Production of Trophoblastic Vesicles Derived From Day 7 and 8 Blastocysts of In Vitro Origin and the Effect of Intrauterine Transfer on the Interestrous Intervals in Japanese Black Heifers

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Abstract. This study was undertaken to produce trophoblastic vesicles (TVs) by using blastocysts of in vitro origin and to estimate the effect on the interestrous interval after transfer of 4 TVs into the uteri of heifers on Day 7. Morphological examination under a stereoscopic microscope revealed that the total formation rate of TVs prepared from IVP expanded blastocysts was 80.5% and that there was no difference in the formation rates of TVs derived from blastocysts between Day 7 (83.5%) and Day 8 (78.9%). After intrauterine transfer of TVs, observation of the corpus luteum (CL) by transrectal ultrasonography together with measurement of the plasma progesterone concentration confirmed that 2 of 4 recipients (50%) had a longer interestrous interval, 33.5 and 35.0 days, while the other 2 recipients had normal cycles, 20.0 and 24.5 days. Consequently, the average number of days after intrauterine transfer of TVs compared with the 2 consecutive cycles just before the treatment was longer than in the controls (6.1 ± 2.4 days vs. -0.8 ± 1.1 days, P<0.05). These results indicate that preparation of TVs from blastocysts of in vitro origin is a useable method and that TVs from blastocysts may have the capacity to maintain CL function after intrauterine transfer.

Key words: Blastocyst, Corpus luteum, Estrous cycle, Trophoblastic vesicle

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A prolonged cycle and delay of luteolysis has been confirmed by injection of homogenates of Day 17–18 bovine embryos into the uteri of heifers on Day 14–15 of the cycle [9]. Furthermore, trophoblastic vesicles (TVs), which are derived from Day 14 embryos of in vivo origin, have been transferred into the uteri of heifers on Day 12 [10]. Transfer of TVs produced a longer cycle (4–16 days delay) compared with the interestrous intervals obtained after twice daily intrauterine injection of homogenates of embryos (3–4 days delay) [9, 10]. Moreover, intrauterine transfer of demi-embryos with TVs, which are derived from conceptuses cultured in vivo following transfer of embryos of in vitro origin, enhances the pregnancy rate in the early stage (Day 26–43) of pregnancy (day from estrus), but not in the mid (Day 38–73) and late stages (Day 280–299) [11]. However, preparation of TVs from conceptuses cultured in vivo is still labor intensive. Therefore, it is necessary to establish a technique to produce TVs without culture in vivo and that is less labor intensive.

Previously, viable TVs derived from in vitro Day 13 and 20 blastocysts have been established [12, 13]. In addition, a trophoblastic cell line has been established using hatched and cultured blastocysts to produce TVs [14]. During the pre-elongation stages of blastocyst development in vitro, IFN-τ can be detected in the non-hatched Day 7 blastocyst [15]. The expression of IFN-τ mRNA has been detected as early as blastocyst formation, soon after the blastocyst expands and just prior to the time that the zona pellucida ruptures and hatching occurs in vitro [16]. The level of IFN-τ secretion from the embryo temporarily increases during the preim-
placental transfer of TVs prepared from Day 7 and 8 blastocysts of \textit{in vitro} origin into the uteri of heifers on Day 7 may mimic the change of IFN-\(\gamma\) secretion \textit{in vivo} and have the potential to support IFN-\(\gamma\) secretion adequately when used for co-transfer with embryos. However, there are no studies available concerning production of TVs from \textit{in vitro} expanded blastocysts and verification of the proper time-point at which to use expanded blastocysts (Day 7 or 8) for production of TVs. Moreover, the effect of intrauterine transfer of TVs prepared from expanded blastocysts of \textit{in vitro} origin on the interobserver interval in the heifer has not been estimated. The purpose of the present study was to produce TVs derived from Day 7 and 8 IVP blastocysts and evaluate whether they have the capacity to maintain CL function and prolong the interobserver interval.

