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Potential of preimplantation genomic selection using the blastomere separation technique in bovine *in vitro* fertilized embryos

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Abstract. Preimplantation genomic selection combined with an *in vitro* embryo production system is expected as a means of accelerating genetic improvement in cattle. While micromanipulation-based biopsy approaches are often used to collect embryonic cells for genetic testing, they require expensive equipment and sophisticated skills, hindering the adoption of this system. In the present study, to develop a simple method for preimplantation genomic selection using the blastomere separation (BS) technique in bovine *in vitro* fertilized embryos, we examined the accuracy of single nucleotide polymorphism (SNP) genotyping and optimal cryopreservation method in demi-blastocysts produced by the BS technique. We demonstrated reliable SNP genotyping using DNA derived from demi-blastocysts. We indicated a suitable equilibrium time in vitrification solution for demi-blastocysts and succeeded obtaining pregnancies by the transfer of vitrified demi-blastocysts. In conclusion, our findings suggest that the BS technique provides a simple method for preimplantation genomic selection in bovine *in vitro* fertilized embryos.

Key words: Blastomere separation, Bovine, Embryo, *In vitro* fertilization, Preimplantation genomic selection

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Genomic selection in cattle based on single nucleotide polymorphism (SNP) genotypes is an accepted method of accelerating genetic improvement, resulting in a shortened generation interval and increased reliability of predicted breeding values [1]. In Holstein and Japanese Black cattle, a genomic selection system has been established and introduced for the prediction of economically important traits, such as lactation or carcass characteristics [2–4]. Additionally, we developed a preimplantation genomic selection system that combines genomic selection with multiple ovulation and embryo transfer (MOET) and preimplantation genetic testing [5]. This system is expected to render cattle breeding more efficient through the selective production of genetically superior animals. Alternatively, the use of ovum-pick up-*in vitro* fertilization (OPU-IVF) technology has notably increased worldwide in recent years compared to that of the MOET program [6], due to the higher production efficiency of embryos [7]. Combining the preimplantation genomic selection system and OPU-IVF technology will enable further acceleration of cattle breeding [8].

The collection of embryonic cells is a prerequisite for preimplantation genomic selection. Microblade biopsy using a micromanipulator is often used to obtain embryonic cells. However, micromanipulation requires the use of expensive equipment and sophisticated skills, which prevents the widespread application of preimplantation genomic selection.

Blastomere separation (BS) is a well-established technique to produce monozygotic twin embryos/calves by separating the blastomeres of IVF embryos at the 2–8-cell stages [9, 10]. Blastomere separation does not require expensive equipment and skilled manipulation; therefore, it can potentially be used in a preimplantation genomic selection system in IVF embryos with simple procedure. Particularly, one of monozygotic demi-embryos is used for genomic evaluation, while the other is used for calf production.

In the present study, for the development of a simple method for preimplantation genomic selection using the BS technique in bovine IVF embryos, we examined the accuracy of SNP genotyping and the optimal cryopreservation method in demi-blastocysts produced by the BS technique.

First, we evaluated the developmental competence of separated blastomeres derived from two-cell stage embryos (BS group) and intact two-cell stage embryos (control group) using a commercial well of the well (WOW) culture system. In the BS group, the rate at which re-cleavage occurred 24 h post BS and blastocyst formation on day 7 (IVF = day 0) was 92.0 (195/212) and 78.3% (166/212), respectively. The rate of re-cleavage and blastocyst formation in a pair was 84.9 (90/106) and 67.9% (72/106), respectively. Tagawa *et al*. [9] and Hashiyada *et al*. [10] reported that the ratio of a monozygotic blastocyst pair from a two-cell embryo using the WOW culture system was 62.0 and 48.0%, respectively. We confirmed that the production efficiency of monozygotic twin embryos in the current study compares favorably with previous reports. Additionally, we observed that the rate at which a couple of blastomeres developed into a blastocyst and to a ≥ 16-cell stage embryo (thought to be applicable for reliable SNP genotyping [5]) at day seven was 73.6% (78/106), which was not different from the rate of blastocyst formation at day

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seven in the control group (81.3%, 87/107). This result indicates that BS treatment does not reduce the efficiency of the preimplantation genomic selection system compared to the microblade biopsy approach using intact blastocysts.

