—Original Article—

Production of calves by the transfer of cryopreserved bovine elongating conceptuses and possible application for preimplantation genomic selection

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Abstract. Preimplantation genomic selection based on single nucleotide polymorphism (SNP) genotypes is expected to accelerate genetic improvement in cattle. However, genome-wide genotyping at the early embryonic stage has several limitations, such as the technical difficulty of embryonic biopsy and low accuracy of genotyping resulting from a limited number of biopsied cells. After hatching from the zona pellucida, the morphology of the bovine embryo changes from spherical to filamentous, in a process known as elongation. The bovine nonsurgical elongating conceptus transfer technique was recently developed and applied for sexing without requiring specialized skills for biopsy. In order to develop a bovine preimplantation genomic selection system combined with the elongating conceptus transfer technique, we examined the accuracy of genotyping by SNP chip analysis using the DNA from elongating conceptuses (Experiment 1) and optimal cryopreservation methods for elongating conceptuses (Experiment 2). In Experiment 1, the call rates of SNP chip analysis following whole genome amplification in biopsied cells from two elongating conceptuses were 95.14% and 99.32%, which were sufficient for estimating genomic breeding value. In Experiment 2, the rates of dead cells in elongating conceptuses cryopreserved by slow freezing were comparable to those in fresh elongating conceptuses. In addition, we obtained healthy calves by the transfer of elongating conceptuses cryopreserved by slow freezing. Our findings indicate that the elongating conceptus transfer technology enables preimplantation genomic selection in cattle based on SNP chip analysis. Further studies on the optimization of cryopreservation methods for elongating conceptuses are required for practical application of the selection system.
Key words: Cattle, Cryopreservation, Elongating conceptus, Embryo transfer, Preimplantation genomic selection

Genomic selection in cattle based on high-throughput microarray platforms for genotyping tens of thousands to hundreds of thousands of single nucleotide polymorphisms (SNPs) is expected to accelerate genetic improvement with a shortened generation interval and increased reliability of predicted breeding values [1–3]. Genomic selection has been introduced for the selection of young bulls with positive impacts for facilitating a reduced generation interval and

selection of traits with low heritability, such as fertility, lifespan, and the udder health of daughters [4, 5]. On the other hand, reproductive technologies, such as multiple ovulation, ovum pick up and *in vitro* fertilization, and embryo transfer enable the acceleration of genetic material proliferation from a selected dam, and combine well with genomic selection in cattle [6, 7]. In addition, preimplantation genetic diagnosis (PGD) enables efficient production of progeny by increasing the selection pressure and efficient use of recipient animals. To further increase the efficiency of genetic improvement in cattle, the establishment of a genomic selection system by PGD is strongly required.

An embryonic biopsy is a prerequisite for PGD and is usually performed during the morula and blastocyst stages in the bovine embryo. As an enlarged biopsy leads to reduced pregnancy rate after cryopreservation and transfer [8], the number of biopsied cells from the early bovine embryo is limited to approximately 10. Although the PGD of limited loci, such as those related to sex and

genetic disorders, is possible using a few biopsied cells [9–11], SNP chip analysis based on microarray platforms for genomic selection requires a larger amount of DNA template. DNA amplification by whole-genome amplification (WGA) techniques may enable SNP chip analysis using a small amount of DNA template. However, using a limited number of biopsied cells decreases the efficiency of WGA, which leads to missing calls and allele dropouts, resulting in decreased accuracy of genotyping [12]. Therefore, genomic selection by PGD in the early embryonic stages, such as morula and blastocyst, is yet to be used as a practical technique for genetic improvement in cattle.

Following successful fertilization, the bovine embryo enters the uterus on day 4 to 5, following which it forms a blastocyst by day 7. After hatching from the zona pellucida, the morphology of the bovine blastocyst changes from spherical to filamentous, in a process known as elongation, and is termed as the 'conceptus'. The elongating conceptus grows a few millimeters to centimeters in length by day 14 of gestation, and reaches tens and several centimeters by day 16 [13]. Recently, Kimura *et al.* [14] reported the successful nonsurgical transfer of bovine elongating conceptuses. They showed that pregnancy rates in transfer experiments did not differ between day 7 embryos and elongating conceptuses. Furthermore, biopsy of the trophectoderm fragment containing several thousand cells for sexing did not affect the pregnancy rate after transfer. These findings led us to apply the elongating conceptus transfer technique for genomic selection by PGD, which requires a large amount of DNA template.

On the other hand, since at least several weeks are required for genotyping by SNP chip analysis, preservation of bovine elongating conceptus is necessary for preimplantation genomic selection. However, methods for *in vitro* culture and cryopreservation of bovine elongating conceptuses are not yet established. Although slow freezing and vitrification based methods are widely used for cryopreservation of bovine early embryos [15–17], the usefulness of these methods for bovine elongating conceptuses is still unknown.