\textbf{Materials and Methods}

\textit{In vitro maturation and fertilization}

Bovine ovaries obtained from a slaughterhouse were transported to the laboratory in sterile Dulbecco’s phosphate buffer saline (D-PBS) and were stored overnight at 15 \(\degree\)C. In Japan, the use of bovine materials, including ovaries, is prohibited by the Abattoirs Law until bovine spongiform encephalopathy (BSE) testing results prove negative. The cumulus-oocyte complexes (COCs) were aspirated from the follicles (3–7 mm in diameter) with an 18-gauge needle attached to a 10-ml syringe. The COCs were washed in TCM-199 (Invitrogen, Grand Island, NY, USA) containing 0.3\% polyvinylpyrrolidone. 100 IU/ml penicillin G potassium and 100 \(\mu\)g/ml streptomycin. The COCs were then washed three times in TCM-199 supplemented with 10\% heat-inactivated fetal calf serum (FCS, Cansera International, Canada), 0.02 AU/ml porcine FSH (Denka Pharmaceutical, Kawasaki, Japan), 0.055 mg/ml sodium pyruvate, 100 IU/ml penicillin G potassium and 100 \(\mu\)g/ml streptomycin by using a 35-mm plastic petri dish (Falcon 1008; Becton Dickinson, Franklin Lakes, NJ, USA). Finally, the 20 COCs were transferred in 100-\(\mu\)l drops covered with liquid paraffin (Nakarai Tesque, Kyoto, Japan) and cultured for 21 to 24 h at 38.5 \(\degree\)C in a humidified atmosphere of 5\% CO\(_2\).

Frozen-stored semen from a Japanese Black bull in a 0.5-ml straw was thawed at 37 \(\degree\)C. The semen was suspended in 10 ml of Bracket and Olipant’s (BO) solution [17] containing 10 mM caffeine (Sigma, St. Louis, MO, USA). After washing twice by centrifugation for 5 min at 600 \(\times\)g, the concentration of the spermatozoa was adjusted to 1 \(\times\)10\(^7\) cells/ml. The sperm suspension was then diluted two-fold with BO solution containing 20 mg/ml BSA (Sigma) and 10 IU/ml heparin (Aventis Pharma, Tokyo, Japan). After 21 to 24 h of maturation, 20 COCs were washed three times in BO solution containing 5 mM caffeine, 10 mg/ml BSA (Sigma) and 5 IU/ml heparin and then transferred into 50-\(\mu\)l drops of sperm suspension for adjusting to 5 \(\times\)10\(^6\) sperm/ml. \textit{In vitro} fertilization was carried out for 6 h at 38.5 \(\degree\)C in a humidified atmosphere of 5\% CO\(_2\).

After insemination (=Day 0), the COCs were rinsed and transferred in Charles Ronsenkrans (CR1aa) medium [18] containing 0.3 \% BSA (BSA-CR1aa). The cumulus cells were then individually removed without enzyme using a handmade pipette by mouth pipetting. The 20 denuded oocytes were transferred into 100 \(\mu\)l BSA-CR1aa at 38.5 \(\degree\)C in a humidified atmosphere of 5\% CO\(_2\), 5\% O\(_2\) and 90\% N\(_2\). On Day 2, early embryogenesis was checked morphologically using a stereoscopic microscope. The embryos were transferred into TCM-199 containing 0.3\% BSA on Day 5, and then only excellent and good quality embryos were selected and cultured until Day 8.

\textit{Preparation of trophoblastic vesicles}

Bovine TVs were prepared from Day 7 or 8 excellent and good quality expanded blastocysts with the zona pellucida and a clear inner cell mass. The zona pellucida of each blastocyst was attached to the base of a 90-mm plastic petri dish in 100-\(\mu\)l drops of D-PBS (+) containing 0.1 M sucrose to increase the tolerance for pressure exerted by cutting [19, 20]. The blastocyst was cut in half at a clear line between the trophoblastic tissue and inner cell mass by hand with a surgical blade (No.14; Futaba, Tokyo, Japan) under a stereoscopic microscope within 2–3 min (Fig.1A). The inner cell mass was then removed using a handmade pipette by mouthpipetting. During the handling, the 90-mm plastic petri dish was on a heat board kept at 38.5 \(\degree\)C. The trophoblastic tissues (Fig.1B) were cultured at 38.5 \(\degree\)C in 100-\(\mu\)l drops of TCM-199 supplemented with 0.1 mM cysteamine (Sigma) and 10\% FCS under controlled gas conditions, 5\% CO\(_2\), 5\% O\(_2\) and 90\% N\(_2\). The trophoblastic tissues that changed into spherical vesicles within 6–24 h of culture were considered to be trophoblastic vesicles (TV, Fig.1C).

\textit{Animals}

The animals used in this study received humane care as outlined in the Guide for the Care and Use of Experimental Animals (Animal Care Committee, National Institute of Livestock and Grassland Science). All animals were housed in a free stall barn with a concrete floor and had access to a pasture area. Four recipients, Japanese black heifers, were selected based on palpation per rectum of genitalia and ovaries to assess normality by transrectal ultrasonography (SSD-900; Aloka, Mitaka, Tokyo, Japan) with a 7.5-MHz linear probe. They had at least 2 estrous cycles of normal length before being used. For estrous synchronization, luteolysis was induced by intramuscular (i.m.) injection of 2.0 ml of a PGF\(_{2\alpha}\) analogue (Estrumate\textsuperscript{TM}; Shering-Plough Animal Health, Tokyo, Japan), and ovulation was ensured by ultrasonography during the pretreatment and treatment estrous cycles in all animals.

