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The contribution of efficient production of monozygotic twins to beef cattle breeding

Yutaka HASHIYADA[1)]

[1)]*National Livestock Breeding Center, Fukushima 961-8511, Japan*

Abstract. Production of sires with high breeding potential is indispensable for prompt and reliable breeding using their semen in the cattle industry. Currently, in Japan, we aim to further the production of Japanese black sires via a new breeding system that uses genetically homologous monozygotic twins so that better growth performance and carcass traits can be translated to the increased production of beef with higher economic value. Several studies have reported that monozygotic twins are produced by embryo bisection. On the other hand, with the evolution and stabilization of *in vitro* fertilization technology, it has become possible to produce multiple monozygotic twin calves from blastomeres separated from a cleavage-stage embryo. This review attempts to clarify breeding practices through revalidation of the factors that affect the production efficiency of monozygotic twin calves by embryo bisection. Furthermore, the establishment of a system for monozygotic twin embryo production via the simplified technique of blastomere separation is reviewed while showing data from our previously performed studies.

Key words: Blastomere separation, Bovine, Embryo bisection, Monozygotic twin

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Since monozygotic twins are genetically homologous, it is possible to obtain two individuals with excellent traits, using two demi-embryos originating from a single embryo, which never result in free-martins. Utilization in research, there is the advantage that the number of animals can be reduced without decreasing accuracy [1]. Various comparative investigations have been performed on the basis of the similarity of monozygotic twins [2–5]. Monozygotic twins have been used as control in previous studies to estimate heritability of genetic variation [6] as well as in epigenome analysis of somatic cell nuclear transfer clones [7]. However, the most effective and valuable utilization of monozygotic twins is their introduction in the breeding and selection systems for sires, where they supply semen for artificial insemination, and thus, are directly related to the production field. In such systems, sires can be selected efficiently, which provides an alternative to the conventional progeny testing. Briefly, the genetically homologous individual from amongst the monozygotic twins is selected as a sire, based on the post-fattening carcass evaluation obtained from castrating the other twin in the pair. In a comparison of the period for sire selection, the conventional breeding progeny testing requires nearly six years; however, the new approach reviewed in this paper allows the process to be carried out in about half that period [8, 9]. It is expected that breeding improvement of beef cattle can be promptly promoted owing to its low cost and labor saving. Currently, in Japan, several Japanese Black sires are produced via this system in the National Livestock Breeding Center.

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Correspondence: Y Hashiyada (e-mail: y1hashiyada@nlbc.go.jp)

Overview of Monozygotic Twin Production in Cattle

There are two different approaches or techniques to produce monozygotic twin embryos that can later be adapted in the field. One of these techniques is the bisection of embryos 6–8 days after fertilization. The second technique is the separation of blastomere during the early cleavage stage of embryos. An advantage of embryo bisection is that the technique can be adapted to work on embryos derived from both *in vivo* and *in vitro* methods. Nowadays, techniques, such as vertical-pressure cutting, have been simplified in the form of ready-made metal blades that use only a single micromanipulator without the need to hold a pipette [10–13]. Similarly, the zona pellucida used to protect the demi-embryos during culture and transfer can be removed in cattle [14, 15], sheep [16], goat [17] and pig [18]. A disadvantage of embryo bisection is that the number of cells and fertility tend to decrease due to the physical damage incurred by cutting [18–21].

In the case of blastomere separation, embryos that are at an early stage of cleavage are used. In protocols that are conventionally used for this separation, complicated steps are required, such as surgical collection of *in vivo* embryos from the oviduct, encapsulation of the isolated blastomeres with an empty zona pellucida and agarose-gel, provisional transfer of embedded blastomeres into the recipient for *in vivo* culture, recovery of developed embryos, and removal of embryos from agarose-gel to transfer [22]. Due to the cumbersome nature of these processes, production of twins using blastomere separation did not attain practical feasibility until *in vitro* fertilization technology was established. However, in recent years, it has become possible to develop the technique for production of monozygotic twins via blastomere separation based on stabilized *in vitro* embryo

production technology. As an additional advantage of the blastomere separation technique, even monozygotic quadruplets were produced from a 4-cell embryo derived *in vitro* [23]. As compared to embryo bisection, this technique of producing twins is expected to ensure higher fertility due to less damage to cells.

On the basis of the above background and knowledgebase, this review aims to describe and clarify the factors affecting the efficient production of monozygotic twin embryos and twin calves in these two production systems.

Clarification of Factors Affecting Embryo Bisection

In various animals, embryo bisection has been performed using blades made of metal or glass [11, 14, 16–18, 24–32] or glass needles [15, 16, 26, 33–36]. No difference was observed in twin pregnancy occurrence from the transferred demi-embryos between these types of micro tools [37]. In addition, it has been reported that the insertion of demi-embryo into the zona pellucida has no effect on fertility [14, 15, 17, 18, 29, 37].

For high-yield production of monozygotic twins, it is important to minimize damage to the embryo and maintain the number of embryonic cells at the time of cutting to produce demi-embryos with more potential for normal development, conception, and fetal growth. Studies on mouse [38] and monkey embryos [30] subjected to cutting reported that the reduction in the number of cells was extremely low, with the cell number in the bisected demi-embryos being approximately half of that in the intact embryos. On the other hand, there has been a report that 26 to 33% of cells were lost in early blastocysts and blastocyst stages of porcine embryos [18] following bisection. In ruminants, 9% [39] of cells were reduced in day 7 (day 0 = day of fertilization) cattle blastocysts and 13% in day 8 sheep blastocysts [19].