In the present study, we investigated the accuracy of genotyping by SNP chip analysis using biopsied cells from bovine elongating conceptuses (described as Experiment 1) and the optimal cryopreservation method for bovine elongating conceptuses (described as Experiment 2) to develop a bovine preimplantation genomic selection system combined with the elongating conceptus transfer technique.

Materials and Methods

All experiments using animals were approved by the Animal Ethics Committee, Animal Research Center, Hokkaido Research Organization.

Production of bovine blastocysts and elongating conceptuses
Japanese Black cattle and Angus cattle maintained at the Animal Research Center Hokkaido Research Organization were used as donors for the production of blastocysts and elongating conceptuses. At any stage of the estrous cycle, controlled internal drug release devices (CIDR®1900, Pfizer Japan, Tokyo, Japan) were inserted into the vaginas of donors. After 2–4 days, 0.5 mg of estradiol benzoate (EB, Kyoritsu seiyaku, Tokyo, Japan) was administered to each cow. Four days after the EB injection, in total 20 IU of follicle-stimulating hormone (FSH) (Antrin®R-10, Kyoritsu Seiyaku) was intramuscularly

administered to each cow twice daily in decreasing doses over 3 days. The CIDR was removed 48 h after initiation of FSH treatment, and prostaglandin F2α (cloprostenol 0.5 mg/cow, Resipron®-C, ASKA Animal Health, Tokyo, Japan) was intramuscularly injected to induce luteolysis. Donor cows were bred by artificial insemination (AI) at 12–24 h after the onset of estrus using frozen-thawed semen. Blastocysts and elongating conceptuses were non-surgically recovered by uterine flushing using balloon catheters (Fujihira Industries, Tokyo, Japan) on day 8 and day 14, 15, or 16 (the day of AI = day 1), respectively. For the recovery of elongating conceptuses, catheters processed to have enlarged side holes (10 mm length, 2 mm width, Supplementary Fig. 1: online only) were used. Blastocysts were classified according to the International Embryo Technology Society (IETS) manual [18], and Code 1 blastocysts were used for the experiment. For elongating conceptuses, lengths were measured, and morphological conditions (intact or fragmented, and existence of the embryonic disc) were assessed.

Biopsies of elongating conceptuses
Biopsies of elongating conceptuses were conducted under a stereomicroscope. From one end of each elongating conceptus, approximately 0.5–1.0 mm of the trophectoderm fragment was manually removed using a surgical razor blade (No. 14, FEATHER Safety Razor, Osaka, Japan) in modified phosphate-buffered saline (PBS, Invitrogen, Carlsbad, CA, USA) supplemented with 10% new born calf serum (NBCS, Thermo Fisher Scientific, Kanagawa, Japan).

DNA extraction
Elongating conceptuses and biopsied cells were individually collected into 1.5 ml tubes with 200 µl of PBS. DNA extraction was performed using the DNeasy Blood & Tissue Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. The final volume of the extracted DNA was 200 µl. Extracted DNA was quantified using the NanoDrop spectrometer (ND-1000; Thermo Fisher Scientific).

WGA and genotyping of SNPs
Genotyping of SNPs was performed using the GGP Bovine LD (v3) BeadChip (Illumina, San Diego, CA, USA, 26,151 SNPs) or GeneSeek bovine 30K BeadChip (GeneSeek, Lincoln, NE, USA, 30105 SNPs). WGA was performed using a multiple displacement amplification (MDA)-based method built into the Infinium® HD Assay Kit (Illumina) according to the manufacturer's instructions to prepare the DNA sample for the GGP Bovine LD (v3) BeadChip. WGA for the GeneSeek bovine 30K BeadChip was performed by GeneSeek Industries. The call rate was defined as the proportion of target SNPs providing positive signals, regardless of whether the called genotype was correct.

Cryopreservation of bovine blastocysts and elongating conceptuses
Slow freezing: Slow freezing was performed according to the method described by Dochi *et al.* [16] with a brief modification. Blastocysts and elongating conceptuses were directly transferred to a solution consisting of 1.5 M ethylene glycol (Wako pure chemical industry, Osaka, Japan), 0.1 M trehalose (Wako pure chemical industry), and 18% NBCS in PBS. Blastocysts were then allowed to

stand for 10 min for equilibration (EGT10). Elongating conceptuses were allowed to stand for 10 min or 60 min for equilibration (EGT10 and EGT60, respectively). After equilibration, the blastocysts and elongating conceptuses were individually loaded into 0.25 ml plastic straws (Fujihira Industries), and placed into an alcohol bath in a programmable freezer (ET-3; Fujihira Industries) precooled to −7.0°C, and then ice-seeded. After 15 min, the straws were cooled to −35°C at a rate of −0.3°C/min, maintained at −35°C for 5 min, and were then plunged into liquid nitrogen. During the thawing process, the straws were warmed in air for 10 sec, following which they were plunged into a water bath at 30°C for 30 sec.