\textit{Transfer of trophoblastic vesicles}

In each replicate for both the experimental and control groups, transfers were performed on Day 7 (estrus=Day 0) of the induced cycle under the same treatment conditions used for estrous synchronization. Transfers were performed non-surgically using a Cassou gun (IMV Technologies, L’Aigle Cedex, France) [21] with 4 TVs (130–250 \(\mu\)m) prepared from Day 7 and 8 blastocysts, which might be equivalent to 2 embryos. The TVs were loaded into a 0.25-ml plastic straw with D-PBS containing 10\% calf serum (CS, Invitrogen Corp.) and transferred into the uterine horn ipsilateral to the CL. The side of the ovary, left or right, that contained the CL was detected by ultrasonography. A control group of 4 recipient heifers was injected with 10\% CS-D-PBS only by the same
After transfer, estrous was detected by checking each recipient (experimental or control) for signs of estrous twice daily (dawn and dusk). The longer axis of the CLs were examined by transrectal ultrasonography on Day 6, 12, 15 and 18 and every second day thereafter until standing estrous; subsequently, they were examined every day until the next ovulation. Standing estrous was the criterion used for determination of the interestrous interval.

Simultaneously, blood samples were collected from the jugular vein for P₄ measurement every day from Day 1 and were centrifuged for 20 min at 2000 × g. The plasma samples were then immediately frozen at −30°C until further analysis.

**Hormone determination**

The concentration of P₄ was determined in duplicate by enzyme immunoassays (EIA) after diethyl ether extraction using 96-well ELISA plates (NUNC-Immuno Plate, NUNC™ Brand Products, Roskilde, Denmark). The extraction and EIA for determination of the plasma P₄ concentration were performed as previously described [22]. The standard curve ranged from 0.05 to 50 ng/ml, and the effective dose (ED₅₀) of the assay was 2.4 ng/ml. The intra- and interassay coefficients of variation (CVs) averaged 6.2 and 9.3%, respectively. The recovery rate of P₄ was 85%.

**Statistical analysis**

The prolonged number of days after intrauterine transfer of TVs or D-PBS was calculated by subtraction of the average of 2 interestrous intervals in the pretreatment period from that during treatment. All data are expressed as means ± SEM. The statistical significance of differences was assessed by one-way ANOVA followed by Bonferroni’s multiple comparison test. Probabilities less than 5% (P<0.05) were considered significant.

**Results**

**Effect of the days of blastocyst development used for preparation of trophoblastic vesicles**

Development of *in vitro* produced blastocysts for preparation of TVs and the proportion of TV formation are described in Table 1. In 18 replicates, a total of 3139 oocytes were chosen for production of *in vitro* blastocysts. The average percentage of cleavage was 79%, and the average percentage of blastocysts per cleavage embryos was 42.3%. The total TV formation rate was 80.5%, and there was no difference in the proportion of TV formation from blastocysts between Day 7 (83.5 %, n=133) and Day 8 (78.5 %, n=114). Based on this data, the transfer of TVs was examined using TVs from both Day 7 and 8 blastocysts.

**Effect of vesicle transfer on the interestrous interval and maintenance of the corpus luteum**

The interestrous intervals during pretreatment and treatment in the experimental and control heifers are shown in Table 2. Two of the 4 experimental recipients of TVs exhibited prolonged estrous cycles (33.5 and 35.0 days). However, in the 4 control recipients, cyclic luteolysis was completed normally 22.3 ± 0.9 days after the previous estrous. The interestrous interval tend to increase after TV transfer compared with the average interestrous interval of the 2 pretreatment estrous cycles (23.1 ± 1.0 vs. 29.3 ± 2.9; P=0.0614). In addition, the average number of prolonged days was longer in the experimental group than in the control group (6.1 ± 2.4 days vs. −0.8 ± 1.1 days; P<0.05). The changes in the longer axis of the CL and plasma P₄ concentration during the treatment period are shown in Fig. 2. In the animals with prolonged estrous cycles, regression of the CL occurred on Day 33.5 and Day 35, as was reflected in the
This study demonstrated production of TVs derived from Day 7 and 8 IVP expanded blastocysts. In our data, there was no difference in the proportion of TV formation between Day 7 and 8 expanded blastocysts. We also demonstrated that the interestrous interval may be prolonged after transfer of in vitro produced TVs into the uteri of heifers on Day 7, suggesting that TVs derived from Day 7 and 8 blastocysts of in vitro origin may have the capacity to maintain the CL after intrauterine transfer.