Materials and procedures used for cattle embryo bisection

Basically, the process of bisection is carried out in a simple way with the aim of cutting vertically from the top of the zona pellucida [11, 13], using an inverted microscope and a three-dimensional hydraulic joystick micromanipulator fitted with a metal blade. This is done without suction fixation of the embryos in Dulbecco's phosphate buffered saline (DPBS) supplemented with 20% calf serum (CS) under room temperature. The purpose of CS supplementation in the splitting medium is to prevent adhesion of embryos to the dishes and to facilitate handling of embryos during micromanipulation [27]. Initially, each splitting medium is applied as drops on a plastic petri dish with or without a covering of paraffin oil. The procedure of embryo bisection is as follows. The blade is placed on the midline of the embryo. Thereafter, while grasping the embryo by compressing the upper part of the zona pellucida with a blade and pressing it towards the bottom of the dish, the blades are gradually pushed downwards to cut equally, especially in the regions of the inner cell mass (ICM) and the trophoblast cells from early blastocyst development stage. In the case of two demi-embryos that exist with a zona pellucida that is not completely halved, they may adhere and fuse again during culture [26, 34]. As a countermeasure, at least one embryo from the pair is removed from the zona pellucida by dissection. Subsequently, demi-embryos are cultured for a few hours *in vitro* so they may recover their form without enclosing them in the zona pellucida.

Table 1. The effect of embryo quality on development of good demi-pairs in cattle embryo bisection

Embryo quality	No. of bisection	No. (%) of Good demi-pairs
Code 1	176	134 (76.1) [a]
Code 2	68	27 (39.7) [b]

Values with different superscripts are significantly different ($P < 0.05$).

Table 2. The effect of development stage of embryos on fertility of the demi-embryos after transfer in cattle

Developmental stage of embryos	No. of transfer	No. (%) of	
		Pregnancy	Twin pregnancy
Compact morula	139	54 (38.8) [a]	16 (11.5)
Early blastocyst	94	50 (53.2) [b]	17 (18.1)
Blastocyst	33	12 (36.4) [ab]	2 (6.0)
Expanded blastocyst	10	3 (30.0) [ab]	1 (10.0)

Values with different superscripts are significantly different ($P < 0.05$).

Quality and developmental stages of embryos for bisection

For stable production of monozygotic twins by embryo bisection, it is essential to divide the embryo into two equal parts with minimum damage. Embryo quality and developmental stage were determined by morphological observation according to the standards of International Embryo Transfer Society [40].

Utilization of higher grade embryos i.e., with high quality, after their morphological classification contributes to the success of splitting them in equal portion. A high proportion of demi-embryos pairs with good morphology and of "Excellent" and "Good" grades rather than "Fair" and "Poor" grades could be produced by bisection using metal blades and holding-pipettes [41]. The results of our vertical-pressure cutting showed similar trends, where Code 1 embryos yielded a significantly superior quality of demi-embryos after 3 hours of culture following bisection as compared to Code 2 embryos (Table 1).

For the preferred developmental stages of intact embryos to bisection, Bredbacka *et al*. [36] reported that the bisection of blastocysts were significantly less than morula in surviving cells of the split embryos by staining evaluation. According to our investigation, pregnancy rate from two demi-embryos transferred in the recipient was significantly higher in the early blastocyst stage than in the compact morula stage. The twin pregnancy rate was lower, when blastocysts were transferred (Table 2); this may indicate that blastocysts can be easily damaged by cutting. Williams *et al*. [25] indicated the pregnancy rate to be the highest in early blastocyst stage, which was corroborated by our results (Table 2), but twin pregnancy rates tended to be higher in blastocyst, which was opposite to what we observed. Moreover, pregnancy rates were found to be similar among compacted morula, early blastocyst, and blastocyst stages in other report [35]. The reason for these different results is attributed to be the difference in the methods of holding embryos and cutting them in the horizontal direction, which was different than what we employed vertical-pressure cutting in our research. In general, since blastocysts

have no perivitelline space, degenerate cells are difficult to identify, which limits the selection of high quality embryos.

Micromanipulation in embryo bisection

Micromanipulation technology plays an important role in the basic research and development of embryonic manipulation techniques in reproductive technology. In assisted reproductive technology in humans, training, including that on micromanipulation techniques, is performed systematically [42, 43]. However, in fields of animal study, including livestock experiments and technology development, techniques are applied and improved in individual laboratories based on experience and self-practice of researchers. It is understandable that technical expertise in micromanipulation affects the accuracy of the bisection process. Nevertheless, there are no reports that clarify the relationship between the degree of micromanipulation skill and the success of bisection. Data on bisection success was collected as all instances of equally isolated demi-embryos over four years for a beginner operator in our team. At the early stage of technique learning, the operator received technical guidance from a skilled technician. This operator bisected approximately 70–200 embryos per year, although there was a difference in the exact number depending on the year. Technical improvement of 3–5% was observed in each year as the number of bisections performed increased (Fig. 1). It was confirmed that the success of embryo micromanipulation is determined by the technical level or expertise, which is, in turn, dependent on experience.