Vitrification: Vitrification was performed according to the method described by Inaba *et al*. [17] and Higaki *et al*. [19] with modifications. Elongating conceptuses were placed into an equilibration solution consisting of 7.5% ethylene glycol, 7.5% dimethyl sulfoxide (DMSO, Wako pure chemical industry), and 20% NBCS in TCM199 (Thermo Fisher Scientific, Waltham, MA, USA) for 3 or 10 min, and then transferred into a vitrification solution consisting of 15% ethylene glycol, 15% DMSO, 0.5 M sucrose (Wako pure chemical industry), and 20% NBCS in TCM199 for 1 or 5 min (Vit3-1 and Vit10-5), respectively. Each elongating conceptus was then placed on a 0.5 × 1.5 cm^2 nylon mesh (Semite, Osaka, Japan), and the excess vitrification solution was removed [19]. Elongating conceptuses on the nylon mesh were directly plunged into liquid nitrogen, and preserved in punctured Cryotube in liquid nitrogen. Vitrified elongating conceptuses were warmed in PBS supplemented with 0.2 M sucrose and 20% NBCS at 37°C for 5 min.

Detection of dead cells in bovine blastocysts and elongating conceptuses after cryopreservation

After freeze-thawing or vitrification-warming, blastocysts and elongating conceptuses were washed three times in PBS supplemented with 4% NBCS, and then cultured in TCM199 supplemented with 10% fetal bovine serum (FBS, MP Biomedicals, Illkirch, France) in 5% CO_2, 5% O_2, and 90% N_2 at 39°C for 5 h, respectively. Some fresh elongating conceptuses were also cultured for 5 h as controls. Re-expanding and shrunken blastocysts after 5 h of culture were classified as viable and degenerated, respectively. Dead cells were stained using the LIVE/DEAD Cell Imaging Kit (488/570) (Thermo Fisher Scientific) according to the manufacturer's instructions. After staining dead cells, the blastocysts and elongating conceptuses were fixed overnight at 4°C in 99.5% ethanol supplemented with 5 μg/ml of Hoechst 33342 (Calbiochem, La Jolla, CA, USA) for nuclear staining, and then mounted on slides in a drop of glycerol. Fluorescence images were obtained on a Nikon E800 fluorescence microscope (Nikon, Tokyo, Japan). The fluorescence areas of nuclei in dead cells (red fluorescence) and in total cells (blue fluorescence) were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The rates of dead cells in blastocysts and elongating conceptuses were calculated as the ratio of nuclear areas in dead cells to total cells.

Transfer of bovine elongating conceptuses

Recipient cows were estrus-synchronized by administration of a CIDR device for 7–14 days and an injection of prostaglandin F2α on the day of CIDR removal. In experiment 2, we used recipient cows at 10 or 12 days after estrus, which were fertile in a preliminary transfer study using fresh elongating conceptuses. A day 14 intact elongating conceptus cryopreserved by the EGT10 method was transferred to a uterine horn ipsilateral to the corpus luteum using a gun for day 7 embryo transfer with a homemade modified sheath. The tip of the sheath was cut off, and the cut end was smoothened with a file [14].

Experimental design

Experiment 1: First, the efficiency of DNA extraction from the elongating conceptus was examined. Elongating conceptuses were recovered at 14, 15, or 16 days post AI. The relationship between the lengths of elongating conceptuses and the amount of extracted DNA was examined.

Second, the effect of the amount of DNA template from elongating conceptuses on the accuracy of genotyping was investigated. DNA was extracted from one fragmented day 16 elongating conceptus (8.1 mm length), and quantified using a NanoDrop spectrometer. The extracted DNA was diluted to 10, 2, and 0.4 ng/μl concentrations. Of each DNA dilution, 4 μl (40, 8, and 1.6 ng of DNA) was processed for WGA using the MDA-based method built into the Infinium® HD Assay Kit. Genotyping was conducted using the GGP Bovine LD (v3) BeadChip, and call rates were calculated.

Furthermore, genotyping was performed using the DNA from biopsied cells of elongating conceptuses. Two intact day 15 elongating conceptuses (elongating conceptus A: 2.4 mm, elongating conceptus B: 4.4 mm) were biopsied (Fig. 1A and 1B), and DNA was extracted from both biopsied samples and the remaining conceptus. From the biopsied cells of elongating conceptus A and B, 37.9 and 8.7 ng of DNA, respectively, were processed for WGA by the method provided by GeneSeek Industries, and the amplified DNA were used for genotyping. An additional portion of DNA from the biopsied cells and DNA from the remaining conceptuses was vacuum-concentrated. Then, 341.1 ng of concentrated DNA from biopsied cells of elongating conceptus A, 266.2 ng of DNA from biopsied cells of elongating conceptus B, 1588.8 ng of concentrated DNA from remaining conceptus A, and 1360.8 ng of concentrated DNA from remaining conceptus B were directly used for genotyping. Genotyping was performed using the GeneSeek bovine 30K BeadChip. The call rates and error rates defined as the proportion of different genotypes relative to the remaining conceptus were calculated.

