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—Original Article—

Successful Nonsurgical Transfer of Bovine Elongating Conceptuses and Its Application to Sexing

Koji KIMURA[1)] and Shuichi MATSUYAMA[1)]

[1)]*Animal Feeding and Management Research Division, NARO Institute of Livestock and Grassland Science, Tochigi 329-2793, Japan*

Abstract. The objectives of the present study were to establish a nonsurgical transfer method for elongating bovine conceptuses and to combine this method with biopsy and sexing. Bovine conceptuses were recovered from donor cows on days 13–14 of the estrus cycle. In experiment 1, day 13 conceptuses were transferred to recipient cows using a standard day 7 embryo transfer (ET) method. The pregnancy rate of day 13 conceptus transfer (CT) is comparable to that of day 7 ET. In experiment 2, day 14 conceptuses were transferred using modified methods (balloon catheters or ET guns with modified sheaths). Using the standard ET method, no pregnancies were obtained; however, when balloon catheters or ET guns with modified sheaths were used, the pregnancy rates after CT were 48.0% and 44.8%, respectively. In experiment 3, day 14 conceptuses were biopsied without a micromanipulator, sexed using the loop-mediated isothermal amplification method and transferred to recipient cows. The pregnancy rate of biopsied conceptuses was 46.2% and did not differ significantly from that of unbiopsied conceptuses. Moreover, all pregnant cows transferred conceptuses following biopsy and sexing delivered calves with the expected sexes. These results suggested that the nonsurgical bovine CT method was comparable to day 7 ET and that this technique enables biopsy and sexing without expensive equipment such as a micromanipulator or specialized skills.

Key Words: Biopsy, Cattle, Conceptus, Sexing, Transfer

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Preimplantation genetic diagnosis (PGD) is the genetic analysis of embryos at the preimplantation stage. The major objectives of PGD in domestic species are to prevent transmission of genetic disorders, determine the sex of embryos and perform phenotypic characterization of economically relevant traits, such as milk and meat production capacity, prior to embryo transfer. Recent advances in molecular biology and genome projects have provided valuable molecular markers linked to economically relevant traits [1]. Thus, PGD will grow in importance as a tool to improve livestock species.

Biopsy of embryonic cells is prerequisite for PGD and must be carried out using a micromanipulator and microscope, since bovine embryos are small at blastocyst stages (diameter: 150–190 μm). Moreover, to achieve high pregnancy rates after embryo transfer, cells must be biopsied from embryos using minimally invasive techniques [2, 3]. Therefore, this method requires expensive equipment and a high level of skill, both of which prevent the widespread application of PGD to bovine embryos under field conditions.

After reaching the uterus and hatching from the zona pellucida, bovine embryos remain unattached from the uterine endometrium until implantation. During this period, the morphology of the bovine embryo changes dramatically from spherical to filamentous, in a process known as elongation. The conceptus grows from 2 mm in length on day 13 of gestation to 6 mm at day 14, reaching 60 mm by day 16 [4]. Thus, the conceptus is visible to the naked eye during the elongating stage, making it possible to perform biopsies without specialized equipment or skills. Despite the clear advantages of using the elongating bovine conceptus for PGD, few studies have investigated the feasibility of uterine transfer of these conceptuses. In one report, day 10–15 bovine conceptuses were transferred to synchronized recipient cows, yielding a pregnancy rate at day 42 of 50.4%, suggesting that the bovine conceptus can be successfully transferred to recipients [4]. However, in this report, all conceptuses were transferred surgically, a technique that is difficult to apply under field conditions.

The objective of this study was to establish an easy nonsurgical method of transferring elongating bovine conceptuses following biopsy that does not require expensive equipment such as a micromanipulator.

Materials and Methods

All experimental procedures involving animals were approved by the Committee for the Care and Use of Experimental Animals at the National Institute of Livestock and Grassland Science. All cows were fed with glass silage on the farm of the National Institute of Livestock and Grassland Science.