We evaluated the accuracy of SNP genotyping in demi-blastocysts derived from separated blastomeres at day eight. As shown in Table 1, the call rates in SNP genotyping using DNA from demi-blastocysts at day eight processed with whole genome amplification (WGA) ranged from 98.3–99.3%. The concordance rate for co-called genotypes between monozygotic demi-blastocysts from embryos No. 1–5 and 7–10 was greater than 99.9%, while that for embryo No. 6 was 99.3%. We reported that the genomic estimated breeding values (GEBVs) for carcass traits, such as carcass weight, ribeye area, and marbling, calculated from the embryonic biopsied cells (approximately 15 cells) with call rates of 98.5–99.3% closely matched those from the corresponding calves produced by embryo transfer in Japanese Black cattle [5]. Furthermore, in a study on dairy cattle, it was observed that the correlation between the GEBV of kg of milk protein (based on 305-day production) in embryonic biopsied cells and the corresponding calves was high ($r^2 = 0.95$) when samples with a call rate >85% were used [11]. Overall, our findings indicate that it is possible to perform accurate SNP genotyping and GEBV calculations using day eight demi-blastocysts produced by the BS technique. On the other hand, a pair of demi-blastocysts from embryo No. 6 showed a lower concordance rate compared to that of other pairs (Table 1). In this pair, 95.9% (324/338) of mismatched SNP genotypes were observed on chromosome 27. Additionally, the rate of SNPs on chromosome 27 whose genotypes were homozygous in one demi-blastocyst was 99.8% (878/880), while that of the other demi-blastocyst was 37.3% (335/898). Hence, it was speculated that one demi-blastocyst from embryo No. 6 was monosomic for chromosome 27, which is caused by post-zygotic errors. Tšuiko *et al*. [12] demonstrated that chromosome stability of bovine IVF embryos is lower than that of embryos produced *in vivo* by studies using SNP arrays. Chromosomal aneuploidy is recognized as a leading cause of pregnancy failure in humans [13]. An approach that performs simultaneous genomic evaluation and aneuploidy testing based on SNP genotyping will enhance the efficiency of the preimplantation genomic selection system in IVF embryos by improving the pregnancy rate, as reported by Turner *et al*. [14].

Since several days are required for SNP genotyping and GEBV calculation, cryopreservation of genotyped embryos is necessary for the preimplantation genomic selection system. Although it has been reported that separated blastomeres of early stage (2–8-cell stage) bovine IVF embryos could be successfully vitrified using a Cryotop device [15], the optimal vitrification protocol for demi-blastocysts derived from separated blastomeres remains unclear. Therefore, in the present study, we investigated the effects of equilibrium time during the vitrification process on the viability of day seven demi-blastocysts after warming. For the BS group, the equilibrium times in equilibration and vitrification solutions were, respectively, set for 3 min and 60 sec (BS-3/60), for 2.5 min and 50 sec (BS-2.5/50), for 2 min and 40

Table 1. The call rates in SNP genotyping in demi-blastocysts derived from the separated blastomeres and the concordance rate for co-called SNP genotypes between the monozygotic twin demi-blastocysts

Embryo No.	Sample type	Call rate * (%)	The concordance rate for co-called SNP genotypes † between the monozygotic twin demi-blastocysts (%)
1	One	99.3	99.9
	The other	99.3	
2	One	99.3	99.9
	The other	99.3	
3	One	99.3	99.9
	The other	98.8	
4	One	99.3	99.9
	The other	99.3	
5	One	99.2	99.9
	The other	99.3	
6	One	99.2	99.3
	The other	99.2	
7	One	99.0	99.9
	The other	99.3	
8	One	99.3	99.9
	The other	99.2	
9	One	99.2	99.9
	The other	99.0	
10	One	99.3	99.9
	The other	98.3	