Experiment 2: Optimal cryopreservation methods for bovine elongating conceptuses were investigated based on the detection of dead cells after cryopreservation. First, to confirm the usefulness of the evaluation method for dead cells used in the present study, we compared the rate of dead cells among fresh blastocysts and the viable and degenerated blastocysts after cryopreservation by EGT10. Next, the rates of dead cells were compared among fresh elongating conceptuses (Fresh), cultured fresh elongating conceptuses (Cultured), and elongating conceptuses cryopreserved by EGT10, EGT60, Vit3-1, and Vit10-5 methods.

Moreover, three of day 14 intact elongating conceptuses were cryopreserved by EGT10 and directly transferred to recipient cows at 10 or 12 days after estrus.

Statistical analysis

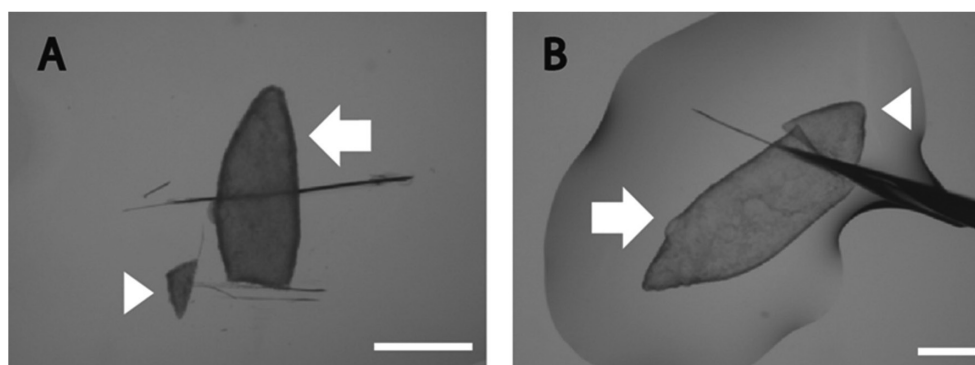In Experiment 1, the relationship between the lengths of elongating

Fig. 1. Representative photographs showing the biopsy of the elongating conceptus. Two bovine elongating conceptuses (A and B) were biopsied manually with a surgical razor blade under a stereomicroscope. Arrowheads indicate the biopsied cells. Arrows indicate the remaining conceptus. Scale bar is 1 mm.
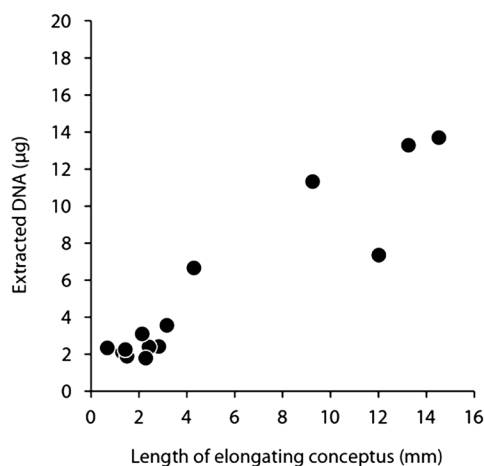

Fig. 2. Relationship between the lengths of elongating conceptuses and the total amount of extracted DNA. The correlation coefficient was 0.94 (n = 15, P < 0.001).

Table 1. The call rates on genotyping by SNP chip analysis using different amount of DNA from bovine elongating conceptus

DNA amount (ng)	Call rate [a] (%)
40	99.69
8	99.55
1.6	98.54

[a] Call rate was defined as the proportion of target SNPs giving positive signals on GGP Bovine LD (v3) BeadChip analysis following WGA.

conceptuses and the amount of extracted DNA was analyzed by Pearson's correlation coefficient. In Experiment 2, data for the rates of dead cells were subjected to arcsine transformation. Differences in the rate of dead cells were analyzed by the Kruskal-Wallis test followed by multiple pairwise comparisons using Scheffe's method. Differences in the average lengths of elongating conceptuses were analyzed by the Kruskal-Wallis test. A probability value P < 0.05 was considered statistically significant. Data were reported as mean ± SE.

Results

Experiment 1: Efficiency of DNA extraction from elongating conceptuses

The lengths of elongating conceptuses ranged from 0.68 to 14.52 mm. As shown in Fig. 2, the correlation coefficient between the lengths of elongating conceptuses and the total amount of extracted DNA was 0.94 (P < 0.001). The average amount of extracted DNA per millimeter of elongating conceptus was 1.31 ± 0.67 µg.