Medium used for embryo bisection

There are many reports that confirmed embryo splitting medium utilizes DPBS supplemented with 5–20% serum [11, 16–18, 25, 26, 32, 35, 44, 45]. In order to reduce physical damage to the embryo at the time of cutting, Ca^{2+}-free PBS was used during splitting [37] and during pretreatment by exposure [34, 36]; this inhibits Ca^{2+}-dependent intercellular adhesion and cell binding in Ca^{2+}-free state [46]. Additionally, sucrose, which shrinks embryos via osmotic pressure, was added in the splitting medium [13, 27, 29]. In addition, there has been a report which described bisection in a cytochalasin B solution to demonstrate production of demi-embryos [47]. The cytochalasin B (CCB) solution is used for enucleation of oocytes during nuclear transfer [48] and blastomere isolation from embryos at cleavage stage [30, 49, 50] because it inhibits maintenance of cytoskeleton by acting on actin filaments of cells. Keeping these observations in perspective, the influence of bisection medium on the production efficiency of demi-embryos was studied using blastocysts derived *in vitro* and expanded blastocysts by comparing different solutions [51]. In bisections performed in both CS+DPBS after exposure to CCB and in CS+DPBS supplemented with 0.2 M (w/v) sucrose, lesser ejection of cells from fractured zona pellucida was observed due to shrinking of the embryos; in addition, majority of the demi-embryos formed had good morphology. Moreover, demi-embryos derived from CCB-treated embryos showed a tendency to have a greater number of cells, suggesting that CCB helped suppress damage to embryos upon cutting. In contrast, low survival rate and lesser cell number were observed in bisection done using PBS (–) solution, and thus, this solution is considered to be ineffective for these developmental stages of embryos derived *in vitro*.

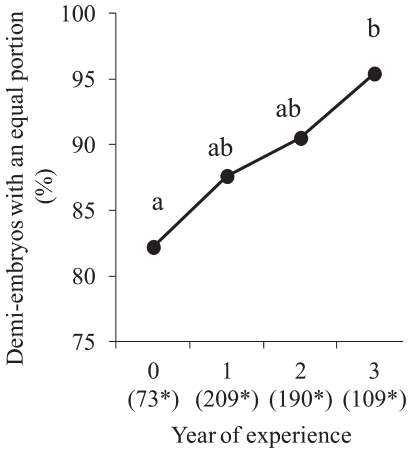

Fig. 1. Improvement in bisection skills of beginners based on portion of evenly separated cattle demi-embryos. * Number of embryo bisections. Values with different superscripts (a–b) differ significantly ($P < 0.05$).

In the embryonic physiology and structural functions, an embryo produced *in vitro* is different from embryos derived *in vivo* in its intercellular adhesion [52] and zona pellucida hardness [53–55]. It is thus necessary to continue to verify whether the above results can be applied to bisection of embryos derived *in vivo*. Transferable blastocysts can be increased through bisection even in embryos derived *in vitro* [56]. To increase the yield of monozygotic twin calves, adapting embryos produced *in vitro* for bisection enhances the efficiency of twin production.

Short-term treatment of damaged demi-embryos

In demi-embryos, any exposed damaged cells on the cut surface detach easily from the embryo. Therefore, embryos are cultured for restoration for a few hours after cutting [13, 24, 26, 29]. For effective recovery of damaged cells, the culturing of demi-embryos using tissue respiration activator has been performed in previous research. “Solcoseryl”, a tissue respiration activator, is a standardized deproteinized hemodialysate derived from calf blood. Its medicinal function is to improve healing in animals and humans. In reproductive biology, the efficacy of Solcoseryl has been confirmed on mouse embryos by culturing and fertility testing [57]. In farm animals, Solcoseryl can substitute BSA in sheep embryo culture [58]. In cattle embryo bisection, improvement has already been reported in the twin pregnancy rate [45] and production of demi-embryos [59]. Furthermore, Solcoseryl was used on biopsied Water Buffalo embryos to recover damaged cells in them efficiently as well [60].

In our study, Code 1 and Code 2 grade embryos collected from Japanese Black donor cows on day 7 (day 0 = day of fertilization) were bisected and cultured in DPBS supplemented with 20% CS and 0.1% (v/v) Solcoseryl for 3 h. A pair of monozygotic twin embryos was transferred bilaterally or unilaterally to Holstein/Japanese Black cross-bred recipients. Pregnancy diagnosis was performed on day 25 from the estrus, while embryonic and fetal losses were monitored for 100 days of gestation at 20-day intervals by ultrasound scanning.

Table 3. The effect of culture containing tissue respiration activator (Solcoseryl) on the fertility of two demi-embryos after bisection

Culture media	No. of transfer	No. (%) of		
		Pregnancy	Twin pregnancy	Pregnancy loss
With Solcoseryl	109	54 (49.5) [a]	20 (18.3) [c]	7 (13.0)
Without Solcoseryl	130	43 (33.1) [b]	212 (9.2) [d]	11 (25.6)

Values with different superscripts (a–b) and (c–d) within each column differ significantly (P< 0.01 and P < 0.05, respectively).

Table 4. The effect of transfer method of two demi-embryos after bisection on fertility and twin birth

Transfer method	No. of transfer	No. (%) of						
		Pregnancy	Twin pregnancy	Single pregnancy loss	Twin pregnancy loss	Delivery in twin pregnancy	Stillbirth in twin delivery	Twin birth in twin pregnancy
Bilateral	101	37 (36.6)	13 (12.9)	2 (5.4)	5 (38.5)	8 (61.5)	3 (37.5)	5 (38.5) [a]
Unilateral	73	29 (39.7)	13 (17.8)	6 (20.7)	0 (-)	13 (100)	1 (7.7)	12 (92.3) [b]

Values with different superscripts (a–b) within each column differ significantly (P < 0.05). Modified from Hashiyada *et al*. (1996) [71].