* Call rate is defined as the proportion of target SNPs giving positive signals on XT Chip (LIAJ custom_50v1, 50,019 SNPs) analysis. † SNPs called in both one and the other demi-blastocysts.

sec (BS-2/40), or for 1.5 min and 30 sec (BS-1.5/30). For the control group, the equilibrium times in equilibration and vitrification solutions were set for 3 min and 60 sec, respectively. As shown in Table 2, the survival rate after 24 h culture post vitrification-warming in the BS-1.5/30 group was significantly ($P < 0.05$) lower than that in the control group, while those in the BS-3/60, BS-2.5/50, and BS-2/40 groups did not differ from those in the control group. There was no difference in the total cell numbers in blastocysts after 24 h culture post vitrification-warming among all the BS groups, while the total cell numbers in blastocysts in all the BS groups were significantly ($P < 0.0001$) lower than those in the control group (Fig. 1A). On the other hand, the rate of dead cells in the blastocysts after 24 h culture post vitrification-warming in the BS-2.5/50 group was not different from that in the control group, while those in the BS-3/60, BS-2/40, and BS-1.5/30 groups were significantly ($P < 0.05$) higher than those in the control group (Fig. 1B). These findings indicate that equilibration for 2.5 min in the equilibration solution and 50 sec in the vitrification solution is suitable for the vitrification of zona-free demi-blastocysts produced by the BS technique under the conditions of this study. The demi-blastocysts derived from the separated blastomeres lack a zona pellucida and have fewer cell numbers and overall size compared with that of intact blastocysts. Consequently, a shorter equilibrium time compared with that in the control group might have a beneficial effect on the viability of the demi-blastocyst after vitrification-warming by reducing the toxicity caused by a high concentration of cryoprotectants. Following this, six vitrified-warmed demi-blastocysts from the BS-2.5/50 group (Fig. 2) were transferred to recipient cows, and three pregnancies were successfully obtained. No abortions or abnormal fetal sizes were encountered in the pregnant cows by day 65 of gestation. Although additional studies with larger sample sizes are required, our results showed that the conception rate of vitrified demi-blastocysts

Table 2. Effect of equilibrium time on viability of demi-blastocysts after vitrification-warming

Experimental group	Equilibrium time		No. of embryos	No (%). of viable ‡ embryos
	Equilibrium sol * (min)	Vitrification sol † (sec)		
BS	3	60	20	18 (90) [ab]
	2.5	50	20	19 (95) [ab]
	2	40	20	18 (90) [ab]
	1.5	30	20	14 (70) [b]
Control	3	60	20	20 (100) [a]

Experiments were replicated six times. * Equilibrium sol: TCM-199 containing 7.5% EG, 7.5% DMSO, and 20% NBCS. † Vitrification sol: TCM-199 containing 15% EG, 15% DMSO, 0.5 M sucrose, and 20% NBCS. ‡ Re-expanded blastocysts after 24 h of culture post-warming. [a, b] Different letters indicate a significant difference ($P < 0.05$).