Effect of the amount of DNA template from elongating conceptuses on the accuracy of genotyping by SNP chip analysis

As shown in Table 1, the call rates on genotyping using 40, 8, and 1.6 ng of DNA from elongating conceptuses were 99.69, 99.55, and 98.54%, respectively.

Genotyping by SNP chip analysis using DNA from biopsied cells of elongating conceptuses

As shown in Table 2, the call rates in unamplified DNA from biopsied cells of elongating conceptus A and B (Fig. 1) were 76.37 and 89.89%, respectively. In case of amplified DNA from biopsied cells, the call rates were 95.14 and 99.32%, which were comparable with those of the remaining conceptuses (97.27 and 99.43%). The error rates in unamplified DNA from biopsied cells were 19.30 and 4.00%, whereas the error rates in amplified DNA from biopsied cells were 0.40 and 0.03%, respectively.

Experiment 2: Investigation of optimal cryopreservation methods for bovine elongating conceptuses

The rate of dead cells in degenerated blastocysts after cryopreservation by EGT10 was significantly higher (P < 0.05) than that in fresh blastocysts and viable blastocysts after cryopreservation by EGT10 (Fig. 3A and B).

Table 2. The call rates and error rates on genotyping by SNP chip analysis using DNA from biopsied cells of bovine elongating conceptuses

Elongating conceptus [a]	Sample type	DNA amount (ng)	WGA	Call rate [b] (%)	Error rate [c] (%)
A	biopsied cells	341.1	−	76.37	19.30
	biopsied cells	37.9	+	95.14	0.40
	remaining conceptus	1588.8	−	97.27	N/A
B	biopsied cells	266.2	−	89.89	4.00
	biopsied cells	8.7	+	99.32	0.03
	remaining conceptus	1360.8	−	99.43	N/A

[a] Elongating conceptuses A and B correspond to Fig. 1A and 1B, respectively. [b] Call rate was defined as the proportion of target SNPs giving positive signals on GeneSeek bovine LD30K BeadChip analysis. [c] Error rate was defined as the proportion of different genotype calls relative to that of the remaining conceptus.

The average length of elongating conceptuses in Fresh, Cultured, EGT10, EGT60, Vit3-1, and Vit10-5 groups was 3.3, 3.2, 4.1, 4.6, 3.6, and 3.9 mm, respectively, and there was no significant difference among the groups. No difference in the rate of dead cells was observed among the Fresh, Cultured, EGT10, and EGT60 groups (Fig. 3C and D). The rate of dead cells in elongating conceptuses cryopreserved by Vit3-1 and Vit10-5 was significantly higher ($P < 0.001$) than that in Fresh, Cultured, EGT10, and EGT60 (Fig. 3C and D).

Transfer of bovine elongating conceptuses cryopreserved by slow freezing

The lengths of three transferred elongating conceptuses were 1.5, 1.9 (Fig. 4A), and 14.3 mm, respectively. Two recipients that were transferred with 1.5 and 1.9 mm elongating conceptuses were pregnant, and delivered male calves (Fig. 4B). The calves stood up after birth, and grew normally until at least three years.

Discussion

In the present study, we examined the accuracy of genotyping by SNP chip analysis using biopsied cells from bovine elongating conceptuses to develop a bovine preimplantation genomic selection system combined with the elongating conceptus transfer technique. We could obtain accurate genotyping data that could enable estimation of the genomic breeding value by this technique. Furthermore, we investigated the optimal cryopreservation methods for bovine elongating conceptuses, and succeeded in producing calves from bovine elongating conceptuses cryopreserved by slow freezing.

Although SNP chip analysis generally requires hundreds of nanograms of DNA template, WGA of the trace amounts of DNA extracted from biopsied cells of early embryos might allow genomic selection by PGD using SNP chip analysis. However, Lauri *et al.* [12] reported that the rate of missing calls and allele dropouts was drastically increased when a low amount of DNA template corresponding to less than 30–40 biopsied cells from blastocyst was used, even though WGA was performed. They also proposed *in vitro* culture of biopsied cells as a means of increasing the amount of DNA template and the reliability of genotyping. However, another researcher indicated that the cell culture approach is unacceptable from a commercial point of view because larger biopsies (20 to 30 cells) were required for successful cell proliferation in culture,

leading to significant reduction in embryo viability [20]. The use of elongating conceptuses allows a large number of cells to be biopsied without any reduction in viability; therefore, we attempted to develop a genomic selection system by PGD combined with the elongating conceptus transfer technique.

