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Technology Report

Generation of viable calves derived from developmentally mature blastocysts produced by on-gel culture

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Abstract. Conventional culture systems for bovine embryos are unable to support sustained embryonic development until the developmentally mature blastocyst stage. Although we have previously developed an on-gel culture system that enables bovine blastocysts to complete cell segregation events at day (D) 10 following *in vitro* culture, the development of D10 blastocysts to term has yet to be achieved. In this study, we attained full-term development of D10 mature blastocysts produced using an on-gel culture system. Two calves derived from on-gel-cultured embryos were vaginally born, showing normal birth and placental weights and no obvious morphological abnormalities. Moreover, we detected no abnormalities in blood metabolic profile analyses. Our findings indicate that on-gel culturing can be used to facilitate the development of developmentally mature blastocysts to term, and produce healthy viable calves. This culture system could make a valuable contribution to cattle production and would enable a range of analyses for characterizing bovine-specific pre-implantation development.
Key words: Blastcyst, Embryo transfer, Full-term development, On-gel culture

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In vitro culture (IVC) techniques can be used to facilitate the development of mammalian embryos from the single-cell to blastocyst stages [1], and *in vitro* culturing of embryos has become well established as an essential reproductive technology for animal production and developmental biology. In cattle, the transfer of embryos to the maternal uterus generally requires morphologically intact blastocyst embryos for non-surgical operations, and in this context, the use of IVC is often combined with other reproductive technologies, including *in vitro* maturation (IVM), *in vitro* fertilization (IVF), and cryopreservation, to ensure efficient *in vitro* production (IVP) of blastocysts.

During the process of blastocyst formation, mammalian embryos undergo two rounds of cell segregation [2]. First, cells differentiate to yield either a pluripotent inner cell mass (ICM) or trophectoderm (TE), forming extra-embryonic tissue [3]. In the subsequent second cell segregation, the ICM undergoes epiblast (Epi) differentiation, thereby forming a primitive endoderm (PrE) [4]. Thereafter, Epi and PrE give rise to the embryo proper and yolk sac, respectively. During the late blastocyst stage, the PrE layer is arranged in the laminae adjacent to the blastocoel. Consequently, these two rounds of cell segregation contribute to the development of a compact Epi, alignment of the PrE, and generation of the TE in the outermost layer, thereby giving rise to a developmentally mature blastocyst.

However, when using an IVC system, mouse embryos can develop into mature blastocysts at approximately embryonic day 4.5 [5]. It is not possible to generate mature bovine blastocysts by day (D) 8 using a conventional culture system [1], indicating that the IVC system currently in use for bovine embryos is, in certain respects, insufficient. Given that bovine embryos undergo a second cell segregation at approximately D9.5 [6], it is necessary to develop a stable IVC system that ensures the development and differentiation of bovine embryos after D8.

In this latter respect, we recently developed a novel bovine IVC system, in which, premature D8 blastocysts were cultured on agarose gel (on-gel culture) and were thereby exposed to both gaseous and liquid phases [6]. Accordingly, it was established that on-gel-cultured embryos survived *in vitro* to beyond D10, during which, the second cell segregation yielding Epi and PrE was completed [6]. D10 on-gel-cultured embryos were characterized by the presence of a TE lacking expression of the ICM marker OCT4, a PrE aligned toward the blastocoel cavity with the expression of SOX17, and Epi expressing pluripotency markers such as OCT4, NANOG, and SOX2. Given that these characteristics correspond to those typically observed in *in vivo*-derived embryos, this would indicate that on-gel culturing would represent an effective approach for facilitating the development of embryos that are comparable to their *in vivo* counterparts.

To validate the viability of the on-gel-cultured embryos, we performed embryo transfer (ET) analyses in accordance with the schedule shown in Table 1. Among the five sessions conducted, we established that two recipient Holstein heifers had become pregnant, although we were unable to confirm the full-term development of the corresponding conceptuses. Furthermore, given that IVC is considered a factor contributing to the development of large offspring syndrome (LOS), the physical condition of newborn calves