Abortion was confirmed by the return of estrus and discharge of the conceptus. Both single and twin pregnancy rate were superior in the Solcoseryl supplementation group than in the control group without Solcoseryl. In addition, incidence of pregnancy loss tended to be lower in the supplementation group, however, there was no significant difference (Table 3). Thus, the results from our research clarified the positive effects of Solcoseryl on restoration of damaged embryos.

Transfer method for monozygotic twin demi-embryos

In cows pregnant with twins, as compared to single pregnancy, abortion [61–63], dystocia [64–67], and postnatal death [61, 66] occurs with a high probability. There are two ways of embryo transfer for twin production. One of them is to transfer an embryo into the uterus on the side of the corpus luteum (CL) and another embryo to the opposite side of the uterus. Another method is to transfer two embryos into the uterus on the ipsilateral side of the CL. In these two methods, it is considered that the pregnancy rate, embryo or fetal loss, and parturition accidents might be different. In studies that reported transfer of two intact embryos, there was no difference in fertility and twin pregnancy between bilateral and unilateral transfers [68, 69]. Furthermore, for embryo transfer following artificial insemination, there was no difference between single and twin pregnancy rate, when the embryo was transferred to the uterus on the ipsilateral or contralateral side of the CL [70]. In our study, twin birth rates were compared for bilateral and the unilateral transfers performed using standard non-surgical transfer equipment. Pairs of Japanese Black demi-embryos were transferred into Japanese Black/Holstein cross-bred recipients. As a result, both pregnancy and twin pregnancy rates did not differ significantly between the two transfer methods (Table 4) [71]; this has been corroborated by previous reports [68–70]. In an interesting report, the elongation of conceptuses on day 14 after fertilization was observed to not be affected, when embryo was located on the side of the uterus that was ipsilateral or contralateral to the CL [72]. Thus, it became clear that the location of the embryo within the uterus might not affect its own survival. However, when comparison was made focusing on twin pregnant animals in our study, fetal losses between 25 and 100 days of pregnancy and birth accidents at twin-bearing stage were higher in bilateral transfer. Finally, twin birth rate was significantly superior in unilateral transfer (Table 4) [71]. This observation is different from previous reports, which showed that there is no difference in twin delivery between the two transfer methods [69, 70]. The reason may be the relationship between the capacity of the uterus in recipients and the size of the fetuses. They transferred embryos to the same breed of the recipient cattle or an unknown breed derived from IVF [69, 70], but we transferred Japanese Black beef cattle embryos with smaller body size to bigger cross breed that had enough capacity to maintain and bear twins. In cattle, the survival rate of embryo is extremely low in the uterus contralateral to the CL [73, 74]. We observed that when the fetus was lost in the ipsilateral side of the CL, its loss also occurred in the uterus present on the opposite side. In sheep, when pregnancy was established with a single embryo, embryonic death was observed to increase, when embryo was in contralateral to the CL rather than being present in the ipsilateral uterus [75]. From these facts, it was concluded that the interruption of twin pregnancy occurred with high frequency in bilateral uteruses as compared to unilateral uteruses. There is a remarkable report [76] that presents another noteworthy consideration. Embryo migration in cattle uterus was reported to be more than 30% higher in transfer of two embryos as compared to transfer of a single embryo; despite two embryos being involved in unilateral transfer, conceptus was observed in each uterus in half of the pregnant cattle. In our experiments, assuming that half of the unilaterally transferred twin demi-embryos migrated, and as their report shows, later became twin pregnancy in bilateral, the abortion and accident rate at twin-bearing and production stage are estimated to be no different between unilateral and bilateral transfers.

Enhancement of fertility for demi-embryos by co-transfer with trophoblastic vesicles

The viability of bisected demi-embryos can decrease because

of cell damage and/or reduced cell number as compared to intact embryos [19, 39]. Considering that interferon tau (IFN-τ) is the embryonic signal secreted from trophoblast cells for establishment of pregnancy through the maternal-fetal recognition [77], IFN-τ from trophoblast cells would decline more along with a reduced cell number in demi-embryos. Accordingly, improvement in the pregnancy signals by co-transfer with trophoblastic vesicles (TVs) might enhance the pregnancy rate of demi-embryos. Heyman *et al*. [78] showed the effects of luteolysis inhibition by TVs transfer. Additionally, improvement in pregnancy rate following co-transfer of demi-embryos with TVs has also been reported [79].

Trophoblastic vesicles of *in vitro* origin were evaluated for their capacity to maintain CL function and prolong the interestrus interval [80]. In our investigation, the preparation of TVs was different from their full *in vivo* production. The TVs were produced by dissection of elongated embryos collected on day 14 (day 0 = day of fertilization) after *in vitro* fertilization and *in vivo* culture for 7 days in the uterus by embryo transfer. After 24 h of *in vitro* culture, the few TVs that were formed were then transferred to the uterine horn ipsilateral to the CL in Japanese Black/Holstein cross-bred recipients along with Japanese Black monozygotic twin demi-embryos. The transition of pregnancy rates after transfer of the two demi-embryos was compared for the transfers performed with TVs and without TVs (control). The pregnancy rate was observed to be significantly higher in the TV co-transfer group as compared to the control group at the time of the first pregnancy diagnosis carried out approximately 25–40 days into the gestation period. Afterwards, pregnancy losses were observed in the co-transfer group at the second diagnosis point conducted approximately 40–70 days into gestation. However, final pregnancy rates according to delivered calves were still higher in the co-transfer group. Calves in the co-transfer group showed normal morphology, while their birth weights and gestation lengths were same as those of the calves in the control group. The genetic identities of calves from co-transfer treatment were confirmed to be derived from transferred embryos and not affected by the TVs [32]. These results indicate that co-transfer with TVs of *in vitro* origin might enhance the fertility of bisected demi-embryos during early stages of gestation. In addition, we have reported previously that conception rate improves even in co-transfers in which TVs were frozen together in a straw with intact embryos [81] using the direct transfer method [82]. Hence, to improve the fertility of demi-embryos, use of freeze-stored TVs for co-transfer is considered to be highly effective.