In this study, there was no difference in TV formation rate between Day 7 (83.5%) and Day 8 expanded blastocysts (78.5%). Morphological evaluation under a stereoscopic microscope showed that both Day 7 and 8 expanded blastocysts may be at the proper time-point for use in producing TVs. TVs were prepared from Day 7 and 8 expanded blastocysts without specific facilities, such as a manipulator. TVs have previously been established from approximately Day 14 conceptuses cultured in vivo [10, 11]. It is possible to produce TVs not only by using conceptuses cultured in vivo, but also by using in vitro expanded blastocysts. However, further studies are needed to evaluate the production of IFN-τ in TVs prepared from Day 7 and 8 IVP expanded blastocysts for selection of good quality TVs and to compare them with TVs derived from in vivo conceptuses, which are already known to produce IFN-τ. Recently, IFN-τ has been shown to play a role in the progression of embryo development in culture [23]. Otherwise, IVP blastocysts that form earlier, on Day 7 or 8, are much lower producers of IFN-τ than those that form later, on Day 9 or 10 [24]. Additionally, there is evidence that transfer of faster developing blastocysts of in vitro origin may have the capacity to maintain the CL after intrauterine transfer.

### Discussion

This study demonstrated production of TVs derived from Day 7 and 8 IVP expanded blastocysts. In our data, there was no difference in the proportion of TV formation between Day 7 and 8 expanded blastocysts. We also demonstrated that the interestrous interval may be prolonged after transfer of in vitro produced TVs into the uteri of heifers on Day 7, suggesting that TVs derived from Day 7 and 8 blastocysts of in vitro origin may have the capacity to maintain the CL after intrauterine transfer.

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origin has a tendency to increase the pregnancy rate [25]. These findings indicate that an adequate level of IFN-τ is critical for each time-point of the embryo’s growth. Hence, TVs derived from Day 7 and 8 IVP blastocysts may secrete an adequate amount of IFN-τ when it is needed for embryo growth and the pregnancy recognition signal after intrauterine transfer at the same time as embryo transfer.

In the present study, the interest boxes intervals of the recipients were prolonged after intrauterine transfer of 4 TVs. Two of the 4 recipient animals in the experimental group showed significant maintenance of the CL structurally and functionally. These data may imply that the TVs secreted IFN-τ in utero to prevent luteolysis in our study. Moreover, these data were consistent with a previous report, which showed that the CL and plasma P4 level were also maintained after transfer of TVs derived from more aged (Day 14) embryos of in vivo origin into the uteri of heifers on Day 12 [10]. Although we did not examine the uteri of the recipients who had a prolonged cycle after transfer of TVs, elongation of TVs was investigated in the uterus on Day 25–28 of the estrous cycle in a previous study [10]. The antiluteolytic effects of IFN-τ include inhibition of endometrial estrogen receptor mRNA expression, thereby inhibiting expression of oxytocin receptor and production of PGF2α pulses [26–28]. Additionally, IFN-τ suppresses TNFα-induced PGF2α by decreasing COX2 gene expression in the stromal cells of the bovine endometrium [29]. These findings suggest that the TVs we transferred may have developed until an adequate time and secreted IFN-τ, after which IFN-τ inhibited the uterine PGF2α in the heifers with a prolonged cycle.

However, in the present study, 2 heifers into which TVs were transferred did not have a prolonged cycle nor did they maintain the CL. There is possibility that failure of CL maintenance might be caused by the lack of a receptor and insensitivity to IFN-τ as suggested in a previous report [30]. Additionally, degeneration or loss of TVs may occur shortly after intrauterine transfer. In our study, we selected TVs by morphological criteria that changed into spherical vesicles from trophoblastic tissues within 6–24 h of culture after the expanded blastocysts. This may have induced an unequal secession of IFN-τ from the TVs we produced; consequently, the effect of TV transfer on CL maintenance may be unstable due to secretion of IFN-τ as antiluteolysin. We did not evaluate the production of IFN-τ in the TVs prepared from Day 7–8 IVF blastocysts. Visible TVs having the capacity for IFN-τ secretion can be derived from IVF blastocysts on Day 13 [12, 13]. In addition, a previous report showed that expanded blastocysts produce IFN-τ [31]. These reports suggest that the TVs derived from expanded IVF blastocysts in the present study have the capacity to produce IFN-τ.

In conclusion, we established a method to produce TVs from Day 7 and 8 IVF blastocysts. This process was simple and has the potential to enable co-transfer of TVs with less viable embryos such as biopsied, vitrified and frozen embryos. Moreover, these TVs may have the capacity to maintain the CL after transfer to the uteri of heifers. Further studies are clearly required to apply TVs from blastocysts of in vitro origin to improvement of the pregnancy rate after embryo transfer.

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