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Technology Report

First Kiso pony foal produced via transfer of long-distance shipped fresh embryo to Hokkaido native pony

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Running head: The first Kiso pony via embryo transfer

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Abstract

Japanese native horses, which consists of 8 breeds, are threatened with extinction. Embryo transfer (ET) has been used for the purpose of reproducing endangered animals in various mammalian species. We aimed at performing ET using Kiso and Hokkaido native ponies as the donor and recipient, respectively. The ET operation included a long-distance transport of non-cryopreserved embryos from Nagano prefecture to Hokkaido. Embryos were transported 1500 km over 9 hours in a container maintained at 22℃. After transferring two embryos to two recipients, one mare delivered a healthy live foal. These results demonstrated that reciprocal ET with the long-distance transportation of fresh embryos between the isolated breeds may allow for proliferation of the Japanese native horses.

Key words: Embryo transfer, Hokkaido native pony, Kiso pony, Long-distance transportation

Japanese native horses are Hokkaido pony (Hokkaido), Kiso pony (Nagano), Taishu pony (Nagasaki), Misaki pony (Miyazaki), Tokara pony (Kagoshima), Miyako pony (Okinawa), Yonaguni pony (Okinawa) and Noma pony (Ehime). Kiso pony is the only native horse breed of Honshu, Japan's main island. The name “Kiso” was originated from Kiso area in Nagano prefecture, a homeland of this breed for more than a thousand year. Kiso pony horses were used in the past for farming, transportation, and on the battlefield. Since the Meiji era (1868 – 1912), the number of pure Kiso ponies has drastically decreased. The few remaining pure breed ponies were used for Kiso pony breeding after 1945. Nonetheless, according to the report of Japan Equine Affairs Association (https://www.bajikyo.or.jp/regist_05.html; Changes in the number of native horse ‘在来馬頭数の推移’), population of Kiso pony was counted as 134 in 2021, and it is now in danger of extinction, like other Japanese native horse breeds [1–3].

To increase the population of Kiso pony, assisted reproductive technology including embryo transfer (ET) could be used. The term "embryo transfer" describes the flushing of an embryo from a donor mare's uterus and transferring it into the uterus of a recipient mare, who will then carry the pregnancy to term [4–6]. Equine ET has not only advantage of producing multiple foals from a donor mare per year, but also advantage of overcoming inability of donor mares to carry foals due to reproduction and musculoskeletal problems, as well as, producing foals from donor mares in athletic competition [5]. Japan is the pioneer of successful ET in horses, resulting the birth of the first foal through cervical by-pass ET method in 1973 [7]. After 46 years, the first Hokkaido native pony foals were produced in Japan via non-surgical ET method after artificial insemination (AI) using frozen semen of Irish Connemara pony stallion [8]. Cost of maintaining recipient herd on the site of donor is the major economic burden of ET program, and embryo shipment is much cheaper than transporting recipient mares [5,9]. There is no reported data on long-distance shipment of equine fresh embryo and transferring to the recipient mare in Japan. Aim of the current study was to produce Kiso pony foals by shipping the fresh embryo from donor mares in Nagano and transferring them to recipients in

Hokkaido.

Accurate synchronization of donor and recipient mares is vital for transferring fresh embryos when there is small number of recipient mares. Between June and August (2021), we synchronized 3 Kiso pony mares with 5 Hokkaido native pony mares (total of 5 estrous cycles). Both the donors and recipients had a single ovulation per estrous cycle, and there were no hemorrhagic follicles. Equine embryos should be transferred into recipient mares that ovulated between the day before the donor mare, or up to 2 days after the donor mare [5]. In this study, recipient mares ovulated within the synchrony window of the donor mares for three cycles (day +1, 0, and -1). Although, two recipient mares ovulated out of the synchrony window to the respective donor mares (day -8 and -5). Up to 7 days negative asynchronization with donors can result pregnancies in recipient mares after ET (38%) [10]. However, the recipient which was out of synchronization (day -8) was not used in ET as no embryo was recovered from the respective donor mare.