Establishment of Efficient Production System for Monozygotic Twin Embryos by Blastomere Separation

The bovine multiple fetus production was done successfully in the early 1980s by blastomere separation [83]. This was achieved almost in the same time period as twin production by embryo bisection [24, 25, 84]. One set of triplets and one pair of twins were successfully produced from each of the four embryos formed from the pairs of blastomeres that were separated from embryos at the 8-cell stage [83]. At that time, however, IVF technique had not been established. Therefore, embryos in the early stages of development were surgically collected. Embryos were then taken out from dissected zona pellucida micro-surgically. Afterwards, blastomeres were separated by pipetting followed by their insertion into the surrogate zona pellucida prepared beforehand from porcine oocytes obtained at the slaughter house. Moreover, these were embedded in agar and then transferred surgically to the sheep oviduct for *in vivo* culture. After one or two days, embedded embryos were recovered, released from the agar, and finally transferred to the recipient cattle, through an extremely complicated and labor-intensive process. Nowadays, *in vitro* fertilization technology based on individual-development cultures has been established successfully in bovine animals; oocytes can be stably collected from surviving animals using transvaginal ovum pickup (OPU) technique. From this perspective, blastomere separation might be a useful technique for the efficient production of monozygotic twins, where damage to the embryo cells is lesser and high fertility is expected as compared to embryo bisection.

Protocol for blastomere separation in monozygotic twin production

For the establishment of a monozygotic twin production system by blastomere separation, the key to success is a stable *in vitro* embryo production technique that allows the development of blastocysts that are derived from isolated blastomeres with totipotency during the early stage of embryo development. To function as an efficient method for production of monozygotic twin embryos by blastomere separation, simple protocols that do not require special equipment, advanced technology, and skillful manipulation need to be devised. The development of such technology was conducted as follows: We used early cleavage-stage embryos with 2- to 8-cells post fertilization. Their zone pellucida was removed by enzymatic treatment using 0.25% pronase in DPBS for 2–3 min, and blastomeres were separated by gentle pipetting. Further manipulation was performed on a warmed plate because embryos are sensitive to low temperature in their early stages as previously described [23, 85]; warming also enhances enzymatic action. Individual culture for aggregation of blastomeres was performed using a microwell plate as an alternate zona pellucida to follow the concept of well-of-the-well (WOW) individual embryo culture. Half the number of blastomeres from amongst the total number of cells in the embryos was introduced in each microwell. Oocytes were prepared from the ovaries collected at an abattoir, except for the study that used OPU-derived embryos to assess the fertility of developed blastocyst. Until blastomere separation was performed, *in vitro* maturation and fertilization of oocytes as well as development of embryos were carried out in groups in a microdroplet based protocol specified in the National Livestock Breeding Center [86].

Developmental stage of embryos for blastomere separation

In the initial study using 2-, 4-, and 8-cell sheep embryos derived *in vivo*, half the number of blastomeres from intact embryos were inserted into the empty zona pellucida and cultured in the oviduct after embedding in agar. Following this experiment, blastocyst formation and fertility performance was found to be equivalent among all three cellular development stages [87]. However, in cattle embryos that are derived from *in vitro* methods, a suitable developmental stage of embryos for blastomere separation is not clear. We studied this knowledge gap using embryos at the 2-, 4-, and 8-cell stage obtained at 24–27 h, 30–36 h, and 48–54 h, respectively, post insemination based

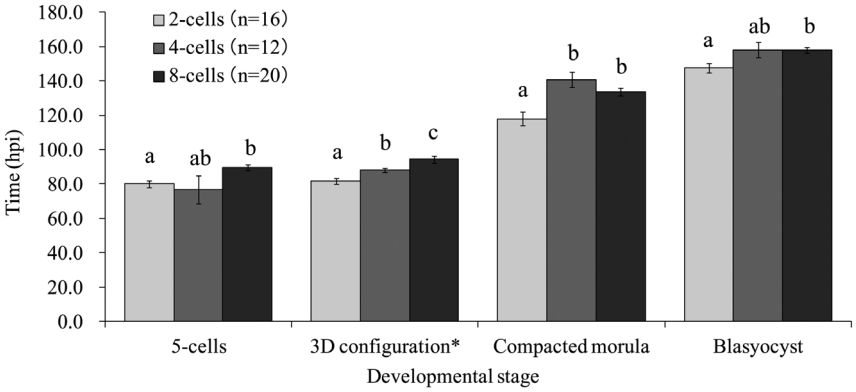

Fig. 2. Time (hours post insemination) to attain each developmental stage of cultured blastomeres that are separated from 2-, 4-, and 8-cell stage embryos. * The stage at which blastomeres formed a three-dimensional structure from their initial planar placement. Values are indicated as means ± SEM. Values with different superscript within the same developmental stage groups differ significantly (a–b, b–c; $P < 0.05$, a–c; $P < 0.01$).

on our previous study [88]. Results of comparative investigation on *in vitro* cultures showed that blastocyst formation rate and incidence of blastocysts in pairs, both were approximately 10% higher in the blastomeres derived from embryos at 2-cell stage than in those derived from 4- and 8-cell stage embryos. Furthermore, during the development of blastomeres to blastocysts, the time taken in hours post insemination to reach the 5-cell, the three-dimensional, the compact morula, and the blastocyst stages were examined using time-lapse cinematography. Photographs of the blastomeres and/or embryos were taken every 15 min using a real-time cultured cell monitoring system with multiple-point imaging captures. The time taken to reach each stage was significantly lesser in blastomeres derived from 2-cell stage embryos than in 4- and/or 8-cell stage embryos (Fig. 2).