Although there are no previous studies quantifying the amount of DNA that can be recovered from a bovine elongating conceptus, Puseteri *et al.* [21] reported that > 10 μg of DNA could be recovered from a porcine day 12 elongating conceptus. In the present study, the average total extracted DNA amount per millimeter of elongating conceptus was 1.31 ± 0.67 μg. Kimura *et al.* [14] showed that the average length of bovine day 14 elongating conceptuses was approximately 20 mm, and that a 0.2–0.5 mm trophectoderm fragment could be biopsied without any reduction in the pregnancy rate after transfer. Therefore, it is considered that at least 0.26 μg of DNA can be obtained by the biopsy of a day 14 elongating conceptus. Furthermore, we could obtain a sufficient call rate on genotyping following the WGA, even though 1.6 ng of DNA template was used. Considered together, our findings suggest that it is possible to obtain accurate genotyping data that can be used to estimate the genomic breeding value by using biopsied cells from bovine elongating conceptuses. In fact, we confirmed that a high call rate (> 95%) and low error rate (< 1%) was observed in genotyping using WGA DNA from the biopsied cells of elongating conceptuses. The genotyping using elongating conceptuses described in this study offers a distinct advantage in terms of accuracy compared to the use of a blastocyst [12]. In addition, a biopsy of elongating conceptuses can be performed without the need for expensive equipment and a high level of skill, as previously reported [14]. On the other hand, the calls missed and the error rate increased when genotyping was performed without WGA, indicating that WGA is indispensable for genotyping with a high accuracy.

The slow freezing and vitrification-based methods have been widely used for the cryopreservation of bovine early embryos [15–17]. However, studies on cryopreservation of bovine elongating conceptuses are considerably limited. Evaluation of early embryo viability post-thawing is usually performed by observation of re-expansion or hatching after *in vitro* culture [16, 17]. However, an *in vitro* culture system has not been established for bovine elongating conceptuses. In fact, elongating conceptuses that re-expand after a short time *in vitro* culture post-thawing are rare; thus, we concluded
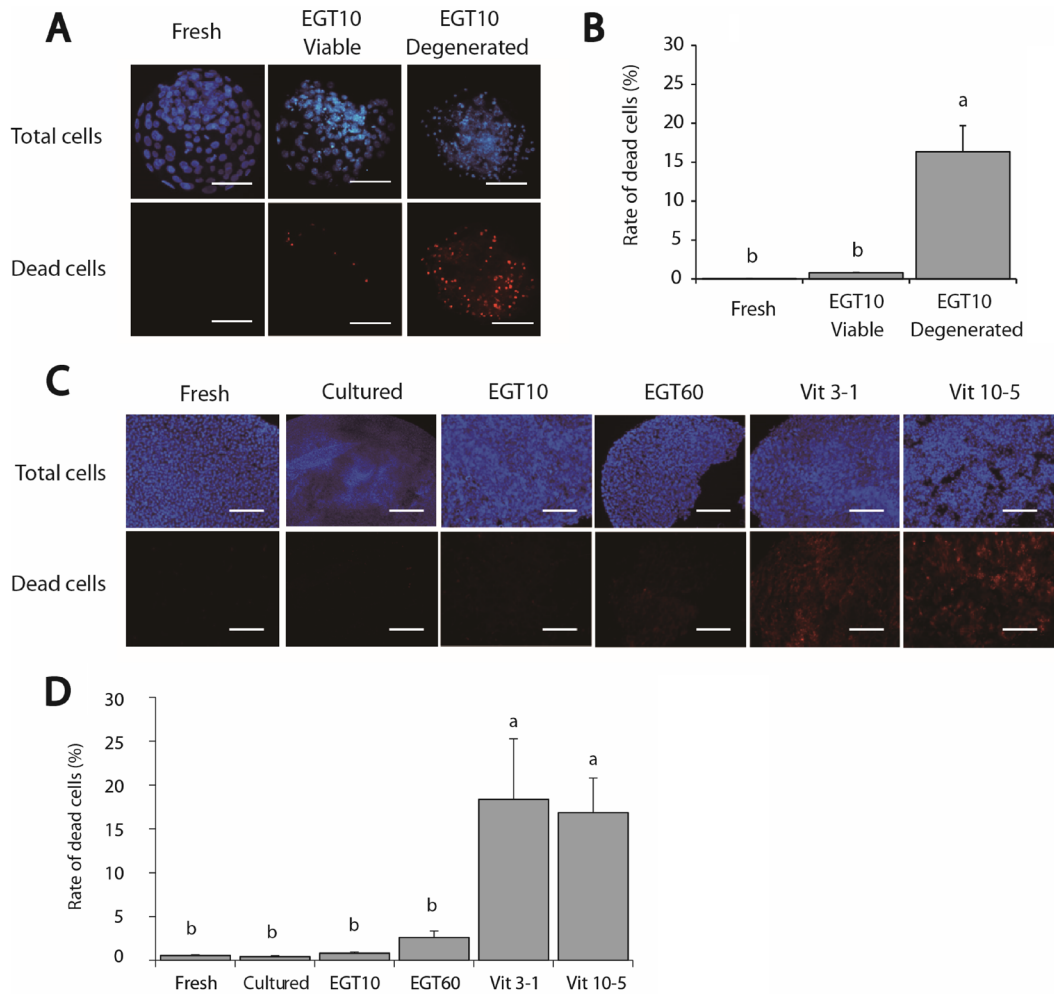
Fig. 3. Detection of dead cells in blastocysts and elongating conceptuses cryopreserved by slow freezing or vitrification. (A) Staining of dead cells (red fluorescence) in fresh blastocysts and viable and degenerated blastocysts after cryopreservation by EGT10. Scale bar is 100 μm. (B) Rate of dead cells in fresh blastocysts (n = 11), and in viable (n = 17), and degenerated (n = 4) blastocysts after cryopreservation by EGT10. Different superscripts indicate significant differences (P < 0.05). (C) Staining of dead cells (red fluorescence) in fresh elongating conceptuses, cultured fresh elongating conceptuses, and elongating conceptuses cryopreserved by EGT10, EGT60, Vit3-1, and Vit10-5. Scale bar is 100 μm. (D) Rate of dead cells in fresh elongating conceptuses (n = 21), cultured fresh elongating conceptuses (n = 8), and elongating conceptuses cryopreserved by EGT10 (n = 42), EGT60 (n = 5), Vit3-1 (n = 5), and Vit10-5 (n = 5). Different superscripts indicate significant differences (P < 0.01). The rate of dead cells (red fluorescence area) was calculated as the ratio of the red fluorescence area to the blue fluorescence area indicating the number of nuclei. Cultured: fresh elongating conceptuses cultured for 5 h. EGT10 and EGT60: slow freezing with equilibration for 10 min or 60 min, respectively. Vit3-1 and Vit10-5: Vitrification with equilibration for 3 and 1 min or 10 and 5 min in equilibrium solution and vitrification solution, respectively.
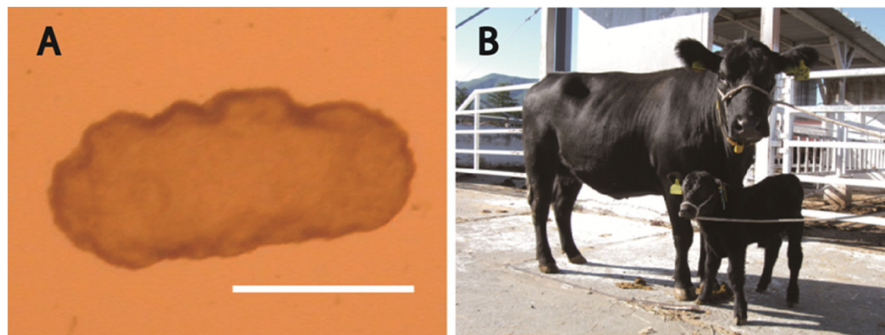