One of the unique features of equine embryos is that they enter uterus 5.5 to 6.5 days after ovulation [5]. We therefore performed uterine flushing of the donor mares on day 7 or day 8, and embryo recovery rate was 60 % (three embryos from five collection efforts). All three embryos were at hatched blastocyst stage (day 8), and morphological assessment showed that all were in the excellent grade. Equine embryos can be held overnight at 5℃ to 19℃ without any detrimental effect [11], and can be transported using commercial embryo holding medium and passive cooling systems [5]. In the present study, all the embryos were successfully transported in embryo holding medium at room temperature (~ 22℃) for 9 h from Nagano to Obihiro (**Fig. 1**). And there was no considerable change in the quality of the shipped embryos. Two out of three embryos were transferred to recipient mares (synchrony of -5 and +1) and one embryo was lost accidentally before ET. To maintain pregnancy in the recipient (-5 synchrony), altrenogest (Regu-Mate® Solution 0.22%, 8 mL, Intervet Inc., NJ, USA) was administered orally before transferring the embryo. Both recipient mares maintained their pregnancy, however only one of them resulted in delivery of healthy live foal. Pregnancy data of the

other mare delivered unhealthy foal was not included in this paper.

Before ET, the day 8 embryo was 1476 μm in size, and its quality was good (**Fig. 2A**). Five days after ET (on day 13), an embryonic vesicle, measuring 11 mm in diameter, was found during trans-rectal ultrasonographic exam. Fetal heart rate measurement is useful tool for monitoring fetal well-being [12], and ultrasonographic exam demonstrated that the embryonic heart beat was within normal range, measured as 171 bpm on day 35 of pregnancy (**Fig. 2B**). In addition, the recipient mare's plasma progesterone concentrations, a crucial hormone for starting and maintaining pregnancy [13], were monitored from the day of ovulation till delivery (**Fig. 2C**). Progesterone concentration was < 1 ng/ml at ovulation, and it increased to 10.31 ng/mL of concentration on day 7. Then, progesterone concentration reached peak concentrations (33.56 ng/mL) on day 44, which was assumed to be related to equine chorionic gonadotrophin secretion and supplementary corpora lutea formation [14]. After day 121, progesterone concentration gradually declined and remained low (3.2 ± 0.2 ng/mL) between day 177 and 261. Precocious increase of progesterone prior to day 308 during late gestation suggests placental dysfunction [15]. In this recipient mare, the last increase of progesterone concentration started from day 317, around three weeks before delivery, indicating healthy pregnancy. The gestational length was 334 days, and recipient mare naturally delivered a healthy colt (**Fig. 3**). The newborn colt stood and began nursing from its surrogate mother within 43 min and 65 min after birth, respectively. The recipient mare had good maternal skills and normal lactation.

In conclusion, this study produced the first Kiso foal through non-surgical transcervical embryo transfer to Hokkaido native pony after 9 h transport from Nagano to Hokkaido. The use of embryo transfer technology would be extremely helpful for the preservation of native Japanese horses and the production of special riding horses.

Methods

In this study, native Kiso pony mares were used as donors (n=3, 10.3±5.0 years old), and pure

Hokkaido native pony and crossbred mares (Hokkaido native pony × Haflinger) were used as recipients (n=5, 10.0±1.0 years old). In accordance with animal care regulation of the university, donor mares were kept in "Kiso-Uma-no-Sato Horseback Riding-center" located in Nagano, and recipient mares were kept in Horse Research Farm at Obihiro University of Agriculture and Veterinary Medicine (OUAVM) in Hokkaido. All experimental methods were approved by Animal Experiment Committee of OUAVM (No. 21-45 and No. 22-22).