At the first cleavage after *in vitro* fertilization in cattle, direct cleavage from one cell to 3–4 cells was observed in approximately 14% [89] and 30% [88] cases. These embryos showed a higher incidence of chromosomal abnormalities [89]. Moreover, slowly cleaved embryos had a higher frequency of abnormal chromosomes as compared to rapidly cleaved ones [90–92]. In our study, it was considered that such abnormally cleaved embryos were contained in the chosen 4- and 8-cell stage embryos without them being observed at the first cleavage stage, and thus, took longer time to develop in the blastocysts. Embryos that cleave faster to 2-cell stage after fertilization indicate a higher blastocyst formation rate than those that cleave more slowly. Furthermore, embryos that developed rapidly showed morphological normality and contained a large number of cells [93]. Two-cell stage embryos can have the number of blastomeres in them be reliably distinguished. Since the blastomere covers a larger volume in 2-cell stage embryos as compared to 4- or 8-cell embryos, there is an advantage that handling can be easily performed. Considering the above result and reasoning, 2-cell stage embryos might be suitable for blastomere separation for the production of efficient monozygotic twin embryos.

Medium for blastomere separation

Several media have been previously used for blastomere separation. Embryo culture medium has been used most frequently [83, 87, 94] owing to its non-toxic nature and ease of preparation. The utilization of Ca^{2+}-free solution for cell disaggregation has also been reported for the purpose of weakening intercellular adhesion [95–97]. In the embryo nuclear transfer procedure, trypsin solution is used to prepare donor cells from cleavage-stage embryos [98, 99]. However, the differences in the effectiveness of these media for blastomere separation and the subsequent effects on embryo development have not yet been clarified. Hence, using 8-cell stage embryos, blastocyst development and cell numbers were compared following the use of three types of blastomere separation media: 0.05% trypsin-0.02% EDTA (Trypsin-EDTA), Ca^{2+} and Mg^{2+}-free PBS containing 0.1% polyvinyl alcohol (PBS(–)-PVA), and CR1aa containing 5% CS (CR1aa-CS), the last of which is the culture medium. Although the blastocyst formation rate and the number of blastocysts classified as Code 1, with favorable morphological properties and paired blastocyst development, tended to higher numerically when using CR1aa-CS than when using the other two media, these differences were not significant. Differential staining of ICM cells and trophectoderm cells indicated that the total cell number of the blastocysts did not differ between the three media; however, the number of ICM cells was significantly lower following the use of both Trypsin-EDTA and PBS(–)-PVA than when using CR1aa-CS (Fig. 3). The ratio of the ICM cells to the total cell number was also significantly lower when using Trypsin-EDTA and PBS(–)-PVA.

A study assessing the effectiveness of dislodging cells using enzymes reported that trypsin can stimulate DNA synthesis in lymphocyte cells and that this increase in stimulation was observed when the cells were exposed to proteases for more than 1 min [100]. Another study reported that treatment using Trypsin-EDTA induced DNA damage during cell isolation, in particular via the action of EDTA [101]. Accordingly, it was suggested that Trypsin-EDTA may affect the developmental competence of blastomeres, following exposure for even a few minutes. Although Ca^{2+}-free solution is most widely used for cell isolation, developmental abnormalities with respect to the growth of blastomeres to the blastocyst stage were indicated in the isolation of 2-cell mouse embryos [102, 103].

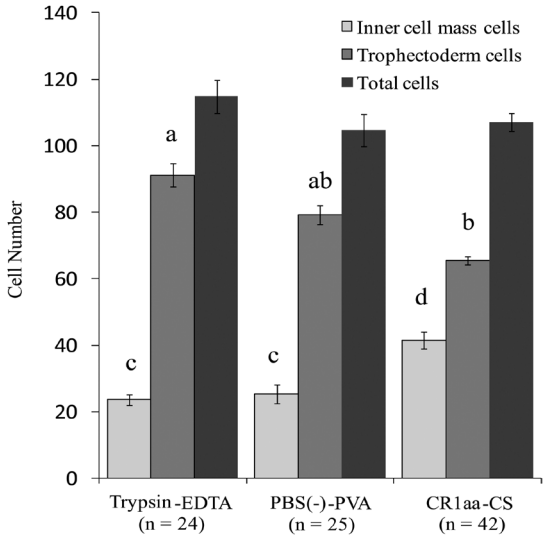

Fig. 3. The number of cells in blastocysts that develop from the blastomeres separated in 0.05% trypsin-EDTA (Trypsin- EDTA), Ca^{2+} and Mg^{2+}-free PBS containing 0.1% polyvinyl alcohol (PBS(–)-PVA), and CR1aa containing 5% CS (CR1aa-CS). Values are indicated as mean ± SEM. Values with different superscripts (a–b) and (c–d) within the same cell type differ significantly ($P < 0.01$ and $P < 0.05$, respectively).

Based on our results comparing the three media, it was suggested that the exposure and/or isolation of early embryo blastomeres in Trypsin-EDTA or PBS (–) medium may have a negative effect on subsequent embryogenesis, even following exposure for a short period of time of a few minutes. In addition, it was considered that the pronase used to remove the zona pellucida reduced the adhesion of the blastomeres, as no difference was found in the difficulty of isolation when using any of the media compared in our investigation.