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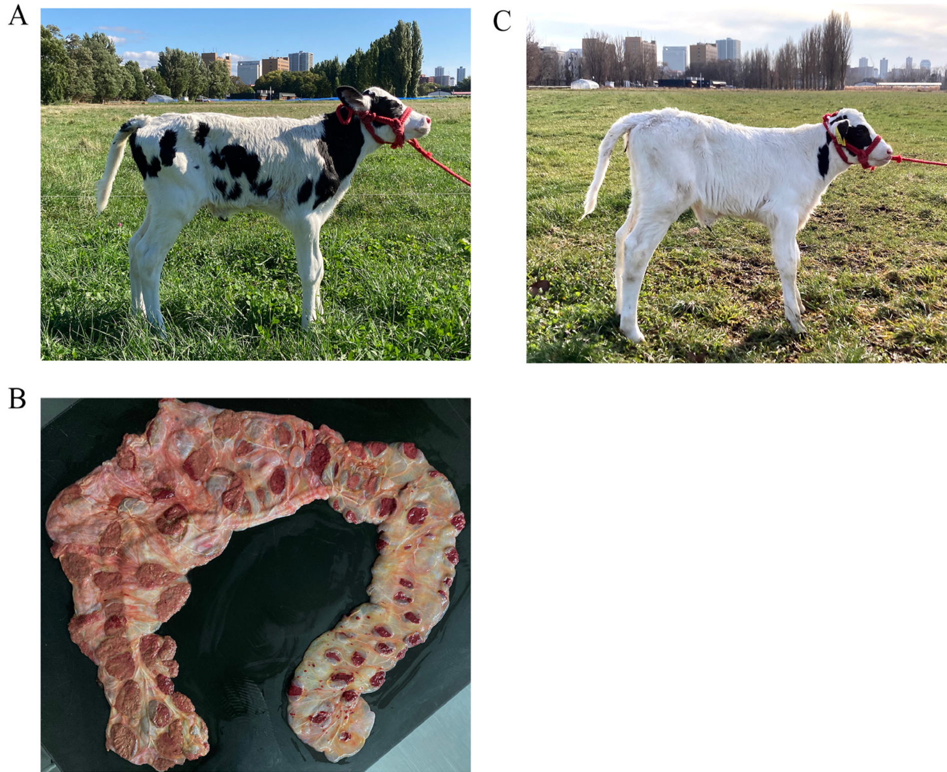

Fig. 1. Calves and afterbirth derived from on-gel-cultured embryos. Viable calves were generated via embryo transfer of three on-gel-cultured D10 embryos to three recipient Holstein cows, two of which subsequent became pregnant. The first calf (Jewel, shown at 3 days of age) was born vaginally on September 21, 2021 (A), and the afterbirth was morphologically normal (B). On this afterbirth, numerous small cotyledons were observed, particularly on the right-hand side of image. The second calf (Jewelry, shown at 9 days of age) was born vaginally on November 29, 2021 (C).

derived from on-gel-cultured embryos needs to be evaluated. LOS is characterized by a large size at birth and placental abnormalities [7–9], which are key concerns with respect to the production of cattle from IVP embryos. Given that on-gel culturing is an IVC system with an extended period of culturing, it is necessary to evaluate the integrity of fetuses and placentas derived from on-gel-cultured embryos for practical application. In this study, we confirmed the full-term development of two on-gel-cultured embryos following ET, for which we determined their birth weights and performed blood biochemical analyses. Furthermore, we monitored post-birth weights and determined the number of afterbirth cotyledons. Our observations indicate that on-gel culturing recapitulates pre-implantation development to D10, corresponding to that seen in their *in vivo* counterparts. Although further trials of ET using on-gel-cultured embryos are necessary to precisely evaluate the delivery rate, these findings indicate that this culturing system can be used to generate developmentally mature blastocysts for animal production and developmental biology research in cattle.

From each of the two pregnant recipients into which one D10 on-gel-cultured embryo had been transferred [6], a calf was vaginally born without assistance on September 21 and November 29, 2021, respectively (Fig. 1A, C), both of which showed spontaneous respiration and were of normal appearance. Their weights at birth (40 and 44 kg) and gestational ages (286 and 285 days) were within the typical ranges (Supplementary Table 1) [10]. At the time of reporting, they were still alive (March 25, 2022) and showed no obvious evidence of disorders. Thus, we confirmed the full-term development of D10 on-gel-cultured embryos via ET and verified the repeatability of cattle production using mature blastocysts produced using an