Embryos or conceptuses collection

Japanese Black cows with an average body weight (BW) of 413 kg and parity of 3.1 were used as donors in this study. Progesterone-releasing intravaginal devices (PRIDs) (Aska Pharmaceutical, Tokyo, Japan) were inserted into cow vaginas at random days in the estrus cycle. After 4 days, cows were given multiple administrations of porcine pituitary-derived follicle-stimulating hormone (pFSH) (24 AU, Antrin R10, Kyoritsu pharmaceutical, Tokyo, Japan) intramuscularly

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Correspondence: K Kimura (e-mail: kimurak@affrc.go.jp)

at 12 h intervals for 3 days with a step down regime [5]. Forty-eight hours after initiation of FSH treatment, the PRID was removed, and a $PGF_{2\alpha}$ analogue (750 µg, Zenoadin C, Zenoaq, Fukushima, Japan) was injected intramuscularly to induce luteolysis. Artificial insemination (AI) was performed at 12 and 24 h after detection of standing estrus with frozen-thawed semen. On 7, 13 or 14 days after the first AI, embryos or elongating conceptuses were collected by nonsurgical uterine flushing [5]. When the day 13 or 14 conceptuses were recovered, catheters with enlarged side holes (10 mm length, 2 mm width) were used, and the uteri were manipulated gently due to the fact that the conceptuses at this stage are fragile. The quality of day 7 morulae or blastocysts were classified according to the International Embryo Transfer Society (IETS) manual [6], and only Code 1 and 2 embryos were frozen by conventional methods using 1.8 M ethylene glycol as a cryoprotectant [7]. The recovered conceptuses were placed within one of three morphological conditions (intact, shrunken or fragmented), and the lengths of day 13 or 14 conceptuses were recorded. The lengths of transferred day 13 or 14 conceptuses were 0.2–10.5 mm. The conceptuses with identifiable embryonic discs in all morphological conditions were used for transfer experiments.

Day 7 embryo transfer (ET)

A total of 81 crossbred cows (Japanese Black × Holstein) were used as recipients (average BW and parity of 421 kg and 2.7, respectively). Frozen day 7 embryos were thawed in a 37 C water bath, and each embryo was cultured in a 10 µl drop of modified synthetic oviduct fluid (mSOF) supplemented with 1.5 mM glucose and 5% FBS under low oxygen conditions (5% O_2) to confirm their viability [8]. After 6–12 h of culture, the conditions of embryos were reevaluated according to the IETS manual [6], and each Code 1–2 embryo was transferred to a uterine horn ipsilateral to the corpus luteum (CL) of a recipient cow synchronized with the embryo using an ET gun and standard sheath (IMV Technologies, L'Aigle, France).

Day 13 or 14 elongating conceptus transfer (CT)

A total of 196 crossbred cows (Japanese Black × Holstein) were used as recipients for the CT experiment (80 cows for day 13 CT and 116 for day 14 CT). The estrous cycles of recipient cows were synchronized with those of the donors. Day 13 conceptuses were transferred to the recipients using ET guns with standard sheaths, as described above. In the case of day 14 conceptuses, three methods were used for transfer. The first method used ET guns and standard sheaths, similar to day 7 ET. The second used a two-way balloon catheter, which is usually used for embryo collection. After introducing the balloon catheter into the uterine horn ipsilateral to the CL, 5 ml of air was injected to inflate the balloon, and then the metal stylet was removed. A 0.5 ml straw containing a conceptus was connected to the plug of the catheter, and the conceptus was introduced to the lumen of the catheter by pushing the straw's cotton plug. Immediately after removing the straw, a syringe containing 10 ml (twice the catheter dead volume) of TCM199 supplemented with 10% FBS was connected to the plug of the catheter, and all the medium was then injected into the uterus. After transferring the conceptus, the air filling the balloon was sucked out, and the catheter was removed from the uterine horn. In the third method, the conceptus was transferred in the same way as in day 7 ET, except ET guns with homemade modified sheaths were used. The tip of the sheath was cut off, and the cut ends were smoothened with a file (Fig. 1).