A previously published technique was employed in this study to synchronize donor and recipient mares. [16]. Briefly, mares were examined by transrectal ultrasonography during the breeding season (June to August 2021) to detect estrous period. To terminate luteal activity and cause early return to estrus, single injection of Cloprostenol (prostaglandin F2 analogue, 0.1 mg, IM, Planate®, Nagase Medicals, Hyogo, Japan) was administered during the mid-luteal phase. When mare had dominant follicle (≥ 35 mm) and substantial uterine edema (grade 3 from a scale of 0–5: endometrial folds can be easily observed) [17], either of human chorionic gonadotropin (hCG, 2,000 IU, Gonatropin®, Asuka Animal Health, Tokyo, Japan) or gonadotropin-releasing hormone (GnRH, 0.75 mg, Deslorelin, Boothwyn Pharmacy, Boothwyn, PA, USA) were administered to induce ovulation within 36−48 h. Recipient mares were selected and injected with ovulation induction agent to maintain synchrony window between +1 and –3 days from donor mares' ovulation. Within 32 h after hCG or GnRH injections, donor mares mated with a native Kiso pony stallion (4 years old). Then, mares were examined daily by ultrasonography for confirming the ovulation. Once ovulation was confirmed in donor mares, embryo collections were performed 7 or 8 days later using non-surgical trans-cervical embryo recovery method as reported previously [16,18]. The recovered embryo was washed in commercial embryo holding media (VIGRO®, Vetoquinol N. - A. Inc., Princeville, QC, Canada) before being put into 0.5 ml straw (01123300, Fujihira Industry Co., LTD, Tokyo, Japan) and sealed with polyvinylpyrrolidone (PVP) powder (FHK straw powder, 01128770, Fujihira Industry Co., LTD, Tokyo, Japan). Then, straw containing embryo was transported in an EquOcyte container (Hamilton

BioVet, Ipswich, MA, USA) across the country by car, bus, and airplane (**Fig. 1**). To keep stable temperature inside the container during the shipment (~22℃, from Nagano to Obihiro), thermal can (Hamilton BioVet, Ipswich, MA, USA) pre-warmed at 37℃ was loaded inside the EquOcyte container. After embryo shipment, embryo was evaluated under stereo microscope (Olympus SZX10, Olympus Corp., Tokyo, Japan), then loaded into 0.5 ml straw. Finally, embryos were transferred into the uterine body of the recipient mares by non-surgical trans-cervical transfer method as described before [16]. Insemination gun (bovine YT gun, Yamanetech Co., Nagano, Japan) or equine ET sheath for 0.5 ml straws with front opening (19290/1050, Minitüb GmbH, Tiefenbach, Germany) were used for transferring embryos depending on the embryo size (<800μm and ≥800 μm, respectively). Five days after the embryo transfer (on day 13), trans-rectal ultrasound exam was performed to detect a spherical embryonic vesicle. On day 35, heart rate (bpm) was evaluated using power Doppler mode of the ultrasound (Noblus, 9 Hitachi Aloka Medical, Tokyo, Japan) as described before [12]. In the early stage of pregnancy, pregnant mares were examined twice weekly for monitoring healthy embryonic development; after that, they were examined once weekly until delivery. In addition, weekly blood sampling from recipient mares was done by jugular venipuncture using heparinized vacutainer from the day of ovulation (day 0) until delivery. Plasma was separated from blood sample and used for measuring progesterone concentration (ST AIA- PACK PROG II, Tosoh Bioscience, Inc., San Francisco, CA, USA) during the pregnancy period in accordance with the methods reported elsewhere [16].