Table 1. Schedule for D10 on-gel-cultured embryo transfer

<table>
<tr><th></th><th>Embryo</th><th>Recipents</th></tr>
<tr><td>Day -10</td><td></td><td>CIDR device insertion
E2 (2 mg)</td></tr>
<tr><td>Day -2</td><td></td><td>CIDR device removal (AM)
$PGF_{2\alpha}$(25 mg twice, AM & PM)</td></tr>
<tr><td>Day -1</td><td>IVM</td><td></td></tr>
<tr><td>Day 0</td><td>IVF</td><td>GnRH 100 µg</td></tr>
<tr><td>Day 1</td><td rowspan="8">IVC in mSOFai medium drop
(Day 1 AM~Day 8 PM)</td><td></td></tr>
<tr><td>Day 2</td><td></td></tr>
<tr><td>Day 3</td><td></td></tr>
<tr><td>Day 4</td><td></td></tr>
<tr><td>Day 5</td><td></td></tr>
<tr><td>Day 6</td><td>CIDR device insertion</td></tr>
<tr><td>Day 7</td><td></td></tr>
<tr><td>Day 8</td><td></td></tr>
<tr><td>Day 9</td><td rowspan="2">On-Gel culture
(Day 8 PM~Day 10 AM)</td><td></td></tr>
<tr><td>Day 10</td><td>Embryo transfer (AM)</td></tr>
<tr><td>Day19</td><td></td><td>CIDR device removal</td></tr>
</table>

Estradiol benzotoa (E2), dinoprost administered ($PGF_{2\alpha}$).

Table 2. Biochemical analysis of peripheral blood in calves derived from AI and on-gel culture derived embryos

Day	0 (birth)	3	7	14	21	28
Glucose (mg/dl)						
Calf from AI (n = 4)	82 ± 19	130 ± 17	106 ± 5	112 ± 9	104 ± 6	93 ± 25
D10 gel #1	50	133	115	127	70	127
D10 gel #2	128	120	118	109	112	93
Blood urea nitrogen (mg/dl)						
Calf from AI (n = 4)	7.4 ± 2.4	7.5 ± 1.7	6.8 ± 1.3	8.5 ± 2.9	9.6 ± 3.7	6.7 ± 1.4
D10 gel #1	11.4	5.5	5.8	6.5	5.1	6.5
D10 gel #2	7.8	13.7	9.2	7.7	6.2	8.5
Total cholesterol (mg/dl)						
Calf from AI (n = 4)	> 50	58 ± 9	79 ± 10	104 ± 32	79 ± 27	98 ± 38
D10 gel #1	> 50	57	74	107	57	109
D10 gel #2	> 50	65	87	74	78	62
Total protein (mg/dl)						
Calf from AI (n = 4)	5.8 ± 1.3	6.3 ± 1.1	6.4 ± 1.0	6.5 ± 0.9	6.7 ± 0.5	6.6 ± 0.5
D10 gel #1	5.2	6.1	6.2	6.8	5.8	6.0
D10 gel #2	7.8	8.0	7.9	8.1	7.5	7.7
Albumin (g/dl)						
Calf from AI (n = 4)	2.8 ± 0.2	3.3 ± 0.2	3.2 ± 0.3	3.6 ± 0.3	3.5 ± 0.2	3.4 ± 0.4
D10 gel #1	3.0	2.8	3.3	3.4	3.3	3.3
D10 gel #2	2.8	2.8	3.3	3.5	3.3	3.9
Calcium (mg/dl)						
Calf from AI (n = 4)	14.1 ± 0.4	14.8 ± 0.8	13.2 ± 0.4	12.8 ± 0.8	12.9 ± 0.7	13.0 ± 0.7
D10 gel #1	12.6	13.2	12.6	12.0	11.4	12.0
D10 gel #2	14.4	14.3	14.7	12.1	13.4	12.2
Inorganic phosphorus (mg/dl)						
Calf from AI (n = 4)	7.2 ± 0.5	8.9 ± 1.1	9.4 ± 0.9	9.2 ± 1.6	9.1 ± 1.1	9.1 ± 1.0
D10 gel #1	7.6	9.8	10.3	10.0	8.5	11.1
D10 gel #2	9.9	9.7	10.8	9.0	10.7	11.0
γ-glutamyltransferase (U/l)						
Calf from AI (n = 4)	330 ± 274	306 ± 276	206 ± 227	99 ± 101	57 ± 46	31 ± 14
D10 gel #1	101	1082	575	187	116	62
D10 gel #2	1200	1200	625	280	147	77
Ammonia (mg/dl)						
Calf from AI (n = 4)	65 ± 17	72 ± 28	61 ± 19	50 ± 16	65 ± 19	61 ± 9
D10 gel #1	76	68	61	76	35	71
D10 gel #2	191	77	54	41	47	52

Results of calf derived from AI embryos are expressed as mean ± standard deviation.

on-gel culture system.