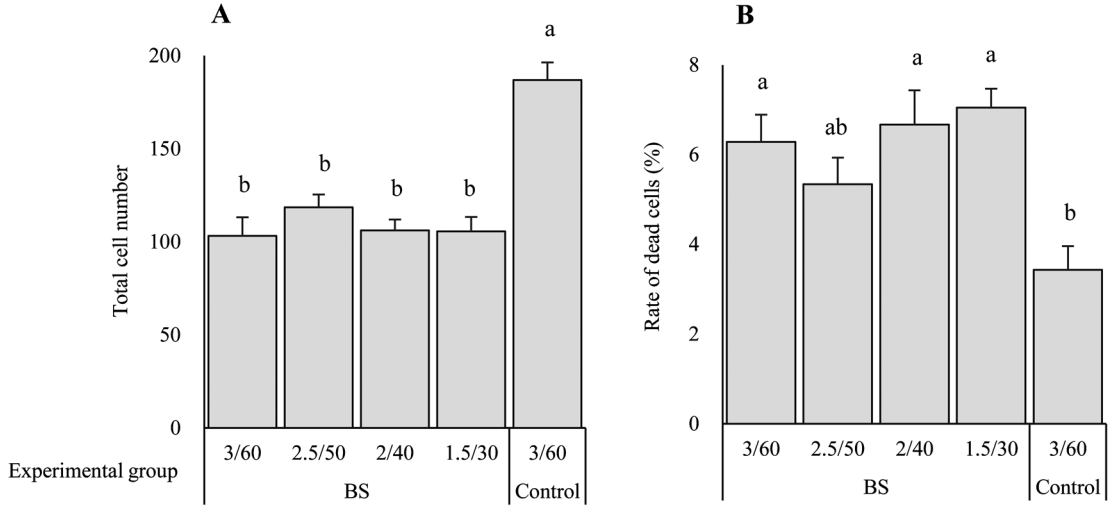

Fig. 1. Mean total cell numbers and rate of dead cells of vitrified-warmed blastocyst in the BS and control groups. (A) Cell numbers were counted after 24 h of culture post vitrification-warming. (B) Dead cell numbers were counted after 24 h of culture post vitrification-warming. The rate of dead cells was calculated as the ratio of dead cell numbers to the total cell numbers. BS group: The equilibrium times in equilibration and vitrification solutions were, respectively, set for 3 min and 60 sec (3/60, n = 17), 2.5 min and 50 sec (2.5/50, n = 18), 2 min and 40 sec (2/40, n = 18), or 1.5 min and 30 sec (1.5/30, n = 13). Control group: The equilibrium times in equilibration and vitrification solutions were set for 3 min and 60 sec (n = 20), respectively. [a, b] Different letters indicate a significant difference (A: $P < 0.0001$, B: $P < 0.05$).

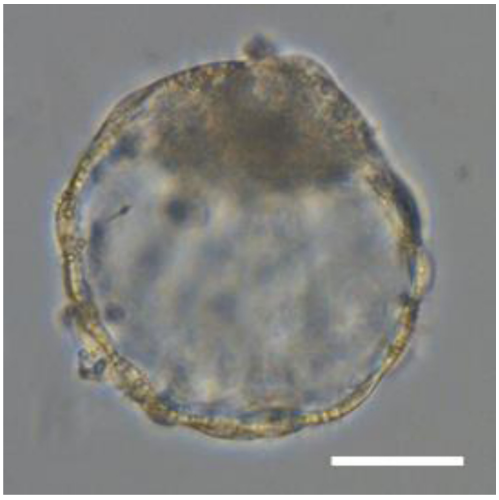

Fig. 2. A representative photograph of the vitrified-warmed demi-blastocyst. Image was captured 3 h after vitrification-warming with the 2.5/50 condition. Scale bar = 50 µm.

derived from a separated blastomere was comparable to that of fresh demi-blastocysts derived from a separated blastomere reported previously [10]. Overall, our results demonstrated for the first time, that demi-blastocysts derived from a separated blastomere could be successfully cryopreserved by the Cryotop vitrification method, and that they exhibited normal developmental abilities following embryo transfer.

In conclusion, we demonstrated reliable SNP genotyping in demi-blastocysts derived from a separated blastomeres. Furthermore, we showed that cryopreservation of zona-free demi-blastocysts can be achieved using the Cryotop vitrification method. The findings of the present study suggest that the BS technique provides a means of preimplantation genomic selection in bovine IVF embryos using a simple procedure. Further studies using OPU-IVF embryos are required to assess the practical implementation of this system.