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgments

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Figures

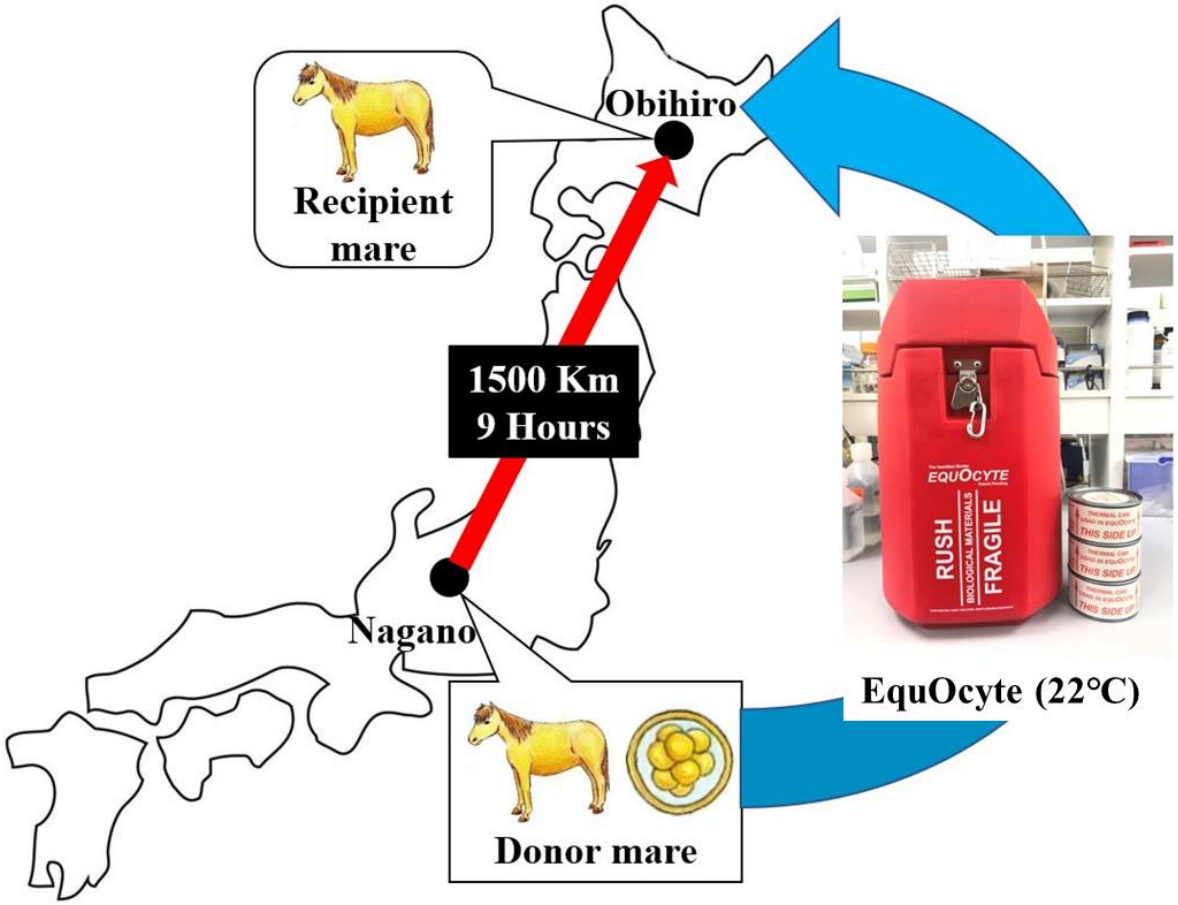

Fig. 1. Procedure of embryo collection and transportation from Nagano in Honshu island to Obihiro in Hokkaido island (1500 km).