Biopsy of conceptuses

Biopsy of conceptuses was carried out under a stereomicroscope or with the naked eye. From one end of the conceptus, a 0.2–0.5 mm long fragment was removed using a surgical razor blade (No. 14, FEATHER Safety Razor, Osaka, Japan) in a 100 µl drop of Dulbecco's mPBS supplemented with 10% FBS. Individual biopsied specimens were transferred to PCR tubes containing 6 µl of autoclaved distilled water and subjected to sexing, as described below. Each biopsied conceptus was transferred to 1ml of TCM199 supplemented with 10% FBS and cultured in 5% CO_2 in air at 38.5 C until it was transferred to the recipients (< 2 h) using a balloon catheter, as described above.

Sexing of conceptuses

Sexing of conceptuses was carried out using a bovine embryo sexing kit (Loopamp Bovine Embryo Sexing Kit, Eiken, Japan), according the manufacturer's instructions [9].

Pregnancy diagnosis

All recipient cows were monitored for standing estrus twice a day after transfer until day 80 post estrus. Pregnancy diagnosis was confirmed by ultrasonography every 10 days from day 30 until day 80 of gestation. Pregnancy was confirmed by the presence of a fetal heartbeat.

Experimental design

In experiment 1, we compared the pregnancy rate and status after day 7 ET and day 13 CT. Moreover, in day 13 CT, the effect of the morphological conditions of conceptuses on pregnancy rate and the relationship between the size of the conceptus and pregnancy rate after transfer were investigated.

In experiments 2 and 3, only day 14 conceptuses were used. In experiment 2, the pregnancy rates following day 14 CT using three different methods (balloon catheter, ET gun with standard sheath, or ET gun with modified sheath) were compared. In experiment 3, we investigated the application of day 14 CT to biopsy and sexing of conceptuses.

Statistical analysis

The data concerning the relationship between the size of the conceptus and resultant pregnancy rate after transfer in experiment 1 were analyzed by Fisher's exact test followed by Tukey's multiple range test. The data gathered on the lengths of conceptuses in experiment 3 were analyzed with the Kruskal-Wallis test using the SAS software (SAS Institute, Cary, NC, USA). The other data were analyzed using a chi-square (χ^2) test. A probability value of $P < 0.05$ or 0.01 was considered statistically significant.

Results

Experiment 1: Comparison of pregnancy status between day 7 ET and day 13 CT

As shown in Table 1, the overall pregnancy rates were 45.7% for

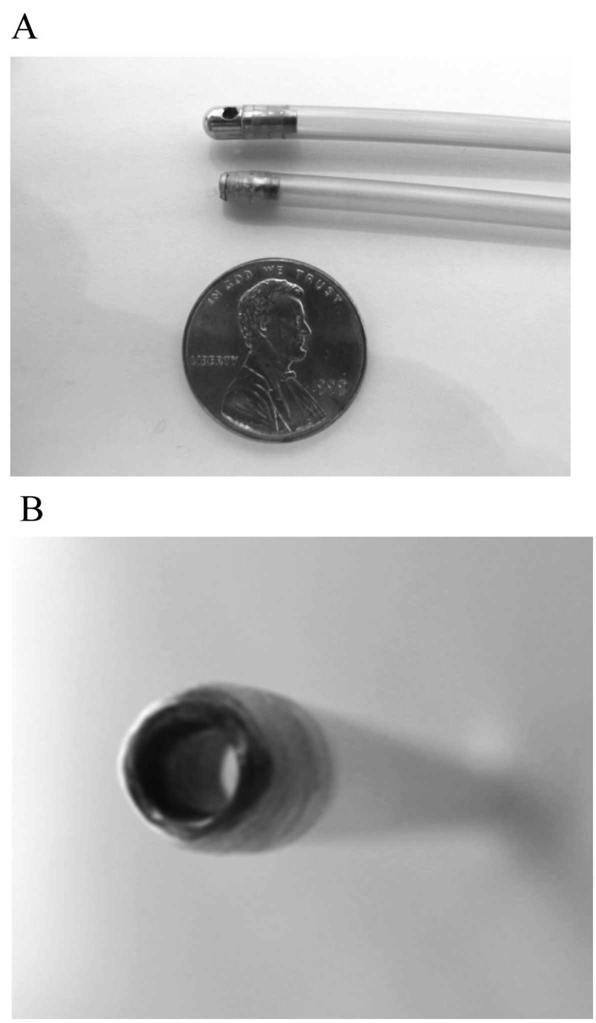

Fig. 1. Photographs of modified sheaths for day 14 CT. The tip of the sheath that was used for standard day 7 ET was cut off, and an opening was made (A). The diameter of the hole at the side of the standard sheath was approximately 1.5 mm. The ridge of the newly made opening was smoothed by a file to avoid damage to the uterine endometrium and conceptus (B). The diameter of the newly made opening was approximately 1.5–2.0 mm.