Methods

Animal care

This study was approved by the Animal Ethics Committee, Animal Research Center, Hokkaido Research Organization.

Production of IVF embryos

Ovaries were obtained from a local slaughterhouse (Obihiro, Hokkaido, Japan) and were transported in physiological saline solution at approximately 20°C. Cumulus-oocyte complexes (COCs) were aspirated from 2–8 mm ovarian follicles. COCs with homogeneous ooplasm and more than six layers of compact and non-atretic cumulus cells were selected and cultured in drops of IVMD101 medium (Research Institute for the Functional Peptides, Yamagata, Japan) covered with mineral oil (Wako Pure Chemical Industry, Osaka, Japan) for 22 h at 38.5°C in a humidified atmosphere of 95% air and 5% CO_2.

Frozen sperm from a Japanese Black bull were thawed at 37°C for 60 sec, and then centrifuged twice at 600 *g* for 5 min in IVF100 medium (Research Institute for the Functional Peptides). After centrifugation, the sperm was adjusted to a final concentration of 5×10^6 sperm/ml using IVF100 medium. After *in vitro* maturation, COCs were transferred to a sperm suspension drop covered with mineral oil and incubated for 6 h at 38.5°C in a humidified atmosphere of 95% air and 5% CO_2.

After IVF, the presumptive zygotes were denuded from cumulus cells and spermatozoa by pipetting in PBS supplemented with 4% newborn calf serum (NBCS, Thermo Fisher Scientific, Waltham, MA, USA), and washed seven times in a commercial one-step culture medium (BO-IVC; IVF Bioscience, Falmouth, United Kingdom). The presumptive zygotes were then cultured in a drop of culture medium covered with mineral oil at 38.5°C in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2. Two-cell stage embryos 27 h post IVF that were equally divided and without any fragments were selected and allocated to the BS and the control (non-separated) groups. Pairs of separated blastomeres and non-separated embryos in the control group were cultured in a WOW culture dish (LinKID® micro25; DaiNippon Printing Co., Ltd., Tokyo, Japan) filled with culture medium covered with mineral oil up to day seven or day eight (IVF = day 0) at 38.5°C in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2.

Blastomere separation

BS was performed according to the method described by Hashiyada *et al*. [10] with some modifications. Two-cell stage embryos in the BS group were immersed in 0.5% protease (Sigma-Aldrich, St. Louis, MO, USA) in PBS (Invitrogen, Carlsbad, CA, USA) for approximately 3 min to remove the zona pellucida. Thereafter, embryos were washed three times in culture medium and separated into pairs of single blastomeres by gentle pipetting in the culture medium.

DNA extraction, WGA, and SNP genotyping

The demi-blastocysts were washed three times in PBS supplemented with 0.02% polyvinyl alcohol (PVA, Sigma-Aldrich), and then washed in PBS without PVA. The blastocysts were individually collected into 0.2 ml tubes containing 1.5 µl PBS (Invitrogen). DNA extraction and WGA were performed using the REPLI-g Single Cell Kit (Qiagen, Manchester, UK) according to the manufacturer's instructions with some modifications. Briefly, 1.5 µl buffer DLB containing dithiothreitol (built into the kit) was added to the tubes containing a demi-blastocyst, incubated at 65°C for 10 min, and then 1.5 µl Stop Solution (built into the kit) was added. Next, 20 µl master mix containing DNA polymerase (built into the kit) was added and incubated at 30°C for 8 h. DNA processed for WGA was quantified using NanoDrop One (Thermo Fisher Scientific, Waltham, MA, USA), diluted with TE buffer (Invitrogen) to a concentration of 106.8–111.3 ng/µl, and stored at −80°C until SNP genotyping.