An inspection of the afterbirths retrieved from recipients after parturition, with cotyledonary weights of 2.6 and 3.0 kg, revealed no evidence of morphological abnormalities (Fig. 1B). The number of afterbirth cotyledons was counted by manually isolating each cotyledon from the afterbirth (162 and 96). Both the afterbirth weight and number of cotyledons were within their respective normal ranges [10]. These findings indicate that TEs in on-gel-cultured mature blastocysts have the capacity to form placentas that support appropriate fetal development to term.

To further confirm the neonatal health of mature blastocyst-derived calves obtained via on-gel culturing, we examined metabolic profiles based on an analysis of peripheral blood samples collected from both on-gel culturing-derived and control artificial insemination (AI)-derived calves. Analyses of nine biochemical indices representing vital signs [plasma glucose, blood urea nitrogen, total cholesterol, total protein, albumin, calcium, inorganic phosphorus, γ-glutamyltransferase, and ammonia] revealed that in each case, concentrations in the on-gel culturing-derived calves were comparable to the values obtained for AI-derived calves at 0, 3, 7, 14, 21, and 28 days after birth (Table 2).

Among the unsolved issues associated with IVP systems for cattle production [7–9, 12], LOS is linked to a heightened incidence of dystocia and retained placentae [9, 13]. Although factors contributing to fetal overgrowth remain insufficiently determined, supplementation of blood serum in IVC has been established to reduce survival following ET and causes abnormal fetal development, including LOS [14–16]. Notably, the two calves derived from on-gel-cultured mature blastocysts showed no abnormal phenotypes during the neonatal period, with birth weight, placental weight, number of cotyledons, and metabolic profiles comparable to those of their AI-generated counterparts. Thus, we speculate that the detrimental effects of serum on fetal and placental growth are reset when using an on-gel culture system, in which, a serum replacer, KSR, is used instead of fetal bovine serum. Although further studies are necessary, we believe that the on-gel culture system has potential utility for

defining the detrimental effects of serum supplementation during bovine term development.

In this study, we demonstrated that on-gel culturing can be used to facilitate the full-term development of developmentally mature blastocysts that have undergone two rounds of cell segregation. Given that on-gel culturing enables bovine embryos to develop with larger cell numbers than does conventional droplet culture alone, combined with blastomere biopsy, this system may represent a novel method for performing genetic evaluations at the embryo preimplantation stage [17, 18]. Moreover, we established that the culture system described herein recapitulates a level of cell differentiation comparable to that observed in its *in vivo* counterparts, at least up to D10 after the onset of IVC. Accordingly, we believe that the on-gel culture system could make a valuable contribution to a range of analyses performed to evaluate bovine-specific preimplantation development [19, 20]. Moreover, given that the extended period of IVC in on-gel culturing enables a more stringent selection of embryos for ET, it is envisaged that this system could be employed to facilitate more efficient cattle production via embryo transfer.

Methods

All experimental procedures were approved by the Regulatory Committee for the Care and Use of Laboratory Animals, Hokkaido University (No. 19-0162).

Full-term development of on-gel-cultured D10 embryos

In our previous study [6], we conducted five sessions of embryo transfer using seven D10 on-gel-cultured mature blastocysts. Prior to ovulation synchronization, recipient Holstein heifers at Hokkaido University were repeatedly examined for normality of ovarian cycles and uterine contents using an ultrasonography device (HS-2100V; Honda Electronics, Aichi, Japan) equipped with a linear probe (10 MHz, HLV-475; Honda Electronics). Endometritis [11] was treated with intrauterine injection of antibiotics, as necessary. Heifers were synchronized for ovulation [21] using an intravaginal progesterone-releasing device (CIDR1900; Zoetis Japan, Tokyo) for seven days (from D10 to D2, with the day of IVF being designated D0) with 2 mg of estradiol benzoate (E2) (Ovahormon injection, ASKA Animal Health Co., Ltd., Tokyo, Japan) and 25 mg of dinoprost administered (prostaglandin $F_{2\alpha}$; $PGF_{2\alpha}$) (Pronalgon F, Zoetis Japan, Japan) at the time of insertion and withdrawal of the device, respectively. On D0, the recipients were dosed with 100 µg of GnRH. Ovaries were examined daily by ultrasonography (5 MHz, HS101V; Honda Electronics), and ovulation was confirmed on D1 in one heifer and on D2 in the other heifer. The ET was performed on D10 in the morning. Table 1 provides the details of the experimental schedule. The CIDR was inserted for two weeks from D6 to facilitate conception [22]. The presence and location of the corpus luteum was confirmed by ultrasonography on D8. The embryo and medium were transferred to a plastic straw (005592; IMV Technologies, L'Aigle, France), immediately loaded into a disposable ET gun (MO5; Misawa Medical Industry Co., Ltd., Ibaraki, Japan), and quickly transferred into the uterine horn ipsilateral to the corpus luteum. Caudal epidural anesthesia with lidocaine hydrochloride (Xylocaine® 2%; Sandoz Pharma K. K., Tokyo, Japan) was administered at the time of embryo transfer. Pregnancy was diagnosed by ultrasonography after 30 days of gestation (HS101V).