day 7 ET and 48.8% for day 13 CT, with no significant difference between the two transfer methods (P > 0.05). The incidence of return to estrus of nonpregnant cows in both transfer techniques is shown in Fig. 2. For day 7 ET, 70.5% of nonpregnant cows exhibited estrus with a normal estrus cycle length (< 25 days). In contrast, with day 13 CT, the rate of nonpregnant cows was significantly reduced (31.7%, P < 0 .05). Moreover, the rates of nonpregnant cows returned to estrus after day 30 in day 7 ET and day 13 CT were 18.2% and 51.2%, respectively (P < 0.01). Half of them had no fetus present, but fetal membranes were evident (data not shown).

Table 1. Comparison of pregnancy rate between day 7 ET and day 13 CT

Transfer methods	No. of cows treated	No. of cows pregnant (%)
Day 7 ET	81	37 (45.7)[a]
Day 13 CT	80	39 (48.8)[a]

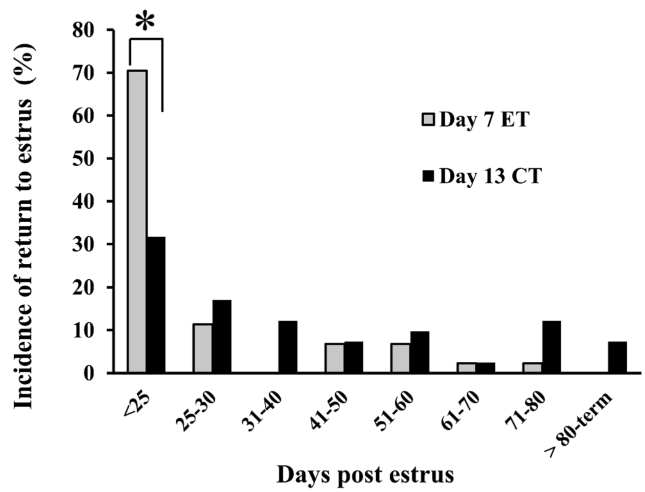

Fig. 2. Incidence of return to estrus in cows after day 7 ET and day 13 CT. The percentages of cows that returned to estrus out of the total number of nonpregnant cows are shown. The asterisk indicates a significant difference among groups (P<0.05). The total numbers of nonpregnant cows after day 7 ET and day 13 CT were 44 and 41, respectively.

Effect of morphological conditions of conceptuses on pregnancy rate after day 13 CT

Elongating conceptuses are not surrounded by zona pellucidae and are consequently more fragile and sticky compared with day 7 blastocysts. Following uterine flushing, 42.6% of conceptuses were shrunken or fragmented (non-intact). Therefore, the pregnancy rates with intact and non-intact day 13 conceptuses following transfer to recipient cows were compared. Fifty-nine intact and 22 non-intact conceptuses were transferred. The pregnancy rates for intact CT (31/59, 47.5%) and non-intact CT (11/22, 50.0%) did not differ significantly (P > 0.05), indicating that the morphological conditions of conceptuses after recovery did not influence pregnancy following transfer.

The relationship between conceptus size and pregnancy rate after transfer

The sizes of recovered conceptuses are variable, but it is unclear whether conceptus size influences the pregnancy rate after transfer. Therefore, the relationship between conceptus size and pregnancy rate after CT was investigated. In this experiment, the average size of transferred day 13 conceptuses was 1.7 ± 0.2 mm (ranged from 0.2 to 10.5 mm). As shown in Fig. 3, the pregnancy rate gradually increased with conceptus length up to 3 mm, but when the length of the conceptus was ≥3 mm, the pregnancy rate was significantly reduced (P<0.05).