Blastomere culture for blastocyst development

One of the functions of the zona pellucida is to maintain the blastomere arrangement to provide blastocysts by aggregation [97, 104]. In the zona pellucida-free embryos, abnormalities in cell arrangement, cell-to-cell contact, and cell number and fertility have been reported [104–106]. When mouse unencapsulated blastomeres were cultured on a flat surface, the blastomeres tended to cleave with a flat and/or open linear conformation without forming a three-dimensional configuration [95, 97]. In such an abnormal arrangement, these blastomeres possibly reach more blastocysts, with fewer ICM cells and lower survival of fetuses after embryo transfer [104]. In contrast, blastocysts can be stably obtained by culture of blastomeres inserted into empty zona pellucida by micromanipulation [22, 23, 33, 83, 85, 87], although the preparation of surrogate zonae pellucidae from oocytes or degenerated embryos is a laborious process. For these reason, several studies have been conducted examining the effect of artificial zona pellucidae on denuded embryos obtained by micromanipulation [106–109]. In recent years, the culture of zona pellucida-free embryos has been attempted using a completely different concept from those of these conventional methods. The basis of this culture is to use a microwell placed at the bottom of the culture dish as an alternative zona pellucida. A culture system of monozygotic twin embryos obtained from isolated blastomeres was developed using individual cultures in microwells with a hole created by a needle at the bottom of the tissue culture dish [86]. This system has also been applied to the preparation of chimeras by cell aggregation [110, 111]. However, the preparation of these wells requires labor-intensive manual manufacturing using a needle [86, 110, 111] or cylinder [112]. More recently, a dish with regular wells for the individual culture of intact embryos has been commercially provided, eliminating cumbersome manual work [113].

In the background of these studies, we conducted experiments aimed at establishing a labor-saving culture system to obtain blastocysts from blastomeres. Blastocyst formation using a needle-depressed dish was compared with that using a commercial WOW culture dish employing single blastomeres derived from 2-cell stage embryos cultured in each microwell used as an alternative zona pellucida. As a result, the blastocyst formation rate tended to be higher in the microwells of commercial WOW dishes than in the needle-depressed dishes. Additionally, the ratio of monozygotic pair blastocysts was significantly higher in this dish (Table 5) [114]. Consequently, the shapes of the wells were irregular in the needle-depressed dish, possibly causing the resultant cultures to become unstable. In future studies, it is also necessary to clarify the relationship between shape, such as the diameter of the wells and the size of the blastomeres, with the developmental stage of embryos used for separation.

Fertility of blastocysts developed from separated blastomeres

In the final stage of the development of a monozygotic twin production system using the blastomere separation technique, embryo transfer was attempted to evaluate the fertility of blastocysts developed from isolated blastomeres using Japanese Black cattle embryos derived from OPU and *in vitro* production. In this investigation, we transferred a pair of blastocysts produced in a system combining suitable conditions as previously described. Two-cell stage embryos developed 24–27 h post insemination were used. Zonae pellucidae were removed using pronase, and blastomeres were separated by gentle pipetting in CR1aa supplemented with 5% CS without enzymatic

Table 5. The effect of culture dish on formation of blastocysts that are derived from separated blastomeres

Culture dish	No. of		No. (%) of	
	Sepalated embryos	Cultured blastomeres	Blastocyst	Blastocyst in pair
Needle depressed dish	38	76	35 (46.1)	10 (26.3) [a]
Commercial WOW culture dish	25	50	30 (60.0)	12 (48.0) [b]

Values with different superscripts (a–b) differ significantly ($P < 0.05$). Modified from Hashiyada *et al.* (2015) [114].

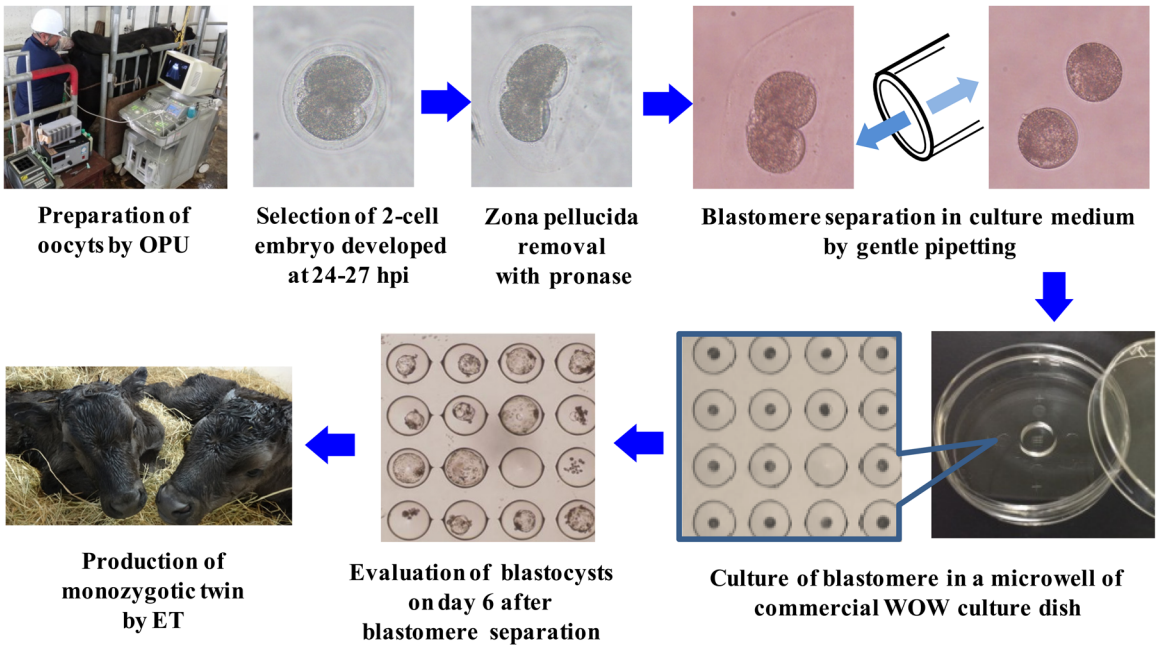

Fig. 4. Outlines for the processes of monozygotic twin calf production using simplified blastomere separation and culture systems.