Fig. 4. Production of calves by transfer of day 14 elongating conceptuses cryopreserved by slow freezing. (A) Representative photograph of transferred day 14 elongating conceptus before cryopreservation by EGT10. Scale bar is 1 mm. (B) Representative photograph of a calf produced from a day 14 elongating conceptus cryopreserved by EGT10. EGT10: slow freezing with equilibration for 10 min.

that observation of re-expansion after *in vitro* culture is unsuitable for the evaluating elongating conceptus viability post-thawing. Therefore, in the present study, we evaluated the viability of elongating conceptuses after cryopreservation by the detection of dead cells. We first examined the usefulness of this evaluation method for viability after cryopreservation by comparing the rate of dead cells between viable and degenerated blastocysts cryopreserved by EGT10. The evaluation method is based on a cell-impermeable dye for staining dead cells characterized by compromised cell membranes. In the present study, we evaluated the viability of blastocysts and elongating conceptuses after a short *in vitro* post-thaw culture to adequately detect the increase in cell membrane permeability in cells injured during the cryopreservation process. The rate of dead cells was significantly increased in degenerated blastocysts cryopreserved by EGT10 compared to that in fresh blastocysts and viable blastocysts cryopreserved by EGT10. These results suggest the usefulness of the evaluation method for cell viability after cryopreservation.