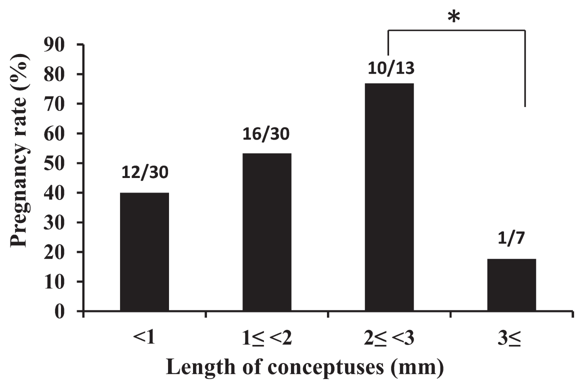

Fig. 3. The relationship between the lengths of day 13 conceptuses and pregnancy rates after CT. The asterisk indicates a significant difference among groups (P<0.05).

Experiment 2: Development of a transfer method for longer conceptuses

As demonstrated in experiment 1, when longer conceptuses were transferred, the pregnancy rate was compromised. To establish a transfer method for longer conceptuses, we used day 14 conceptuses (> 3 mm) and transferred them using a catheter or an ET gun with a homemade modified sheath (a large opening at the tip, Fig. 1). As a control, an ET gun with a standard sheath was used. As shown in Table 2, when the ET gun with the modified sheath or the catheter was used, the pregnancy rates were 48.0% and 44.8%, respectively. In contrast, in the control using the ET gun with a standard sheath, no pregnant cows were obtained after CT. There was no significant difference in conceptus length among groups (P > 0.05).

Experiment 3: The pregnancy rate with day 14 CT after biopsy

In this experiment, we investigated whether biopsy of conceptuses affects the pregnancy rate after CT. As shown in Table 3, even conceptuses that were subjected to biopsy before transfer could establish pregnancy at the same rate as controls. Notably, biopsied specimens were subjected to sexing before transfer in experiment, and all calves derived from CT after biopsy showed the expected sexes and grew normally until at least 6 months after birth.

Discussion

The aims of this study were to develop a nonsurgical bovine CT method and to investigate its application for PGD, such as sexing. Using a nonsurgical transfer method, we obtained pregnancy rates following CT that were comparable with that following standard day 7 ET. The day 14 conceptus is large enough to biopsy without a micromanipulator. Moreover, following biopsy for sexing, the pregnancy rate after CT did not decrease, and we produced calves of the expected sexes.

As shown in experiment 1, the morphological conditions of day 13 conceptuses at recovery did not affect the pregnancy rate after transfer. Another report also demonstrated that damage to the conceptus did not affect the pregnancy rate after transfer [10], suggesting that the conceptus can recover from limited damage after transfer. With day 13 CT, the pregnancy rate after transfer of longer conceptuses (≥ 3 mm) using the standard ET method decreased significantly, and no pregnancies were obtained with day 14 CT using the standard ET method. However, the pregnancy rate of day 14 CT was dramatically improved when the transfer method was modified using a balloon catheter or a modified sheath. The reason why pregnancy was not obtained in longer CT using the standard sheath was not been revealed in this experiment. One possible reason may be the position of the holes. In standard sheath, the holes are located on the side of the tip. We speculated that since the side of the tip of the sheath would attach tightly to the uterine endometrium, irreversible damage might have occurred to longer conceptuses when they were transferred into the uterus from ET guns with standard sheaths. On the other hand, when the balloon catheter or modified sheath was used, there might have been space between the uterine wall and the catheter or ET gun, and the expelled conceptus might not have been damaged seriously.

Biopsy of embryonic cells is essential for PGD and typically requires expensive equipment such as a micromanipulator and a high level of skill. However, we demonstrate here that PGD can be performed with ease and without specialized equipment. In experiment

Table 2. The pregnancy rate following day 14 CT using various transfer tools

	No. of cows treated	No. of cows pregnant (%)	Averages of conceptus lengths (mm ± SEM)
ET gun + sheath with a large opening at the tip	29	13 (44.8)[a]	18.9 ± 2.5
Catheter	25	12 (48.0)[a]	13.5 ± 1.8
ET gun + standard sheath (control)	11	0 (0)[b]	11.3 ± 1.4

The data with different superscripts within the same column differ significantly (P<0.05).

