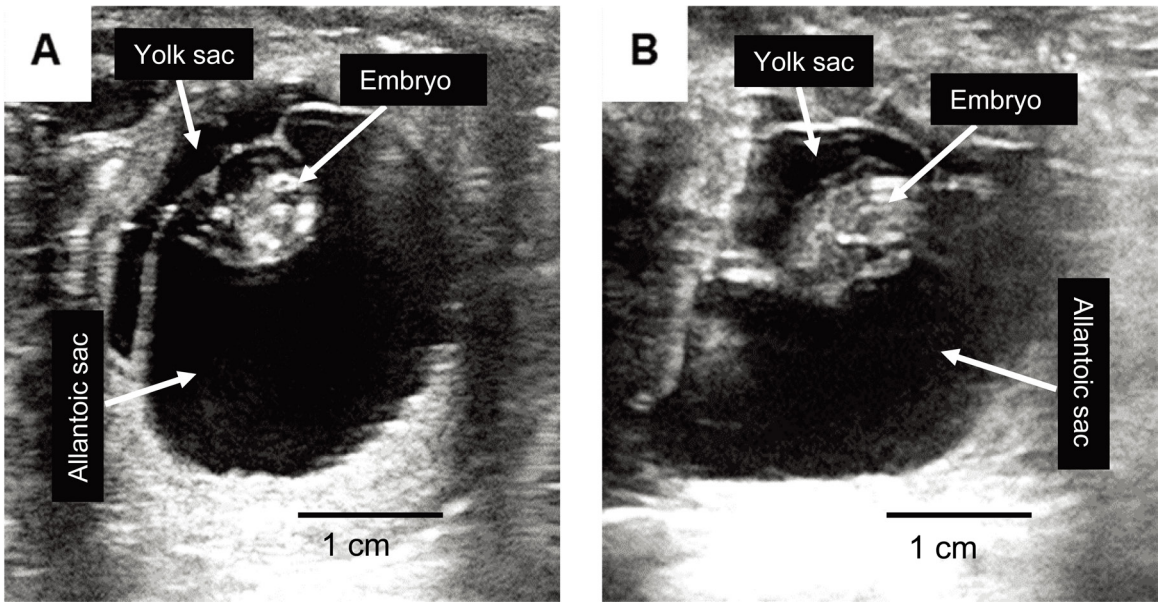

Fig. 2. Ultrasonographic image of the embryonic vesicle at day 35 of pregnancy in recipient mare #1 (a) and #2 (b). Embryo is located in between the allantoic and the yolk sacs.

that ovulation will take place within next 6–12 hr. Ovulation usually occurs 36–42 hr after hCG treatment [4]. However, we did not receive the embryo in another instance where ovulation had occurred spontaneously, and in this case, there was approximately a 24 hr window between the ovulation checks. Therefore, our success with hCG-induced ovulation was likely to be aided by performing AI just few hours (6 to 12 hr) prior to the induced ovulation. Although our observation number is low, we believe that doing artificial insemination within a close window of the ovulation period may improve the success rate and thus recommend monitoring the pre-ovulatory follicle every 6 to 12 hr, particularly when ovulation is not induced. A higher pregnancy rate was observed for mares inseminated 0 to 6 hr after ovulation than for those inseminated between 18 and 24 hr [14]. However, it was argued that checking for ovulation more frequently than 12 hr does not improve the pregnancy rate in a post-ovulatory insemination program [9].

Progesterone is the main hormone that establishes and maintains pregnancy. Progesterone levels in both the recipient mares in the present study were in accordance with previously reported progesterone levels in early pregnant mares carrying viable embryos

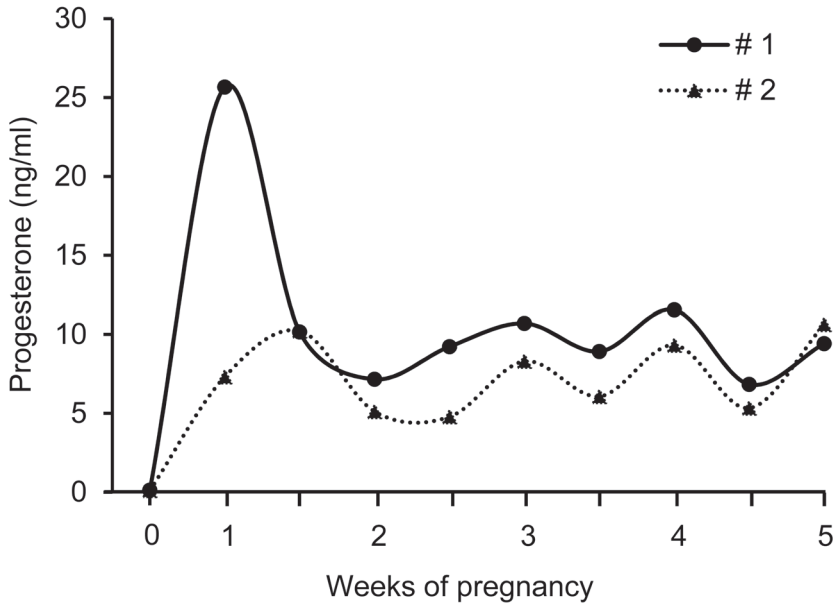

Fig. 3. Plasma progesterone levels of the 2 recipient mares from the day of ovulation until the 5th weeks of pregnancy.

[1, 7], confirming a normal-pregnancy status in the recipient mares in our study. We are hoping that the crossbred offspring (Hokkaido native pony × Connemara pony) from this study will introduce a generation of calmer horses in Japan.

In conclusion, the present study has shown a successful non-surgical collection and transfer of equine embryos for the first time in the last 24 years in Japan. In fact, this is the first report in the history of Japan showing a successful embryo transfer after artificial insemination using frozen semen. We confirmed that both the recipient mares had a normal pregnancy until the end of the study, which was at day 35 of the pregnancy and are hoping to see healthy foals born in spring 2019. The use of embryo transfer technology would be extremely useful for the conservation of native Japanese horses and the production of special riding horses.

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