Table 6. The effect of transfer method of blastocysts that are derived from separated blastomere on monozygotic twin production

Transfer method	No. of		No. (%) of		
	Transferred recipients	Transferred pair of embryos	Pregnancy	Twin pregnancy	Twin production
Single embryo transfer	16	8	8 (50.0)	3 (37.5)	3 (37.5)
Twin embryo transfer	22	22	10 (45.5)	5 (22.7)	3 (13.6)
Total	38	30	18 (47.4)	8 (26.7)	6 (20.0)

treatment. Developing culture for each blastomere was performed in a microwell of the commercial WOW culture dish with the above culture medium. Morphologically normal blastocysts developed in a pair on day 7 post fertilization were selected for transfer (Fig. 4).

For Japanese Black cattle recipients, which generally have small body frames, each demi-embryo of the monozygotic twin embryos was freshly transferred to produce twin calves from a set of recipients. For the Holstein recipients, a pair of twin embryos was transferred into the uterus ipsilateral to the CL. The resulting pregnancy rate was similar for both types of transfer; however, the twin pregnancy rate and twin birth rate based on the pairs of twin embryos were higher when single embryos were transferred than with twin embryos transfer. Overall, the twin-calf production rate was 20% as a result of transfer of embryos derived from this blastomere separation system (Table 6). This result is approximately twice as high as that of transfer of conventional bisected embryos as reported in our previous studies [32, 115]. Interestingly, according to a report using microwells prepared via needle depression [86], the percentage of live twin births (based on calculation from the data presented in their report) resulting from the transfer of a pair of blastocysts derived from tetra-blastomeres isolated from 8-cell stage embryos was almost the same as those reported in our study. Given these two results, blastocysts derived from separated blastomeres cultured in microwells might have only limited fertility potential. In light of this, further research is required to improve fertility, including measures such as the provision of pregnancy recognition signals when performing embryo transfer.

Current status and Future Progress of Monozygotic Twin Production in Cattle Breeding

To date, we have already produced more than 120 pairs of monozygotic twins in breeding projects on both sires and dams using embryo bisection technique. In addition, 10 sires have been selected after evaluation of fattening via the progeny test following preliminary selection through monozygotic twins testing. An excellent sire was selected in 2016 with the highest marbling score till now. This sire was created from parents and paternal granddam produced through the twin production system (Fig. 5). Thus, the utilization of monozygotic twins has greatly contributed to the improvement in breeding of beef cattle. Meanwhile, a newly developed twin production system based on the blastomere separation technique can be practically used in the sire production system instead of the

Japanese Black sire "Mituhirasakae" born in Apr, 2011 at Tottori station of National Livestock Breeding Center.

Sire Mituhirateru*	Sire Yasuhirateru	Sire Yasuhira
	Dam Mituyasu1-1*	Sire Yasufuku165-9
Dam Sakaedoi-1*	Sire Fukusakae	Sire Yasufuku (Gifu)
	Dam Hisafuku	Sire Tanifukudoi

Pedigree chart of "Mituhirasakae".
* Selected by the monozygotic twin testing.

Results of carcass grading in progeny test

Gender (n)	Dressed weight (kg)	Rib eye area (cm^2)	Marbling score[1]	Rate of quality grade 4 and 5[2] (%)
Steer (18)	443	57	8.4	94
Heifer (8)	406	59	7.4	100
Total (26)	425	58	8.1	96

[1]of all 12 classes, [2]of all 5 grades, assessed by Japan Meat Grading Association.

Cross section of loin in one of the progeny tested steers. Yield and quality grade: A-5, marbling score: No. 9.

Fig. 5. Overview of a selected Japanese Black sire with excellent marbling value produced from bisected embryo by monozygotic twin test subsequent progeny test.

embryo bisection technique. In addition, embryo production based on OPU can be widely applied to donor cattle consistently, regardless of their reproductive performance and breeding stage. It is expected that, in the near future, more efficient sire production systems with faster and superior breeding values may be developed by combining OPU with the blastomere separation protocol.

In conclusion, various factors that affect the production efficiency of monozygotic twin calves via bisection of embryos collected from donors treated with multiple ovulation have been described and clarified. On the other hand, a production system for monozygotic twin embryos based on blastomere separation from embryos (derived from *in vitro* fertilization) during early cleavage stage was developed as a simplified technique in a series of similar studies. Furthermore, it has been verified that the methods that use blastomere separation technique produce monozygotic twins more efficiently than the conventional embryo bisection. This production system will thus contribute greatly to improvement in the breeding of beef cattle. Promotion of the adaptation of OPU in this production system will consistently provide high performance sires and will produce cattle with economically valuable characteristics. In order to steadily promote improvement in breeding, further research is necessary to improve production stability of identical twins.

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