Since a bovine elongating conceptus possesses a cavity containing a large amount of fluid and a multi-layered structure, it is anticipated that a longer equilibration time may be required for elongating conceptus cryopreservation. In fact, 36% of the elongating conceptuses cryopreserved by EGT10 and all elongating conceptuses cryopreserved by Vit3-1 remained afloat in the freezing solution after equilibration. Therefore, we conducted cryopreservation by EGT60 and Vit10-5 with a longer equilibration time in addition to EGT10 and Vit3-1 methods that are used for cryopreservation of early embryos. In the experiment for EGT methods, a prolonged equilibration time (EGT60) did not affect the floating or sunken states after equilibration, whereas all the conceptuses in Vit10-5 sank after equilibration. In both the EGT and vitrification methods, equilibration time did not affect the rate of dead cells. Furthermore, in the experiment for EGT10, the rate of dead cells after cryopreservation did not differ between floating and sunken elongating conceptuses after equilibration (data not shown). These results suggest that structural modification such as cell junction, conceptus size and formation of the hypoblast layer may prevent elongating conceptuses from penetration of cryoprotectants. Although the permeability of cryoprotectants in slow freezing was not associated with the rate of dead cells post-thawing in the present study, a further detailed study on the equilibration of cryoprotectants and cryoinjuries is required to optimize the cryopreservation method for elongating conceptuses.

Characteristic distribution of dead cells in the embryonic disc was not observed in the present study. Therefore, we did not evaluate the dead cells in the embryonic disc and trophoblast separately. However, it is thought that evaluation of cryoinjuries in the embryonic disc that directly affect the ability to conceive elongating conceptuses after transfer is important to develop the appropriate cryopreservation method. To perform a more detailed evaluation of cryoinjury in the embryonic disc, establishment of an *in vitro* culture system or *in utero* culture experiments involving elongating conceptus transfer and recollection by uterine flushing are required.

In the present study, the mean rate of dead cells in degenerated blastocysts cryopreserved by EGT10 was $16.4 \pm 3.35\%$. On the other hand, the rates of dead cells in all elongating conceptuses cryopreserved by EGT10 or EGT60 were less than 5%. The elongating conceptus has a multi-layered structure; thus, it is possible that cell-

impermeable dyes could not reach dead cells in the inner layer by sites. Furthermore, since cryopreservation injures the cell membrane as well as organelles, the extent and kind of cryoinjury may differ between blastocyst and elongating conceptuses. Therefore, the rate of dead cells in blastocysts (Fig. 3B) and elongating conceptuses (Fig. 3D) should not be directry compared, and we cannot consider the difference in cryotolerance between blastocysts and elongating conceptuses.

The rates of dead cells in elongating conceptuses cryopreserved by EGT10 and EGT60 were comparable to those of fresh elongating conceptuses, whereas remarkable increases in dead cells were observed in elongating conceptuses cryopreserved by Vit3-1 and Vit10-5. In the present study, it was difficult to place the elongating conceptuses on the cryotop, which is an optimized device for vitrification of early embryos; we therefore used a nylon mesh as a substitute container for vitrification of elongating conceptuses [19]. Higaki *et al*. [19] reported the successful production of fertile zebrafish originated from primordial germ cells in embryos vitrified using a nylon mesh. In addition, our preliminary study showed that the viability of *in vitro* fertilized bovine blastocysts vitrified by Vit3-1 using nylon mesh was comparable to those vitrified by the cryotop method (data not shown). Therefore, it is unlikely that the increases in dead cells in vitrified elongating conceptuses were due to adoption of the approach using a nylon mesh for vitrification. Various factors, such as cell number, size, permeability to water and cryoprotectants, and sensitivity of cells to cryoprotectant toxicity affect the success of cryopreservation. Although the reason for decreased viability in vitrification methods remains incompletely understood, it is speculated that the conditions during the vitrification processes used in the present study were not appropriate for bovine elongating conceptuses. Our findings in the present study suggest that EGT methods are more suitable for cryopreservation of bovine elongating conceptuses in comparison with vitrification methods.

Based on the results of detection of dead cells in elongating conceptuses after cryopreservation, we conducted transfers of elongating conceptuses cryopreserved by EGT10, and succeeded in producing calves. In studies by Betteridge *et al*. [13] and Kimura *et al*. [14], production of calves by the transfer of fresh bovine elongating conceptuses was reported. However, there are no reports on the production of calves from cryopreserved bovine elongating conceptuses. To the best of our knowledge, the present study is the first report on the successful production of calves from cryopreserved bovine elongating conceptuses. On the other hand, the cow which was transferred with the 14.3 mm elongating conceptus was not pregnant. There was a large variation in the size of elongating conceptuses even on the same recovery day. Further research is thus required to clarify the influence of size and biopsy of elongating conceptuses on viability after cryopreservation and transfer.

In conclusion, we demonstrated precise genotyping based on high-throughput microarray platforms using biopsied cells from bovine elongating conceptuses. Furthermore, the present study is the first to report the successful production of calves from cryopreserved bovine elongating conceptuses. Although further experiments for optimization of the cryopreservation method for biopsied elongating conceptus and the concordance of genotypes between elongating conceptus and the subsequent calf are required, the findings of the present study

suggest that the elongating conceptus transfer technique enables its practical application for preimplantation genomic selection in cattle.

Acknowledgements

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