Preparation of bovine embryos by conventional culturing in microdroplets

Bovine oocyte retrieval, IVM, IVF, and subsequent IVC up to the blastocyst stage were performed as previously described [23]. Briefly, cumulus-oocyte complexes (COCs) collected from slaughterhouse-derived Holstein ovaries were cultured in TCM-199 medium (Thermo Fisher Scientific Inc., Waltham, MA, USA) at 38.5°C in a humidified atmosphere of 5% CO_2 for 20–22 h. *In vitro*-matured oocytes were transferred to Brackett and Oliphant (B.O.) medium containing 2.5 mM theophylline (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 7.5 µg/ml heparin sodium salt (Nacalai Tesque, Inc., Kyoto, Japan). Subsequently, freeze-thawed semen was centrifuged at 600 × *g* for 7 min in B.O. medium, and spermatozoa were added to the COCs at a final concentration of 5×10^6 cells/mL. The presumptive zygotes were denuded by pipetting after 12 h of incubation and cultured in mSOFai medium at 38.5°C in a humidified atmosphere of 5% CO_2 and 5% O_2 for eight days.

On-gel culture of bovine embryos after the premature blastocyst stage

Agarose powder (Agarose S; Dojindo Laboratories, Kumamoto, Japan) was solubilized in double-distilled water to a concentration of 1.5% (w/v), autoclaved, poured into 10 cm dishes, solidified at room temperature (22–25°C), and cut into 10 mm × 10 mm × 5 mm pieces. The processed gel was placed in the wells of a four-well dish (Thermo Fisher Scientific, Inc.), to which 0.5 ml of RPMI1640 medium was added (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), supplemented with 30% (v/v) knockout serum replacement (KSR; Thermo Fisher Scientific, Inc.), 1× non-essential amino acids (Sigma Aldrich Japan, Tokyo, Japan), 1× essential amino acids (Sigma Aldrich Japan), and 1× insulin-transferrin-selenium solution (Thermo Fisher Scientific, Inc.), followed by overnight incubation at 38.5°C. Thereafter, the spent medium was replenished with fresh medium in which the gel was immersed to approximately half its height. After washing several times in culture medium, D8 blastocysts suspended in a small volume of medium were placed on the surface of the gel sections using a glass pipette. Typically, five or six blastocysts were placed on each gel section, and subsequent culturing was performed at 38.5 °C in a humidified atmosphere of 5% CO_2 and 5% O_2. During the initial period of culturing, these blastocysts were surrounded by the medium introduced during pipetting; however, this was gradually absorbed by the gel upon which the blastocysts were exposed to both gaseous and liquid phases.

Blood sampling and analyses

At 0, 3, 7, 14, 21, and 28 days postpartum, blood samples were collected from the jugular vein of calves using heparin-sodium tubes (Venoject II; Terumo, Tokyo, Japan) for analysis of biochemical constituents. Following collection, the samples were immediately centrifuged at 1660 × *g* for 15 min at 25°C to separate the plasma, which was stored at −30°C until analysis. The plasma glucose, blood urea nitrogen, total cholesterol, total protein, albumin, calcium, inorganic phosphorus, γ-glutamyltransferase, and ammonia concentrations were measured using a DRI-CHEM 3000V clinical blood analyzer (Fujifilm, Tokyo, Japan). The control calves were Holsteins and were produced using the common AI method.

Conflict of interests: The authors have no conflicts of interest to declare.

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