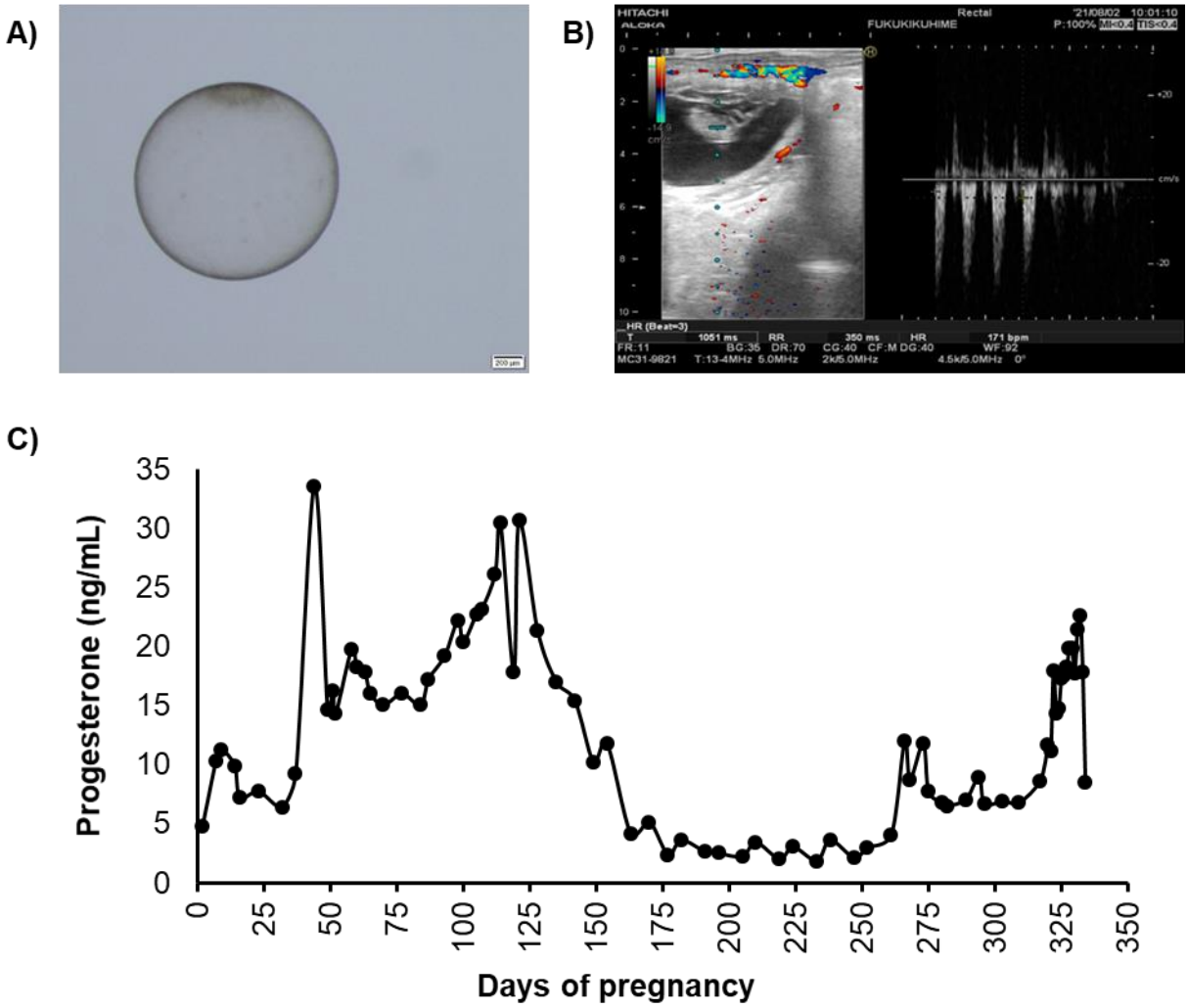

Fig. 2. Image of embryo sizing 1476 μm before ET (**A**), ultrasonographic image of the embryonic heart beat (171 bpm) on day 35 of pregnancy in recipient mare (**B**), and plasma progesterone levels of recipient mare throughout gestation (**C**).

Fig. 3. Image of healthy born colt and his surrogate mother. This is the first Kiso pony foal born through fresh embryo transfer to Hokkaido native pony mare.