Table 3. The pregnancy rate of day 14 conceptuses after biopsy, sexing and transfer

Conceptus	No. of cows transferred	Pregnant (%)	Averages of conceptus lengths (mm ± SEM)	No. of calves of expected sex (%)
Biopsied	26	12 (46.2)	20.7 ± 3.1	12 (100)
Non-biopsied (control)	25	11 (44.0)	19.1 ± 2.1	-

3, the length of conceptuses biopsied ranged between 2.5 to 34 mm (data not shown), which permitted biopsy using a stereomicroscope or by the naked eye. Recently, a novel DNA amplification method, loop-mediated isothermal amplification (LAMP), was developed [11] and applied to bovine embryo sexing [9]. Although this sexing method is fast and easy, biopsy of cells from bovine blastocysts using a micromanipulator is necessary [9]. In the present study, we combined a nonsurgical conceptus transfer technique with this simple sexing method. Twenty-six conceptuses were biopsied, sexed and transferred to recipient cows. Twelve cows were pregnant and delivered calves, all with the expected sex. These results indicate that CT permits use of the LAMP sexing method without the need for expensive equipment or high-level skills. Therefore, this method is adaptable to field conditions.

Along with advances in genome research, the number of genes that will be of interest to ranchers will increase. For multiple gene analysis in PGD, more DNA from biopsied embryonic cells is necessary. However, the number of cells that can be biopsied from blastocyst-stage embryos is limited, since the viability of the embryos decreases after cryopreservation and ET when more embryonic cells are biopsied [2, 3]. To solve this problem, a whole genome amplification (WGA) method has been developed for individual biopsied embryonic cells and has been used to evaluate multiple genotypes [12], but another study suggests that the repeatability of the results acquired from the WGA analysis for genotyping is not high [13]. Here, we biopsied 0.2–0.5 mm fragments of conceptuses that contained 388–7600 cells (data not shown). Moreover, we demonstrated that the biopsy of conceptuses did not affect the pregnancy rate after transfer. Thus, the nonsurgical bovine CT technique we describe combined with biopsy might enable multiple gene analysis without requiring amplification of DNA and decreasing viability.

In the present study, the time of return to estrus of nonpregnant cattle differed between the two transfer methods. The rate at which cows returned to estrus in normal cycles (< 25 days) following day 13 CT decreased significantly compared with that following day 7 ET (Fig. 2). When the conceptuses secrete IFNτ during the period of maternal recognition of pregnancy (days 16–18 post estrus), PGF2α secretion is inhibited, and the estrus cycle is prolonged [14–16]. Taken together, it was suggested that the majority of embryo loss occurred before the period of maternal recognition of pregnancy in day 7 ET. This is consistent with the result of a previous report, in which major embryo loss occurred by day 14 [17]. On the other hand, only 32% of nonpregnant cows transferred day 13 conceptuses, which secrete IFNτ [18], showed estrus in the normal cycle. We previously reported that the expression of interferon-stimulated gene 15 (*ISG15*), which responds to IFNτ, is detected in peripheral blood mononuclear cells during the period of maternal recognition of pregnancy in the nonpregnant cows showing prolonged estrus cycle after AI or ET [19]. Therefore, even if pregnancy is not established, transferred conceptuses might survive for a while and produce IFNτ during the period of maternal recognition of pregnancy, and as a result, the estrus cycle could be prolonged. CT can bypass the period in which major pregnancy loss occurs in day 7 ET, and transferred conceptuses can secrete IFNτ (advantages for pregnancy); nevertheless, the overall pregnancy rate after day 13 CT did not differ from that of standard day 7 ET. This result supports the previous finding that the cause of pregnancy loss in recipients is already present at the time of transfer [20].

Moreover, in the present study, more nonpregnant cows returned to estrus after day 30 in day 13 CT than day 7 ET (51.2% *vs.* 18.2%). Half of them had no fetus present, but fetal membranes were evident. Under field conditions, the presence of nonpregnant cows with extended estrous cycles would adversely affect farm management. Therefore, pregnancy diagnosis using ultrasound scanning is recommended after CT.

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