DNA samples were processed for SNP genotyping using the Infinium XT Assay Kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. SNP genotyping was performed using XT Chip (LIAJ custom_50v1, 50,019 SNPs) customized by the Livestock Improvement Association of Japan (Maebashi Institute of Animal Science, Gunma, Japan).

Vitrification and assessment of viability of vitrified-warmed blastocysts

Vitrification of the blastocysts was performed according to the method described by Inaba *et al*. [16] with some modifications. Blastocysts were placed into an equilibration solution consisting of 7.5% ethylene glycol (EG, Wako), 7.5% dimethyl sulfoxide (DMSO, Wako), and 20% NBCS in TCM199 (Thermo Fisher Scientific) for 1.5, 2.0, 2.5, or 3 min, and then transferred to a vitrification solution consisting of 15% EG, 15% DMSO, 0.5 M sucrose (Wako), and 20% NBCS in TCM199 for 30, 40, 50, or 60 sec. The blastocysts were subsequently placed on a Cryotop® device (Kitazato BioPharma, Shizuoka, Japan) with a small volume (< 1 µl) of vitrification solution, directly immersed in liquid nitrogen, and preserved therein until warming. Vitrified embryos were warmed in PBS (Invitrogen) supplemented with 0.2 M sucrose and 20% NBCS at 38.5°C for 3 min.

Warmed blastocysts were cultured in a culture medium for 24 h at 38.5°C in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 to confirm their survival. Re-expanded and shrunken embryos were classified as surviving and degenerated embryos, respectively. Re-expanded blastocysts were subsequently subjected to dead cell-detection tests. Dead cells were stained using a LIVE/DEAD Cell Imaging Kit (488/570; Thermo Fisher Scientific) according to the manufacturer's instructions. Thereafter, the blastocysts were fixed overnight at 4°C in 99.5% ethanol supplemented with 5 µg/ml Hoechst 33342 (Sigma-Aldrich) for nuclear staining, and then mounted on slides in a drop of glycerol. Fluorescence images were obtained using a Nikon E800 fluorescence microscope (Nikon, Tokyo, Japan). The total and dead cell numbers were determined using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The dead cell rate of the blastocysts was calculated as the ratio of the numbers of dead cells to the number of total cells.

Embryo transfer

Aberdeen Angus cows were used as the embryo recipients. The recipient cows were estrus-synchronized by an injection of 1 mg of estradiol benzoate (Kyoritsu Seiyaku, Tokyo, Japan), administration of a controlled internal drug release device (CIDR, Zoetis JAPAN, Tokyo, Japan) for 10 days, and an injection of prostaglandin F2α (cloprostenol 0.5 mg/cow, Resipron®-C, ASKA Animal Health, Tokyo, Japan) two days prior to CIDR removal. Vitrified-warmed demi-blastocysts derived from the separated blastomeres were transferred (one blastocyst per recipient) to a uterine horn ipsilateral to the corpus luteum of the recipient cows at eight days post estrus using a YT gun (Yamanetech Co., Ltd., Nagano, Japan). Pregnancy diagnoses were performed using ultrasonography at days 35 and 65 of gestation by confirming the existence of conceptuses and their heartbeats.

Statistical analysis

The rates at which a couple of blastomeres developed into a blastocyst and a ≥ 16-cell stage embryo, blastocyst formation in the control group, embryo survival after vitrification-warming, and dead cells after vitrification-warming were subjected to arcsine transformation. The differences in the rate at which a couple of blastomeres developed into blastocysts and ≥ 16-cell stage embryos and that of blastocyst formation in the control group were analyzed using the Mann-Whitney U test. Differences in the rate of embryo survival, total cell numbers, and the rate of dead cells after vitrification-warming among the experimental groups were analyzed by the Kruskal-Wallis test followed by multiple pairwise comparisons using Scheffe's method. Statistical significance was set at $P < 0.05$. Data in Fig. 1 are reported as the mean ± SEM.

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